ANTIBODY DIRECTED PHOTOSENSITIVE LIPOSOMES: THEIR POTENTIAL FOR CYTOTOXIC ACTION

BY

JANET MORGAN

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Department of Haematology
Faculty of Clinical Sciences
University College London

ABSTRACT

The purpose of this study was to design a tumour specific cytotoxic system which will act rapidly and with few side effects. The water soluble sulphonated derivative of the photosensitive dye aluminium phthalocyanine (AlSPc), which is known to be activated by light to produce singlet oxygen, was tested as a cytotoxic agent in antibody targeted liposomes.

The methods used to purify the AlSPc, to encapsulate it in liposomes and to attach targeting ligands, antibody or Protein A, covalently to the liposomes were described.

Preliminary experiments optimised conditions for the greatest differential of cytophototoxicity between targeted and untargeted liposomes. Further series' of experiments using a variety of cells showed that the phototoxic effect was dependent on the concentration of liposomes, antibody and AlSPc as well as the light exposure at specific wavelengths. Results were similar for both directly and indirectly targeted liposomes. The former had specific antibody attached to the liposomes, and the latter had anti-mouse immunoglobulin or Protein A attached to the liposomes which then bound to cell specific antibody in a 'sandwich' technique. Target cells included human T lymphocytes, B cell lines, and human osteosarcoma and colorectal carcinoma cell lines. Specific killing was demonstrated in all cases when relevant antibodies were used. When cells did not express antigen for the antibody or when irrelevant antibody was used there was no phototoxicity. Free AlSPc incubated under the same conditions as liposomes also had no toxic effects.

Different concentrations of AlSPc were encapsulated in liposomes to determine the degree of amplification required for maximum photocytotoxicity. Similarly, purified fractions of di-, tri-, and tetra- sulphonated phthalocyanine encapsulated in liposomes were compared for their phototoxic effects, and the differences observed were explained.

Using a cooled charge coupled device (CCD) camera, a sensitive method for the detection of AlSPc fluorescence, it was demonstrated that the phototoxic effect of AlSPc liposomes was effective without internalisation of the liposomal contents, and also that cell binding in cell mixtures was antibody specific. Some non specific binding of liposomes was also shown but it was insufficient to cause toxicity.

Fluorescent analysis of bone marrow samples incubated with CD3 and anti-B cell antibodies and AlSPc liposomes showed specific targeting of subpopulations of cells and a small population of cells also took up liposomes nonspecifically. When subpopulations of bone marrow were targeted by AlSPc liposomes and also treated with red light, growth of CFU-GM colonies from untargeted progenitors was the same as untreated controls.

The mechanism by which cell death was photodynamically induced by targeted AlSPc liposomes is discussed.

The potential of the techniques used here as a treatment for specific clinical situations such as bone marrow purging and photodynamic therapy of bladder and ovarian carcinoma are discussed.

TO MY HUSBAND AHED

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cancer chemotherapy depends on agents which are selectively active against malignant cells compared with normal tissues, and progress results from the deployment of new compounds with increased selectivity, or from new ways of administering existing agents.

A problem with many chemotherapeutic agents is that they are largely phase or cell cycle specific and therefore inactive against cells in the resting phase of the cycle. Furthermore they often have very toxic side effects due to low specificity for tumour cells. Drugs would be more effective if they were independent of cell growth whilst maintaining specificity to the tumour cells. Antibody targeting of liposome encapsulated drugs is highly specific (Gray et al 1988, Morgan et al, 1989) thus reducing non specific toxicity, but their cytotoxicity is limited by cell cycle kinetics and the rate of internalisation of the liposomes. This varies widely depending on the target tissue, liposome size (Machy and Leserman, 1983) and composition. In some cells, despite excellent liposome attachment, no toxicity occurs because the antigen/liposome complex does not internalise (Machy et al 1982). Provided the right antigen is chosen liposomes can deliver large amounts of drug into cells resulting in an amplification of the toxic effect.

Compounds that are not normally toxic to cells because there is no uptake mechanism, or because of their hydrophilic nature, could exert a cytotoxic effect if delivered to the cell interior by liposomes. Similarly, compounds that are usually considered to

be too toxic could be used in liposomes because the protective lipid bilayer has an insulating effect. There are however limitations on the use of such compounds in vivo, even when delivered in liposomes, because of the uptake of liposomes by the cells of the reticuloendothelial system (Weinstein, 1984).

A liposome encapsulated agent which does not require internalisation but can exert its cytotoxic action from the cell surface would be independent of these variables. If in addition it were also toxic to resting tumour cells a more universal and effective drug targeting system could be designed.

Free radical producing compounds are toxic regardless of the stage of the cell cycle. Agents such as methyl viologen (Paraquat), are readily reduced in cells by electron donation resulting in free radicals which are rapidly reoxidised by molecular oxygen. This redox recycling regenerates Paraquat which can be reduced again and produces the superoxide radical, the chain initiator of subsequent radical reactions which cause cell damage and death (Osheroff et al, 1985). A problem with redox cycling is that it is difficult to control, and in the case of Paraquat, accumulation occurs in lung cells by an active transport system causing damage there (Smith et al, 1979). Other free radical producing drugs, for example anthracyclines such as adriamycin and daunorubicin have similar effects due to accumulation in tissues other than those targeted, in this case the heart, causing cardiotoxicity (Lown, 1984; Cohen and D'Arcy Doherty, 1987). For these reasons, agents acting by redox production of free radicals and reactive oxygen species are not

ideal for clinical use.

Photosensitisers produce active oxygen species and free radicals only subsequent to irradiation by light, and therefore have the advantage of a more controlled production of toxic species, which can be triggered after the compound has reached its target. Moreover, many photosensitive molecules, particularly naturally occurring porphyrins, for example precursors of substances such as haem (uroporphyrin, protoporphyrin, coproporphyrin, Reyftman et al, 1989; El-Far and Pimstone, 1984 and 1984a), or similar synthetic compounds, are not particularly toxic when unactivated.

Phthalocyanine dyes are such a class of compounds. They are relatively non toxic (Reviewed by Spikes 1986) and their cytotoxic action is thought to be primarily by the generation of singlet oxygen on exposure to light of specific wavelengths, (Brasseur et al, 1985). Moreover, because singlet oxygen is able to diffuse a mean distance of 1000-2000 Å during its lifetime (Lindig and Rodgers, 1981; Grossweiner, 1981), there is the possibility that singlet oxygen producing agents may have a toxic effect on cells, even when bound to the outside of the cell.

Sulphonated aluminium phthalocyanine (AlSPc) and other phthalocyanines have been reported to have tumour localising properties in their own right (Rousseau et al, 1983 and 1985; Tralau et al, 1987; Singer et al, 1987 and 1988). This is probably partly mediated by the lipophilicity of the less polar fractions of AlSPc such as some of the di- and mono-sulphonated forms (Paquette et al 1988). While this may facilitate uptake

into tumour cells, there is also the potential for uptake by normal cells, and hence damage on illumination. In vivo the degree of tumour selectivity varies with the tissue. The phthalocyanine uptake ratio of tumour to normal tissue in experimental models is very high in the brain (30:1, Sandeman et al, 1987), but much lower in other tissues such as the muscle (2:1, Tralau et al, 1987a). When the ratio is low there is obviously much less potential for photodynamic therapy of the tumour without producing non-selective damage of normal tissues.

In vitro, too, some selectivity has been shown between acute myeloblastic leukaemia and normal haemopoietic progenitor cells (Singer et al, 1987 and 1988). This could be due to differences in membrane viscosity in the leukaemic cells compared to normal membranes. Acute myeloid leukaemia cells have been reported as having low cholesterol to phospholipid ratios, which results in increased membrane fluidity and may make them more susceptible to increased uptake or retention of photosensitisers (Shinitzky, 1984). Another photosensitive dye, merocyanine 540 has also shown differential phototoxicity towards early (resistant) and late haemopoietic progenitors and leukaemic cell lines (sensitive) (Sieber et al, 1987). Partitioning of merocyanine into cells was thought to be determined partly by membrane surface charge, with the most sensitive cells having the lowest negative charge, and the most resistant the highest (Smith and Sieber, 1989). For both sensitisers, however, the differential between tumour and normal cells was not high, resulting in a very narrow band of parameters for effective

treatment.

Others have shown no differential uptake between normal and tumour cells in vitro (Chan et al, 1987; Brasseur et al, 1988), but differences in dye uptake have also been observed between normal cells of different haemopoietic lineage (Singer et al, 1987). Presumably the differential effects seen in normal cells, cell lines or tumours are due to biological variations in the components of cell membranes which affect uptake (Galeotti et al, 1984). Liposomal bilayer composition (as a cell membrane model) had an effect on the binding of the photosensitisers haematoporphyrin derivative (HpD) and Photofrin II (PF II, a purified component of HpD). Rigidification of the bilayer by increasing the cholesterol content decreased both HpD and PF II partition into the liposome, whereas addition of saturated phospholipid increased PF II but not HpD (Ehrenberg and Gross, 1988). This reflects the situation seen in vivo, in which plasma membranes from hepatomas with differing degrees of differentiation showed variations in lipid content. Less differentiated tumours were more fluid and had lower cholesterol to phospholipid ratios (Masotti et al, 1986). Graschew et al, 1988, have shown that highly differentiated tumours are more sensitive than less differentiated tumours to photodynamic therapy with PF II.

Differences in phototoxicity between cells which do take up the dye could be because of the protection and repair mechanisms of the cells. Glutathione, a tripeptide, and the related enzymes glutathione transferase and glutathione peroxidase are involved

in the repair of oxidative damage and the scavenging of free radicals. Depletion of glutathione levels in cells resulted in increased cell killing in response to photodynamic treatment with dihaematoporphyrin ether, probably by influencing cellular repair mechanisms (Miller and Henderson, 1986). Others have shown that some lung cancer cell lines displayed chemosensitivity which related to levels of glutathione, and activity of several enzymes including superoxide dismutase (Carmichael et al, 1988).

The rate of light delivery is also of importance. Cell survival decreases with increased rates of light delivery, probably because at lower rates, cells are able to repair sub lethal damage (Ben-Hur et al, 1987b). This applies also to irradiation and post irradiation temperatures. Hypothermia is thought to inhibit repair of potentially lethal photodynamic damage (Moan et al, 1979), thus producing more efficient toxicity, although conflicting evidence has been obtained. West and Moore, 1988, found decreased photokilling at low temperatures. Presumably the effects depend partly on the cell type (for example some may be rich and others deficient in the enzymes involved), and the photosensitiser used. Some biomolecules which can protect against singlet oxygen damage either by preventing its production (urate and a fungal product, ergothionane) or by quenching it (carnosine, present in striated muscle) might be implicated in some photo-resistant tissues, or alternatively, could be useful in helping to prevent non-specific damage in photodynamic therapy (Dahl et al, 1988).

It was thought that encapsulating the dye within liposomes

and directing them to tumour cells with antibodies might decrease the non specific uptake by normal cells, as well as improving the tumour localising properties by antibody targeting, thereby increasing the therapeutic ratio considerably.

In this thesis sulphonated aluminium phthalocyanine (AlSPc) was chosen for further study because it had several desirable properties that made it suitable for both encapsulation in liposomes and use as a phototoxic agent. This is discussed further in the next sections.

1.2. PHOTOSENSITISATION

1.2.1. MECHANISM OF ACTION OF PHOTOSENSITISATION

In this section the general mechanisms of photosensitivity will be discussed with particular reference to biological systems.

For a phototoxic effect to occur there are three basic requirements:

1) The presence of a photosensitiser.
2) Excitation of the photosensitiser by an appropriate light source.
3) Oxygen.

For a photochemical reaction to occur, energy from the irradiating light must be absorbed by the photosensitive molecule,which becomes electronically excited. This means that an electron is excited from a ground state to a higher energy singlet state (which has paired electron spins). The excited singlet state is usually too unstable and short-lived to interact

effectively with other species, but the electron can undergo intersystem crossing (ISC) which involves inversion of electron spin to an excited triplet state (which has two unpaired electron spins). The triplet state is longer lived and therefore more likely to interact with other species (Foote 1984).

The presence of oxygen is important because it is ultimately the conversion of molecular oxygen to free radical forms in the photosensitising reactions that produces the phototoxic species which will damage or kill the cells. Oxygen in its ground state (which is a triplet state) cannot easily react with most organic molecules because it contains unpaired electrons of parallel spin, and is restricted to reactions with compounds from which it can accept electrons also of parallel spin to fit the empty orbitals. Most biological molecules contain paired electrons of opposite spin in their covalent bonds so oxidations are not electronically favourable (Gutteridge and Halliwell, 1989). However, light activated compounds can generate highly reactive oxygen species.

There are a number of ways in which an excited electron can decay to the ground state, with a concommitant release of energy from either the singlet or triplet state. Some of these, for example fluorescence, phosphorescence and quenching, are summarised in the Jablonski diagram (Fig 1). However, photosensitisation of biological molecules is mainly concerned with two photoprocesses and both result from the triplet state. They are: Type I: hydrogen or electron transfer, and Type II:

KEY TO FIGURE 1

(a) Fluorescence

(b) Internal conversion

(c) Quenching from singlet state

(d) Phosphorescence

(e) Quenching from triplet state

(f) Quenching of singlet oxygen to ground state

(g) Reaction of singlet oxygen with a substrate

PC - Phthalocyanine

ISC - Intersystem crossing

ET - Energy transfer

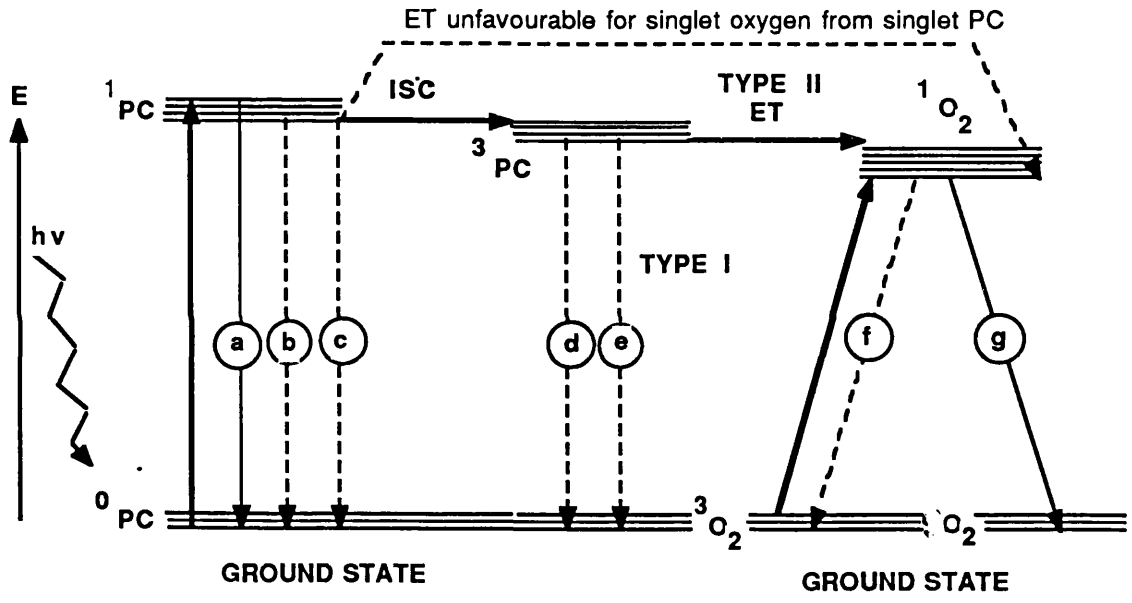
S - Substrate

E - Energy

hv - Incident light

FIGURE 1

DIAGRAM SHOWING PHOTOPROCESSES OF ELECTRONICALLY EXCITED PHTHALOCYANINES



The energy changes are shown in the form of a Jablonski diagram

KEY TO FIGURE 2

PC - Pthalocyanine

ISC - Inter System Crossing

S - Substrate

3 - Triplet state

1 - Singlet state

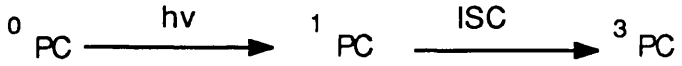
0 - Ground state

ET - Energy Transfer

FIGURE 2

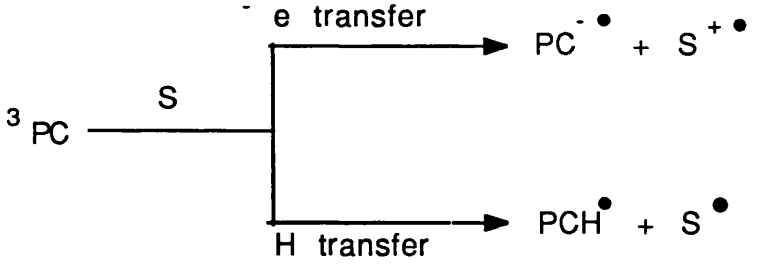
PHOTOPROCESSES INVOLVED IN PHOTOSENSITISING BIOLOGICAL MOLECULES

FORMATION OF TRIPLET STATE

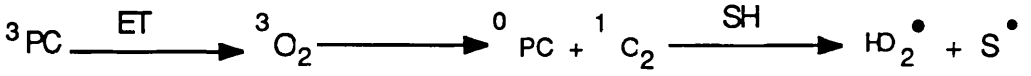


PHOTOPROCESSES POSSIBLE FROM THE TRIPLET STATE:

TYPE I



TYPE II



energy transfer reactions (Foote 1984 and 1988), summarised diagrammatically in Figure 2.

TYPE I PHOTOPROCESS

In the Type I reaction, the triplet sensitiser interacts directly with a substrate and abstracts hydrogen or an electron, producing free radicals. The radicals may then react with either various substrates causing functional changes, or molecular oxygen producing the superoxide radical which is then able to go on and oxidise many other substrates.

TYPE II PHOTOPROCESS

In the Type II reaction, energy is transferred from the excited triplet electron of the photosensitiser to molecular oxygen in its ground state (triplet). Molecular oxygen then becomes excited to the reactive singlet oxygen with electrons of opposite spin. The singlet oxygen thus formed is then able to react directly with biological substrates, for example with unsaturated double bonds in fatty acids. Another Type II reaction which also occurs sometimes, is electron transfer from the excited photosensitiser to oxygen giving the superoxide free radical anion and oxidised photosensitiser (Foote, 1989).

The type of process that occurs or predominates depends on many factors and in particular the microenvironment of the photosensitising system. The Type I reaction is more likely to have a greater contribution at low O_2 concentrations and higher pH (Ferraudi et al, 1988), whereas Type II reactions are favoured

in well oxygenated systems (Langlois et al, 1986; Wagner et al, 1987).

Since the photosensitiser to be used is AlSPc, a water soluble compound which will be encapsulated in the aqueous phase of liposomes, the reactions of most importance in the experiments which follow are more likely to be of Type II. This is because Type I reactions are favoured if the photosensitiser is already complexed to (Davila and Harriman, 1989), or in the presence of high concentrations of the substrate (Foote, 1984), whereas sensitiser free in solution favours the Type II process.

1.2.2. PROPERTIES OF A GOOD PHOTOSENSITISER

A good photosensitiser for the purposes of this investigation will have properties that will favour singlet oxygen production in an aqueous solution. These are:

1) large absorption at an appropriate wavelength since only absorbed light can produce active species. It also means that when necessary, lower light intensities may be used, thus producing less thermal damage.
2) a high quantum yield of triplet formation. This means an efficient inter-system crossing from the short-lived singlet to the longer-lived triplet state per quantum of excitation light.
3) a long triplet lifetime. This increases the possibility of interaction with other molecules (eg. energy transfer to molecular oxygen).
4) a triplet energy close to but above that of singlet

oxygen so that it is able to generate singlet oxygen.

5) stable to self-oxidation and oxidation by singlet oxygen.
6) stable to photodegradation so that a single molecule of photosensitiser will be capable of generating many singlet oxygens without breaking down.
7) stable in aqueous solution.

1.3. SITE OF ACTION OF PHOTOACTIVITY

Much of the work done on elucidating the site of action of phototoxic species produced on irradiation of photosensitisers has been done on haematoporphyrin or some of its derivatives. However, most photosensitisers give similar types of photodamage since they produce the same active species. Basically, toxicity arises from damage to, or destruction of biological molecules important for the integrity, normal function, protection, growth and division of cells by these toxic species.

The most susceptible molecules are those which contain electron-rich sites such as double or more extensively conjugated bonds. Examples of these are unsaturated bonds in phospholipids, cholesterol, purines, pyrimidines and some amino acids, all of which can be photo-oxidised (Spikes, 1984).

The part of the cell in which damage occurs depends on both the properties of the photosensitiser and the cell. More lipophilic photosensitisers are taken up more easily into cells than hydrophilic ones (Moan et al, 1987), and become distributed among the organelles, particularly those rich in lipids such as membranes which are probably the major targets.

In experiments in vitro, longer incubation of cells with photosensitiser results in its increased penetration and distribution from the initial uptake at the cell membrane to mitochondria, lysosomes and finally the nucleus. After irradiation, the effects will depend upon which organelles contain the photosensitiser. In microsomes, cytochrome P-450 is destroyed by photosensitisation with haematoporphyrin derivative (Dixit et al, 1983), and the same cytochrome c oxidase in mitochondria is similarly damaged in vitro and in vivo (Gibson et al, 1984). The oxidase is a critical enzyme, being the terminal oxidase for detoxification of potentially harmful substances such as drugs or carcinogens, as well as endogenous compounds. Its destruction by photo-produced singlet oxygen therefore has potential for great harm.

In solution and in E. coli, haematoporphyrin sensitises reversible strand breaks in DNA by singlet oxygen action (Boye and Moan, 1980). Another compound, chloroaluminium phthalocyanine causes photosensitised DNA strand breaks in Chinese hamster cells which were again largely reversible (Ben Hur et al, 1987). Some cells have equal sensitivity throughout the cell cycle (Ben Hur and Rosenthal, 1985a), whereas others are more sensitive at particular stages (Berg and Moan, 1988; review, Spikes, 1984).

Another method of delivering photosensitiser to cells is via antibody conjugates (Mew et al, 1983; 1985; Wat et al, 1984; Roberts et al, 1987), or bound to substances like low density lipoproteins (Morliere et al, 1987; Zhou et al, 1988) which then bind respectively to antigen or their receptors on the cell. The

mode of action of the photosensitiser will depend on whether or not it is internalised, and its ultimate destination in the cell. Chlorin e_6 conjugated via a carrier to antibody, caused cell membrane photolysis when externally bound to cells (Oseroff et al, 1986). Pyrene incorporated into liposomes and targeted to cells by antibody was taken up by antibody-mediated endocytosis (Yemul et al, 1987). It was proposed that the pyrene entered the lysosomes and irradiation caused damage to their membranes thus releasing degradative enzymes into the cell cytoplasm.

Obviously there are many mechanisms by which cells can be photosensitised. Although damage is initiated by rapid photoactivated events, it may be completed by the subsequent slower reactions of radical chain auto-oxidation, damage to vital enzymes or other proteins, autolysis by lysosomal enzymes or all of these.

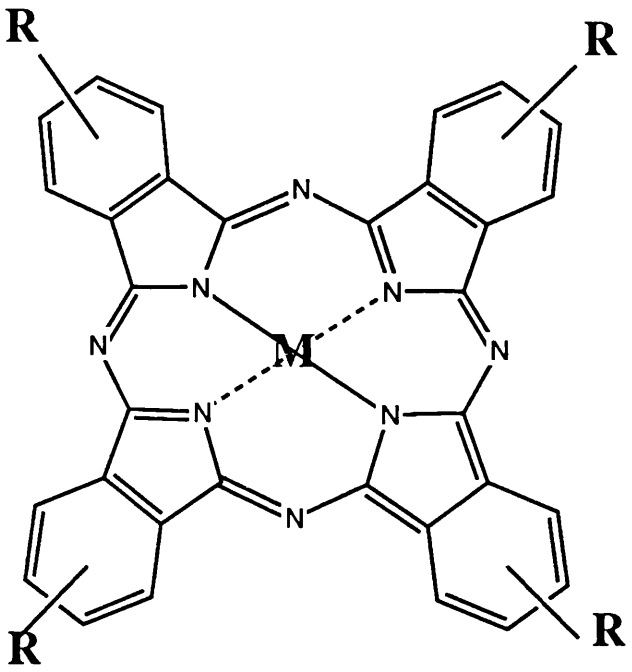
1.4. PHTHALOCYANINES

The properties described in Section 1.2.2 which make a good sensitiser are typical of many phthalocyanines, and they have in addition many other characteristics which enhance their suitability for photodynamic therapy.

The basic phthalocyanine molecule is porphyrin-like in structure and composed of four iso-indole units linked by nitrogen bonds (Dent et al, 1934) as shown in Figure 3. This produces large conjugation pathways in the rings which results in long wavelength absorption peaks. For in vivo photodyanamic therapy this is important since light of longer wavelength has

FIGURE 3

BASIC PHTHALOCYANINE STRUCTURE



M = H_2 or Metal eg. Zn, Al

R = Functional group

eg. sulphonic acid	SO_3^-
amino	NH_3
nitro	NO_2
carboxyl	COOH

Also methyl, alkyl, ester, ether, benzyl etc.

The groups are attached at one of the carbon positions of the ring structure

good tissue penetrating properties (Doiron, 1984). The depth of tissue penetration depends on the tissue type, being less in heavily pigmented tissues such as the liver, and more in the brain (Wilson et al, 1984). Many phthalocyanines fluoresce, which permits their detection and measurement, which is useful for determining targeting and dose. Phthalocyanines are relatively easy to synthesise (Byrne et al, 1934; Linstead and Lowe, 1934; Lever, 1965) and the basic structure can be modified to exploit those properties which make them more suitable for any particular photosensitising situation.

a) The central portion of the phthalocyanine molecule can act as a metal chelator. Depending on the metal substituent, the singlet and triplet lifetimes vary (Langlois et al, 1986), and the absorption spectrum shifts (Sonoda et al, 1987). Some metal substituents (eg. copper) result in photoinactive molecules (Brasseur et al, 1985).

b) The basic or metallo phthalocyanine is not soluble in water which is a disadvantage for its use in biological systems. They have, however, been used successfully by dissolving in small amounts of organic solvents and then diluting in aqueous buffers (Ben-Hur and Rosenthal, 1985b), or incorporating in the lipid phase of liposomes (Reddi et al, 1987). Functional groups such as nitro, amino (Van Lier et al, 1984), carboxylic acid (Shirai et al, 1979), or sulphonic acid (Weber and Busch, 1965), can be added to the outer rings to make derivatives, some of

which are water soluble. This is disadvantageous in one sense because there are many points at which the substituents can attach, resulting in a large number of isomers. Derivation means that the homogeneous insoluble phthalocyanine becomes a heterogeneous mixture and the components have variable solubilities depending on the type, number and position of the substituent. However, many derivatives can be purified by HPLC, and the most useful fractions and isomers separated (Ali et al, 1988).

Many of the typical characteristics of the phthalocyanines have been determined in organic solutions, but in different environments such those found in biological systems, there will of course be many changes due to the increased number of variables. Some will be slight and others of greater consequence, but they must be taken into consideration when deciding which phthalocyanines are most suitable as cell photosensitisers. Some of the variables will affect the photophysical properties of the photosensitiser, and others the photochemical reactions that lead to cell toxicity.

Some of the factors to be taken into consideration are:

a) wavelength shifts in absorption and fluorescence spectra. If these change when photosensitisers are taken into cells or encapsulated in liposomes, then the wavelength of the activating light may need adjusting so that it is as effective as the free sensitiser. The excitation and emission wavelengths for detection of the photosensitiser may also need altering.

b) changes in absorption coefficients.

Higher or lower intensities of activating light may be required for the same amount of phototoxicity.

c) concentration effects such as aggregation and quenching.

These may occur at high encapsulation concentrations in liposomes.

d) changes in singlet and triplet lifetimes.

e) changes in triplet yields.

These (c, d and e) may all result in different photoactivity. The precise effects vary depending on the photosensitiser. For example, some are taken up into, and retained by cells or tissues best in an aggregated form (Dougherty, 1987). However, aggregation results in decreased photoactivity for many photosensitisers because of lower lifetimes and yields of the excited states (Moan and Sommer, 1984). The overall effect probably depends on the eventual fate of the photosensitiser within the cell, tissue or liposome, and its final physical condition (eg. aggregated or not, or bound to substrate) on illumination.

f) pH. The pH governs the charge on the photosensitiser. Increased photokilling of cells by chloro aluminium phthalocyanine with decreased pH was observed by Ben Hur and Rosenthal (1985c). They suggested that it was due to an effect on the interaction of the phthalocyanine with cellular targets, making them more sensitive. Experiments done by Thomas and Girotti (1989) found that greater

phototoxicity was achieved in tumours in vivo which were made more acidic by metabolism of glucose. They found the results were due to increased uptake of photosensitiser in the more acidic environment. It was previously mentioned that pH affects the type of reaction that predominates (Section 1.2.1).

Many of these effects are interdependent and some will be discussed with particular reference to AlSPc.

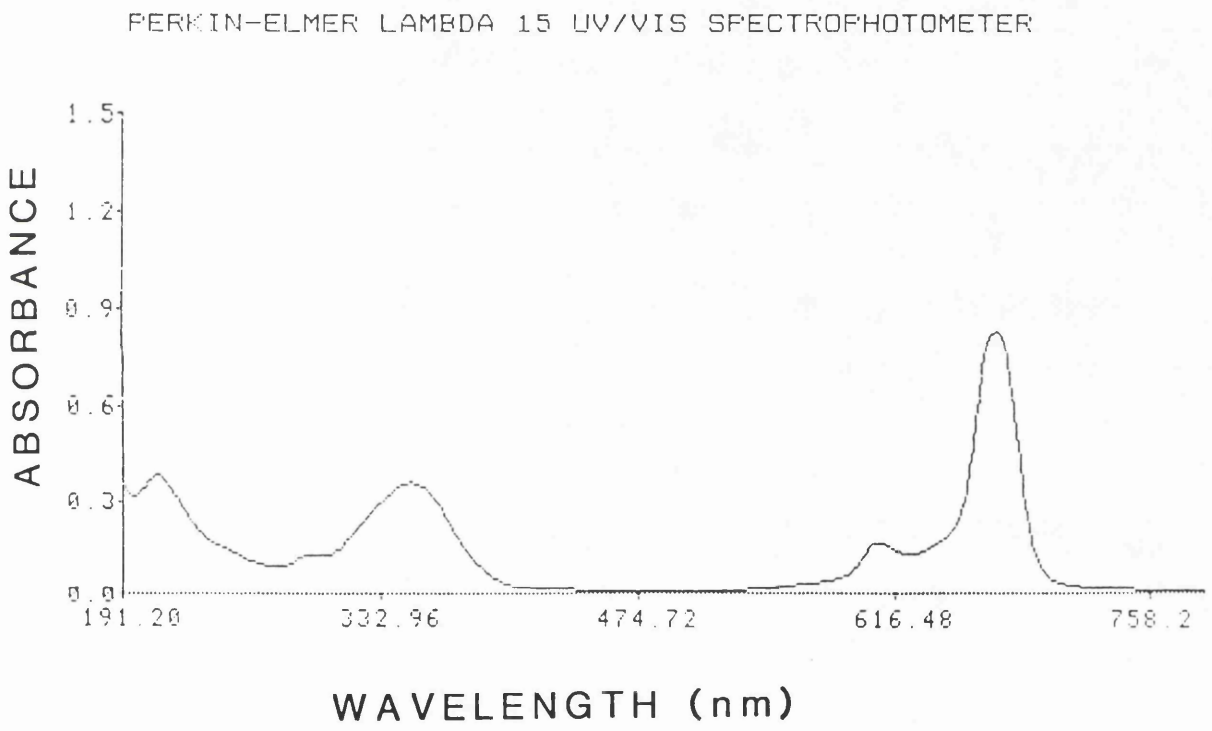
1.4.1. SULPHONATED ALUMINIUM PHTHALOCYANINE

Aluminium phthalocyanine can be sulphonated by boiling with fuming sulphuric acid above 80°C to produce a mixture of differentially sulphonated products (Linstead and Weiss, 1950; 1950a). An alternative method is by the condensation of sulphophthalic acid in the presence of aluminium chloride to provide unambiguous tetra-sulphonated aluminium phthalocyanine, or, by varying the ratio of phthalic to sulphophthalic molecules, isomeric mixtures of the the other sulphonates can be produced (Weber and Busch, 1965). The structure of sulphonated aluminium phthalocyanine (AlSPc) is shown in Fig 3 and its absorption spectrum (from a sulphonated mixture which is the starting product for experiments in this thesis) in Figure 4. The spectrum shows a peak in the uv at about 365 nm and a broad peak in the red with maxima at about 605 and 680 nm, the latter being the largest.

The number of sulphonated groups does not affect the kinetics of singlet oxygen production directly (Wagner et al,

FIGURE 4

ABSORPTION SPECTRUM OF SULPHONATED ALUMINIUM PHTHALOCYANINE



1987), but in aqueous solution most sulphonated phthalocyanines tend to form dimers or larger aggregates which are photoinactive even at low dilutions (10^{-6} to 10^{-5} molar, Harriman and Richoux, 1980; Wagner et al, 1987). AlSPc has the advantage that even at much higher concentrations (10^{-4} molar) there is little dimerisation (Darwent et al, 1982), so it is useful for encapsulation in the aqueous phase of liposomes. The amount of aggregation at higher concentrations depends on the degree of sulphonation, and can be observed as a shift in the 680 nm peak towards lower wavelengths as shown in the spectral scans of purified tetra-, tri- and di-sulphonated aluminium phthalocyanine (Fig 5). Aggregation is highest for the di-sulphonated derivative with a definite peak at 639 nm, and lower for the tri- and di-sulphonated components. The more useful molecules therefore, seem likely to be the more highly sulphonated ones, which are also the most water soluble.

Some of the photochemical properties which help to enhance the suitability of AlSPc as a photosensitiser are:

a) singlet lifetimes = 5 ns

b) triplet lifetimes = 0.5 ms

c) triplet yield = 40%

d) molar absorption coefficient, = 1.6×10^5 $M^{-1}cm^{-1}$ at 6[illegible] nm.

These are not necessarily the best, highest or most efficient values for phthalocyanines (a summary of the photophysical properties of some metallo phthalocyanines is given in Darwent et al, 1982a), or other photosensitisers. However, when other factors such as solubility, absorption maxima, and monomerisation

LEGEND TO FIGURE 5

The absorption scans are of approximately 1 mM AlSPc encapsulated in liposomes. They were scanned using a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer.

A - Tetra-sulphonated AlSPc

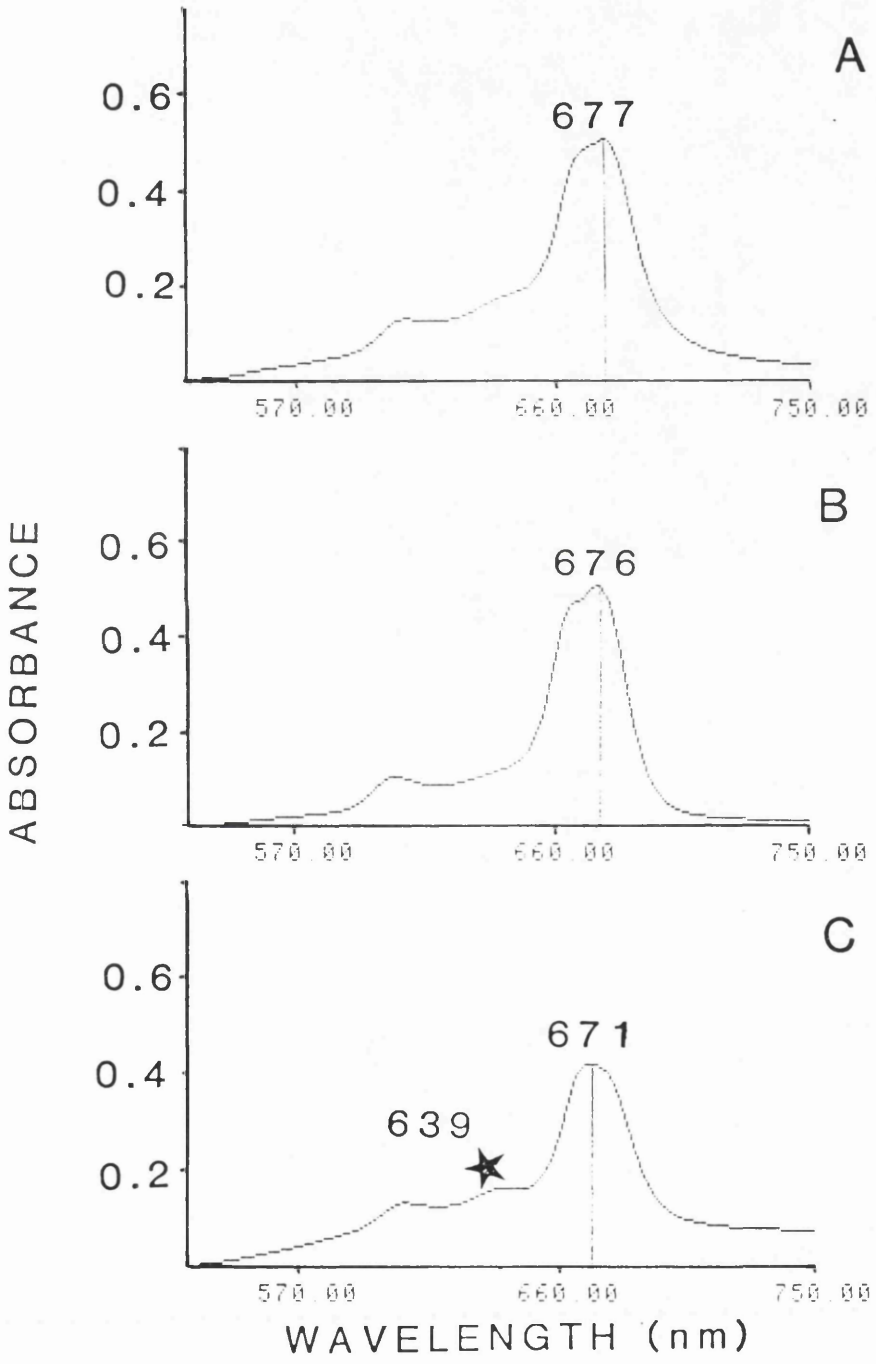
B - Tri-sulphonated AlSPc

C - Di-sulphonated AlSPc

The wavelengths of the peaks of each particular fraction are shown.

FIGURE 5

ABSORPTION SPECTRA OF DIFFERENTIALLY SULPHONATED DERIVATIVES OF ALUMINIUM PHTHALOCYANINE



are taken into consideration, on balance, AlSPc is a relatively efficient photosensitiser overall and is particularly suitable for encapsulating in liposomes. This was further supported by a study on several photosensitisers in which AlSPc, although the least efficient generator of singlet oxygen, was the most efficient at cell killing per weight of sensitiser (Kimel et al, 1989).

1.5. LIPOSOMES

Liposomes are vesicles which consist of a lipid bilayer formed of phospholipids, with or without cholesterol, enclosing an aqueous space. Liposomes form spontaneously when phospholipids are dispersed in aqueous medium because they have both polar and hydrophobic parts which orientate into bilayers. Phospholipids consist of a glycerol backbone with fatty acids at carbon positions 1 and 2 and a phosphate group at position 3 (Figure 6). The polar phosphate headgroups are exposed to the aqueous medium on the exterior and interior of the vesicle and the fatty acid tails form the inside of the bilayer. (Wilschut, 1982). The many different types and sizes of liposomes have been used for a multitude of purposes both in vitro and in vivo. They can be carefully tailored for use in a particular situation by exploiting factors such as the size, charge, lipid composition, number of bilayers and the compound to be incorporated or encapsulated. Liposomes can vary in size from about 25 nm to 10 μm or more in diameter.

Thus:

a) negatively charged medium sized vesicles are best for delivering drugs to the RE system (Poste et al, 1983);

b) large multilamellar vesicles (LMV) delivered intramuscularly are best for slow release of drugs (Culliss et al, 1987);

c) small vesicles delivered subcutaneously are best for targeting the lymph nodes via the lymphatic circulation (Patel, 1985);

d) neutral or positively charged small unilamellar vesicles (SUV), have a longer half life in the circulation than LMV or negatively charged vesicles (Juliano and Stamp, 1975).

The purpose of this thesis is to target liposomes to cells specifically by means of antibody, and several factors were considered when designing an appropriate liposome.

a) Non specific binding by the liposomes themselves should be minimised, binding being conferred by an attached ligand.

b) Water soluble compounds should be stably encapsulated in the aqueous compartment of the liposome.

d) It should be possible to attach suitable ligands to the bilayer surface for targeting to cells, without affecting the stability of the liposome

c) The method of preparation should be reproducible, homogeneous and stable.

The type of liposomes chosen were small unilamellar liposomes (SUV) formed by sonication which had previously been

used to deliver methotrexate to T cells in vitro, using protein A as the targeting ligand to bind to a CD3 antibody (Gray et al, 1988); and as an immunofluorescent probe containing carboxyfluorescein for detection of cell surface antigens, using sheep anti-mouse immunoglobulin to bind to monoclonal antibodies raised against the antigens (Gray et al, 1989). They possessed the characteristics listed above, were stable to serum, and were considered appropriate for encapsulating AlSPc, at least for the initial experiments in vitro, though adaptations might eventually be required.

1.5.1. LIPOSOME COMPOSITION

The lipid components for the chosen liposome were in the following proportions:

Dipalmitoylphosphatidyl choline (DPPC)	66 mol %
Dipalmitoylphosphatidyl ethanolamine (DPPE)	1 mol %
Cholesterol	33 mol %

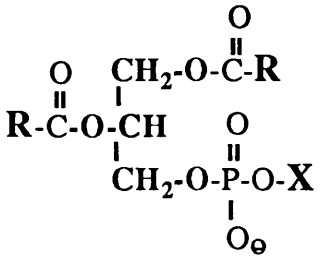
The reasons for the choice and composition of lipids are discussed below and their basic structures given in Figure 6.

The phospholipids were synthetic, with fully saturated symmetrical side chains for good packing in the bilayer. Saturated side chains are less susceptible than unsaturated chains to hydrolysis and peroxidation during storage, since there are no double bonds available for attack..

The main phospholipid, DPPC, has a fairly high transition temperature (Tc) of 42°C (Wilschut, 1982). The Tc is the

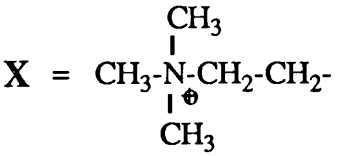
FIGURE 6
STRUCTURE OF THE LIPIDS USED IN THE LIPOSOMES

A) Phospholipid structure

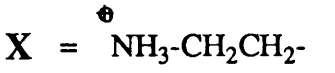


Glycerol backbone (bold)
with fatty acid chains (**R**)
and charged head group(**X**)

R = $CH_3(CH_2)_{14}$-
Palmitic acid

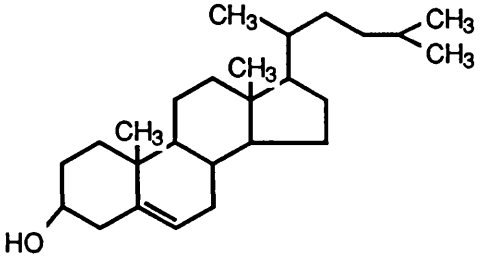


Phosphatidylcholine (PC)



Phosphatidylethanolamine (PE)

B) Cholesterol structure



temperature at which the phospholipids in the bilayer change from a 'solid' or gel state (below 42°C) to a 'liquid' or mobile state (above 42°C). Packing of the molecules is tighter in the solid phase, producing a liposome that is less likely to bind non-specifically to cells, and less permeable than one in the mobile phase. Since some of the incubations with cells were likely to be performed at 37°C, it was reasonable to choose a phospholipid with a Tc above that temperature. DPPC is also a neutral phospholipid which is less susceptible to fusion with cells than negatively charged phospholipids (Papahadjopoulos, 1973).

DPPE contains an amino group which can be reacted with a linking agent for coupling with the ligand to be attached to the liposome (described in Section 1.6). It was used in its derivatised form DPPE-DTP. The optimum proportion of DPPE-DTP in the liposomes was 1%. Levels higher than 1% did not result in more ligand coupling, but increased non-specific binding of liposomes to cells (A.G. Gray, personal communication).

Cholesterol was included in the liposomes because it reduces the permeability of the bilayer by packing between the phospholipid molecules and bringing them closer together (Papahadjopoulos, 1972). This helps prevent leaking of the liposomal contents by producing a less fluid structure and hence decreasing non specific uptake by cells of any leaked, and therefore unencapsulated compound. Ratios of cholesterol to phospholipid higher than 2:1 results in organisation of the cholesterol molecules into distinct domains which disrupts the rigid packing observed at lower ratios (Shinitzky, 1984).

Cholesterol also inhibits liposomal interactions with plasma proteins and lipoproteins (Mayhew et al, 1987; Senior and Gregoriadis, 1982).

SUVs were formed from the lipids described above by ultrasonication at a temperature above the Tc of the main lipid, DPPC. This helped to prevent structural defects in the bilayer during formation which would result in unstable and leaky liposomes (Lawaczeck et al, 1976).

1.6. COUPLING AGENT

The agent used for coupling liposomes with the protein ligand was N-hydroxy succinimidyl 3-(2-pyridyldithio)propionate (SPDP). This is a heterobifunctional linking agent which minimises homopolymerisation of either proteins or liposomes. The agent forms a stable linkage with both DPPE and protein, so the products can be prepared when convenient and stored (at $-20^{o}C$) until required.

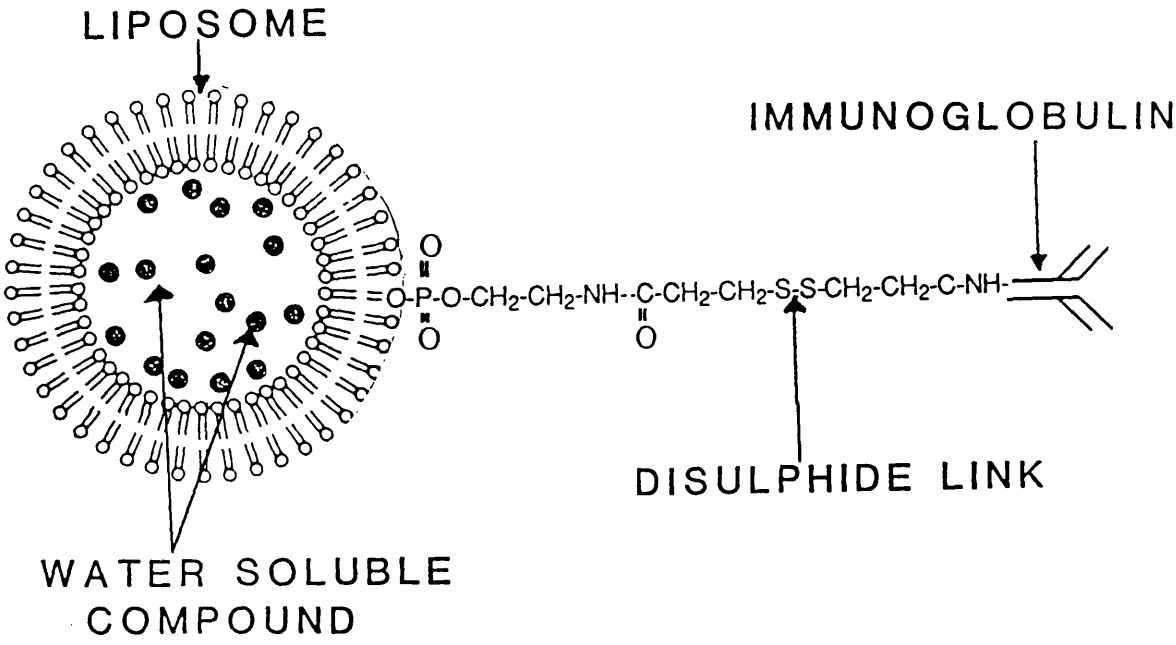
Reaction conditions are mild, thus proteins are not destroyed during the process (Carlsson, 1978), and when antibodies are used, their binding affinity is usually largely unaffected. Binding affinity may be affected if the agent links to amino groups present in the hypervariable region of the antibody, which confers its specificity, but since the agent reacts randomly at all available amino sites on an antibody this is minimal.

When the link is complete there is a spacer of six carbon

FIGURE 7

DIAGRAMMATIC REPRESENTATION OF AN ANTIBODY COUPLED LIPOSOME SHOWING THE DISULPHIDE LINK

(NOT TO SCALE)



atoms and a disulphide bond between the protein and the liposome (Figure 7). The spacer may be important in helping to prevent steric hindrance (when the protein binds to its receptor on the cell), which is more likely to occur if the protein was incorporated into the lipid bilayer, or bound directly to the lipid surface (Heath and Fraley, 1980).

The disulphide bond has been shown to be susceptible to hydrolysis in vivo and in vitro due to the presence of enzymes in serum (Martin et al, 1981). However, this has not appeared to be a problem with incubation conditions used with this liposome previously (Gray et al 1988, 1989). Should it prove to be so in the present work there are alternative linking agents such as succinimidyl(maleimidophenyl)butyrate, in which a thioether bond linking antibody and liposome is protected from enzyme action by a space occupying phenyl group, (Martin and Papahadjopoulos, 1982).

CHAPTER 2

MATERIALS AND METHODS

2.1. GENERAL MATERIALS

2.1.1. GLASSWARE

For general use, glassware was pyrex ware which was routinely washed in detergent and rinsed in distilled water.

For lipid and liposome preparation and assays, glassware was soaked in chromic acid solution for at least 2 hours, rinsed 5 times in distilled water and oven dried.

For use in protein preparations and storage, glass was silicon coated with Sigmacote to prevent any losses by protein binding to surfaces.

OTHER GLASSWARE

Microscope slides.	British Drug House.
Glass vials (for storage)	Camlab.
Pasteur pipettes.	Bilbate Ltd.
1 and 2 ml graduated glass pipettes.	Volac.

2.1.2. CHROMATOGRAPHY PLATES

Linear K silica gel, 5 x 20 cm, 250 μm thick.

PLK-5 silica gel, 20 x 20 cm , 1000 μm thick. Whatmans.

2.1.3. PLASTICWARE

These were of tissue culture grade unless otherwise indicated.

30 ml Universal containers, 50 ml polythene tubes, 5 and 10 ml pipettes	Sterilin Ltd.
Flowpore D 0,2 and 0.45 μm filter units.	Flow Labs Ltd.
Flat bottom 96 well tissue culture plates.	Linbro.

1289 polystyrene tubes with caps for fraction collecting.

LIP.

7 ml Bijou bottles, Microtest flexible assay plates, Tissue culture flasks, 15 ml polystyrene screw cap conical tubes.

Falcon.

LP3 polystyrene test tubes. Luckham Ltd.

1.8 ml Cryotubes. NUNC.

1-50 ml syringes. Sabre Int. Products Ltd.

Disposable plastic pasteur pipettes. Liquipette.

Parafilm.

2.1.4. REAGENTS

All reagents were Analar or tissue culture grade where possible.

Anhydrous sodium sulphate. sodium hydroxide, sodium chloride, sodium acetate, sodium iodide, iodine (resublimed), dimethylsulphoxide, Hepes (N-2-hydroxyethylpiperasine-N-2-ethane sulphonic acid), Ammonium thiocyanate, Ferric chloride hexahydrate. British Drug House.

Sodium azide, L-dithiothreitol, Sigmacote, Trypsin, 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester. Triton-X-100 (octylphenoxypolyethoxyethanol), Tri-ethylamine

Sigma.

EDTA (diaminoethanetetra-acetic acid disodium salt). Fisons.

5(6)-carboxyfluorescein. Eastman Kodak.

Protein A (Staph. aureus), Sephadex G-25M PD-10 columns, Sephadex G-50, Sephadex LH-20, Sepharose CL-4B and CL-6B, Trypan Blue solution (5%). Pharmacia.

Sulphonated Aluminium Phthalocyanine (AlSPc). Ciba-Geigy.

Phytohaemagglutinin (PHA). Wellcome Pharmaceuticals.

2.1.5. SOLVENTS

Methanol, Ethanol, Acetone, Acetic acid, Ammonia (35%), N-butyl alcohol, Formaldehyde, Conc. sulphuric acid. May and Baker.

Scintillator 299. Packard.

2.1.6. RADIOACTIVE COMPOUNDS

^{125}I sodium iodide (1 mCi/ml), ^{3}H leucine (1 mCi/ml), ^{3}H thymidine (1 mCi/ml)

Amersham International.

2.1.7. LIPIDS AND LIPID STORAGE

L -phosphatidylcholine dipalmitoyl (DPPC), Cholesterol. Sigma.

L -phosphatidylethanolamine dipalmitoyl (DPPE). Calbiochem.

Stock solutions of lipids were stored at -20°C in airtight glass bottles under an atmosphere of nitrogen. Cholesterol and DPPC were stored dissolved in 9:1 chloroform/methanol at 25 mg/ml. DPPE was substituted with DTP residues as described in Section 2.2.6. and stored in chloroform at the concentration obtained after its derivation.

2.1.8. MEDIA

All from Gibco.

RPMI 1640 (Roswell Park Memorial Institute) with L-glutamine and with or without 20 mM Hepes was used for washing, culturing and freezing cells.

Eagle's MEM (minimum essential medium) with Earle's salts and L-glutamine and with or without leucine was used for some cell cultures.

IMDM (Iscove's minimum Dulbecco medium) at double strength was used for bone marrow culture.

FCS (foetal calf serum), screened batches that supported growth in culture were used as a growth supplement. FCS was heat inactivated at 56°C for 1 hour, aliquoted, and stored at -70°C until used.

Agar was made up in double distilled water (deionised) at 0.9% for soft agar culture of bone marrow.

2.1.9. RECIPES FOR BUFFERS AND OTHER MADE-UP REAGENTS

PHOSPHATE BUFFERED SALINE (PBS).

This was made up from Oxoid tablets Oxoid Ltd.

One tablet was dissolved in 100 ml distilled water and the pH checked and adjusted to the required pH.

HEPES BUFFER (pH 7.4 or 8.0).

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

2.38 g of Hepes and 8.468 g of NaCl were added to 1 litre of double distilled water and the pH adjusted with 5 M sodium hydroxide (NaOH). It was stored at 4°C.

GLYCINE BUFFER (pH 2.5, 0.2 M).

25 ml 0.2 M glycine and 15 ml 0.2 M HCl were made up to 100 ml with distilled water.

ACETATE BUFFER (pH 5.0).

Solution A (0.1 M).

6.8 g of sodium acetate (NaAc) and 2.93 g of NaCl were added to 500 ml of distilled water.

Solution B (0.1 M).

2.87 ml of glacial acetic acid and 2.93 g of NaCl were added to 500 ml distilled water.

63 parts of solution A were added to 37 parts of solution B to give a final pH of 5.0. Any slight adjustments were made by adding acetic acid or NaOH.

TRIS BUFFER (pH 8.8).

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

FORMALDEHYDE FIXATIVE

Formaldehyde solution (containing 41% HCHO and 10-14% MeOH) was diluted to 1-4% in RPMI or PBS containing 4% FCS

CHROMIC ACID CLEANING SOLUTION

For each 100 mls of cleaning solution, 25 ml of concentrated sulphuric acid was added slowly to 75 ml of distilled water in an ice bath with constant stirring. 10 g of potassium dichromate was dissolved in the solution and it was stored in glass bottles. The solution was used repeatedly until the colour turned from orange to green.

2.2. GENERAL METHODS

2.2.1. CELLS AND CELL CULTURE.

CELLS

Osteosarcoma cell line 791T.

Colorectal carcinoma cell line C170.

791T and C170 were supplied by Prof R W Baldwin of CRC Nottingham DW-BCL and RC-BCL were both Epstein-Barr virus (EBV) immortalised polyclonal B cell lines derived from individuals seronegative to EBV.

CELL CULTURE

791T and C170 cells were grown in culture flasks as a monolayer in a growth medium comprising Eagles minimum essential medium (EMEM), (Gibco) supplemented with 10% (v/v) foetal calf serum (FCS), and subdivided at confluence after treatment with 0.25% trypsin (Sigma) and 0.5% EDTA (Fisons) in EMEM to detach cells from the flask, and two washes.

DW-BCL and RC-BCL cells were seeded at 1 x 10^5 cells/ml in RPMI medium supplemented with 10% FCS and subdivided once a week.

FREEZING AND THAWING CELLS

Cells were suspended at 2-5 x 10^6/ml in 10% dimethylsulphoxide and 20% FCS in RPMI. Aliquots of 1 ml were frozen overnight in cryotubes at -70^{o}C and then transferred to liquid nitrogen for storage.

Frozen cells were thawed rapidly at 37°C in a water bath and diluted by dropwise addition of ice-cold RPMI to 20 ml. They were then washed twice more in cold medium before culturing.

2.2.2. ANTIBODIES

791T/36 (subclass IgG 2b), was raised against an epitope expressed on the 791T cell line. (Supplied by Prof Baldwin, CRC Nottingham). Both cell line and antibody are well characterised and documented (Garnett et al 1983; Pimm and Baldwin, 1984; Perkins et al 1985; Roe et al 1985). 791T/36 also binds with the colorectal carcinoma cell line C170 and other cells (Embleton et al 1981).

UCHT 1 (IgG subclasses 1, 2a and 2b). These are all CD3 cluster antibodies and bind to an epitope expressed on most peripheral T lymphocytes (Beverley and Callard, 1981). (Supplied by Professor P Beverley, Human Tumour Immunology Group, ICRF, UCMSM, London).

8A (IgG subclass 1). Anti B cell antigen expressed on B cells from the pre-B stage of ontogeny through to plasma cells (Tazzari et al, 1987), and also expressed on other cells including some T lymphocytes (personal observations), (Supplied by M Gobbi and A Bontadini, University of Bologna, Italy).

2.2.3. COLUMN PREPARATION AND STORAGE

The following materials were degassed under vacuum, and packed into 1 x 25 cm glass columns containing a plug of glass wool:

a) Sephadex G50 granules swollen in distilled water (for

separating liposomes from unencapsulated material);

b) Sephadex LH-20 powder, swollen in distilled water (for removing lipophilic contaminants from solutions);

c) Sepharose 4B or 6B (for separating liposome coupled and free protein).

The prepared columns were equilibrated with Hepes buffer, at ph 8 for the Sepharose, pH 7.4 for the Sephadex and distilled water for the LH-20 as required for their respective separations.

Ready prepared Sephadex PD10 columns for protein substitutions were similarly equilibrated with the correct buffer, acetate buffer at pH 5 for Ig and Hepes buffer at pH 7.4 for Protein A.

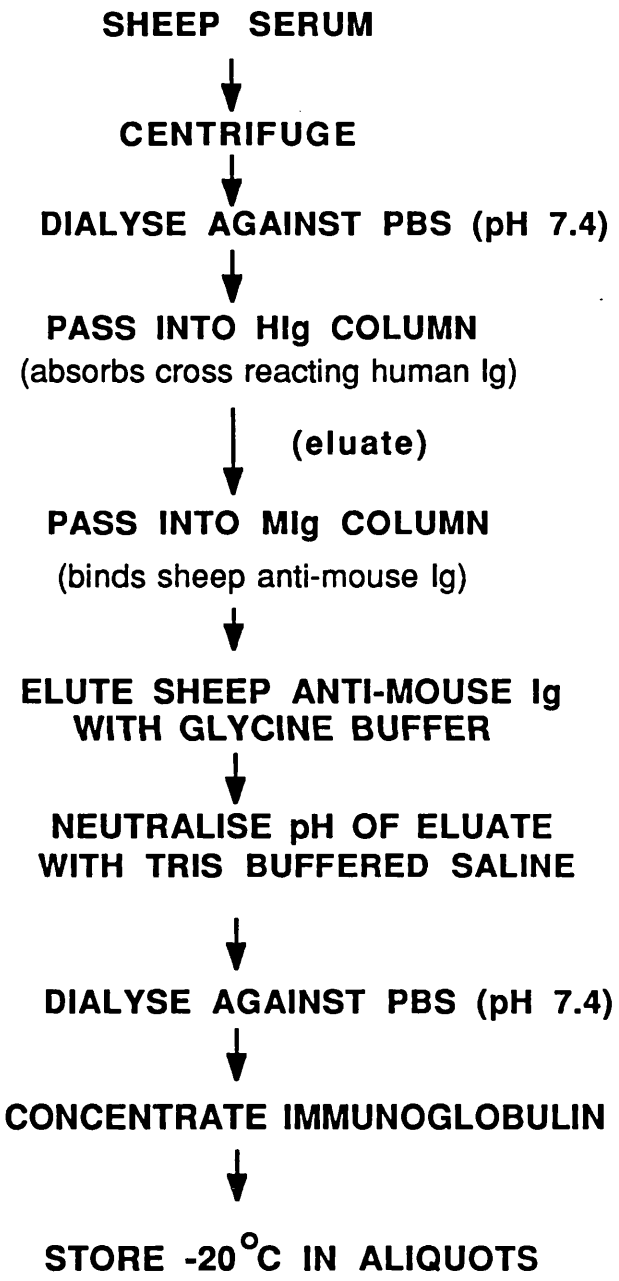
Before the first use, liposomes or protein were passed down the columns to saturate non-specific binding sites on the resins. Between uses, the columns were stored in buffers containing 10 mM sodium azide at $4^{o}C$, which was eluted before use. LH-20 columns were discarded after each use because the bound lipophilic compounds could not be easily removed, but the other columns were used repeatedly. Separate columns were used for each protein and each dye.

2.2.4. PURIFICATION OF POLYCLONAL SHEEP ANTI-MOUSE IMMUNOGLOBULIN FROM SHEEP SERUM

A pure preparation of polyclonal sheep anti-mouse immunoglobulin (S-MIg), without cross-reacting contaminants to human cells was required for binding to liposomes to eliminate non-specific binding when incubating with human cells. This was

FIGURE 8

PURIFICATION OF SHEEP ANTI-MOUSE IMMUNOGLOBULIN



prepared as shown on the flow diagram, Figure 8. All the purification steps were performed at 4°C.

Sheep serum containing antibodies raised against mouse immunoglobulin (a gift from Prof. Peter Beverley, Human tumour Immunology Group, ICRF, UCMSM, London) was centrifuged at 700g for 20 minutes to remove aggregates and debris and dialysed overnight against PBS at pH 7.4. 50 ml of the dialysed serum was circulated overnight through a HIg column (human immunoglobulin coupled to Sepharose, Serotec) equilibrated with PBS, to remove any antibodies crossreacting with human immunoglobulin. The eluate was passed down a MIg (mouse immunoglobulin coupled to Sepharose, Sigma) column in the same way. Any antibodies to mouse Ig bound to the column, whilst other proteins passed through. The column was washed with PBS, and the eluate monitored with U.V. light until no more protein was detected. The eluate was discarded, the bound S-MIg was eluted with glycine buffer at pH 2.5, and the protein containing fractions were pooled and titrated to neutral pH with Tris buffer at pH 8.8. The S-MIg was dialysed for 24 hours with several changes of PBS.

Protein concentration was determined by the absorbance (A) measured at 280 nm and according to the formula:

$$A/1.4 = \text{protein concentration in mg/ml.}$$

(See Appendix 1 for derivation).

The protein was adjusted to 5 mg/ml by concentrating in an Amicon apparatus under N_2 pressure, and stored in aliquots at -20°C until required for coupling to liposomes.

2.2.5. PURIFICATION OF CARBOXYFLUORESCEIN (CF)

Liposomes containing carboxyfluorescein and coupled to S MIg were used as a sensitive fluorescent probe for cell surface antigens as described by Gray et al, 1989. Carboxyfluorescein obtained from Eastman Kodak contained lipophilic impurities which intercalated with the lipid bilayer producing leaky liposomes when encapsulated. To ensure production of stable liposomes, carboxyfluorescein was purified before use, according to the method of Ralston, 1981.

About 5 g of carboxyfluorescein was boiled in 100 ml of ethanol with 5 g of activated charcoal. Some of the impurities became bound to the charcoal. The solution was filtered through Whatman No. 1 filter paper to remove the charcoal, cooled on ice and the carboxyfluorescein precipitated by slowly adding cold distilled water. This step removed most of the polar impurities. The precipitate was washed in a Buckner funnel with more cold water and dissolved in 6 M sodium hydroxide, adding sufficient to give a final pH of 7.4. This solution, the sodium salt of carboxyfluorescein, was passed down a column (1 x 25 cm) of Sephadex LH-20, a preparation which binds lipophilic compounds, and eluted at room temperature with distilled water. The first peak which was brown and non-fluorescent was discarded. The main, orange band was the purified carboxyfluorescein and was collected. Most of the lipophilic contaminants remained on the top of the column or trailed behind the main peak. The carboxyfluorescein was freeze dried and stored at room temperature in the dark in an airtight brown glass jar.

2.2.6. DERIVATION OF DPPE-DTP

In order to couple proteins covalently to liposomes, an anchor in the lipid bilayer was needed. Dipalmitoyl phosphatidylethanolamine (DPPE) contains a free amino group in the polar head region which is suitable for reacting with linking agents to produce a derivative which can react with appropriately modified proteins. The agent used was N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP) which introduces a disulphide bond. When the derived phospholipid is incorporated into liposomes, the disulphide bond in those molecules orientated towards the external surface of the bilayer are available for further reactions. Materials were freshly prepared for each batch, and reacted by the method of Barbet et al, 1982.

The following ingredients were added to a glass test-tube and vortex mixed gently for 2 hours at room temperature.

10 μmoles of DPPE dissolved in 700 μl of a chloroform/methanol mixture (9:1).

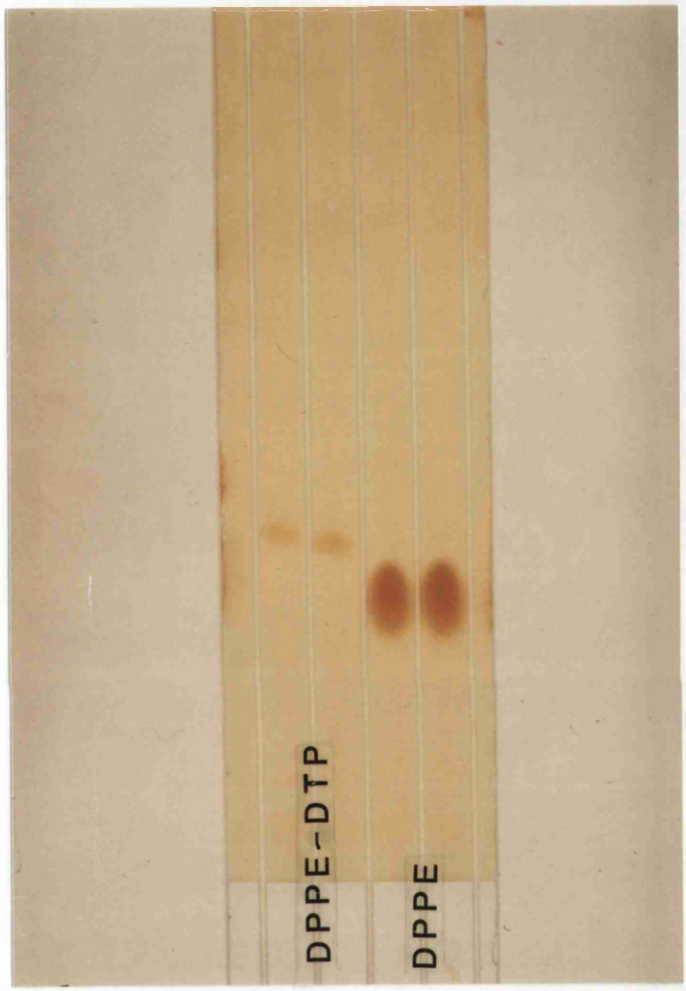
12 μmoles SPDP (300 μl of a 40 mM stock in methanol).

20 μmoles triethylamine (500 μl of a stock of 30 μl triethylamine in 0.5 ml chloroform.

The mixture was washed to remove water-soluble products and unreacted reagents, once with 2 ml PBS and twice with 2 ml distilled water, discarding the aqueous layer each time. Emulsions formed during washing were broken by centrifuging at 700 g for 5 minutes. Finally, drops of methanol were added until the organic phase was clear. Solvent was evaporated under a stream of N_2 and final traces removed by freeze drying for at

FIGURE 9

THIN LAYER CHROMATOGRAPHY OF DERIVATISED PHOSPHOLIPID



The TLC plate shows complete conversion of dipalmitoyl phosphatidylethanolamine (DPPE) to the dithiopyridyl derivative (DPPE-DTP) by the heterobifuntional linking agent SPDP.

DPPE-DTP runs faster in the solvent system used (see text):

Rf values are:

DPPE = 0.36

DPPE-DTP = 0.41

least an hour. The lipid residue was dissolved in 10 ml chloroform and the amount of lipid present assayed as described in Section 2.2.7.

Thin layer chromatography was performed to assess the purity of the derived product and confirm that the reaction was complete. The derived DPPE-DTP was run alongside a DPPE standard on a 5 x 20 cm Whatman linear K silica thin layer chromatography (TLC) plate in a solvent system composed of chloroform/methanol/distilled water in the ratios 14:5:0.7. The lipid spots were stained with iodine vapour and showed that DPPE (Rf value = 0.36) had been completely converted to the derivative DPPE-DTP (Rf value = 0.41), which runs a little further from the origin as shown in Figure 9. The chemical reactions are shown in Figure 14.

2.2.7. PHOSPHOLIPID ASSAY

Phospholipid assays to determine the concentration of liposome and derived DPPE-DTP samples were performed according to the colorimetric method of Stewart (1980). The assay is rapid and sensitive and based on the formation of a 1:1 coloured complex between ammonium ferrothiocyanate and phospholipids such as DPPC. The chromogen obeys Beer's Law up to an absorption of about 0.8, and the concentration of phospholipid in the samples to be assayed were adjusted to fall within this absorption range. The slope differs for some types of phospholipids eg. DPPE, because the complex is 2:1. For assaying single lipids therefore,

separate standard curves must be prepared for the greatest accuracy.

a) Ammonium ferrothiocyanate solution was prepared by adding 27.03 g of ferric chloride hexahydrate and 30.4 g of ammonium thiocyanate to deionised distilled water and making up to 1 litre.

b) Stock standards of DPPC or DPPE at 0.1 mg/ml in chloroform were added in duplicate volumes of 0.1 to 1.0 ml to glass test tubes, and each made up to 2 ml with chloroform. Blanks contained chloroform only.

c) Test samples were treated in the same way using volumes according to the expected phospholipid content, usually 0.1 or 0.05 ml for both liposome and synthesised DPPE-DTP samples.

d) 2 ml of ammonium ferrothiocyanate solution was added to each tube and they were covered with parafilm.

e) The samples were each vortex mixed vigorously for one minute, and the phases separated by centrifuging for 10 minutes at 750g.

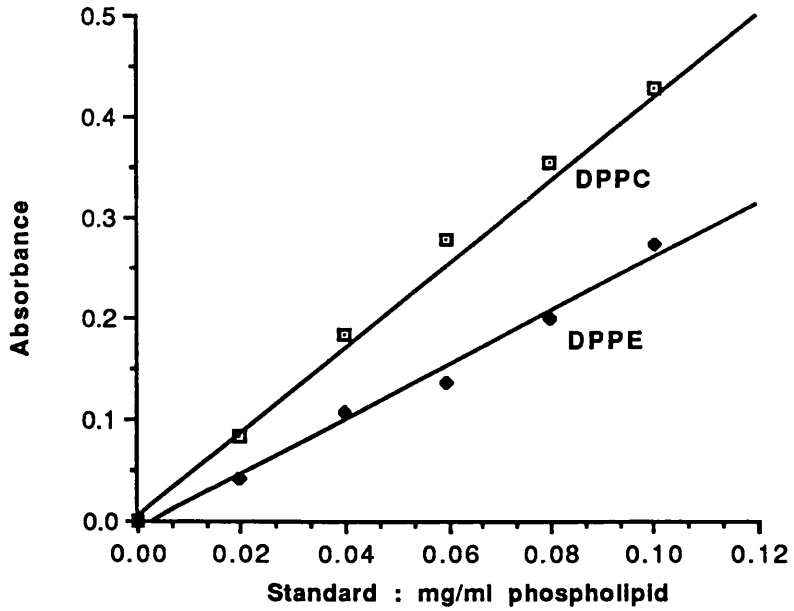
f) The lower organic phase was removed with a glass Pasteur pipette, a small pinch of anhydrous sodium sulphate added, and the absorbance at 488 nm measured.

g) Mean absorbance was plotted against phospholipid concentration, and the samples read from the standard curve. Typical standard curves showing the different slopes are shown for both DPPC and DPPE in Figure 10.

For liposome samples, since DPPC is the major phospholipid, their concentrations were determined by the DPPC curve. There

FIGURE 10

PHOSPHOLIPID STANDARD CURVES



LEGEND.

Phospholipid standard curves were constructed from a series of concentrations of DPPE and DPPC which were reacted with ammonium ferrothiocyanate to produce a chromogen. The absorption of the chromogen was read at 488 nm and plotted against the phospholipid concentration. Phospholipid concentrations of samples were read directly directly from these curves.

would be a small contribution to the absorbance from the DPPE-DTP (about 1% of the total phospholipid), but this was considered negligible.

2.2.8. PURIFICATION OF AlSPc

A) PURIFICTION OF AlSPC BY SEPHADEX LH-20

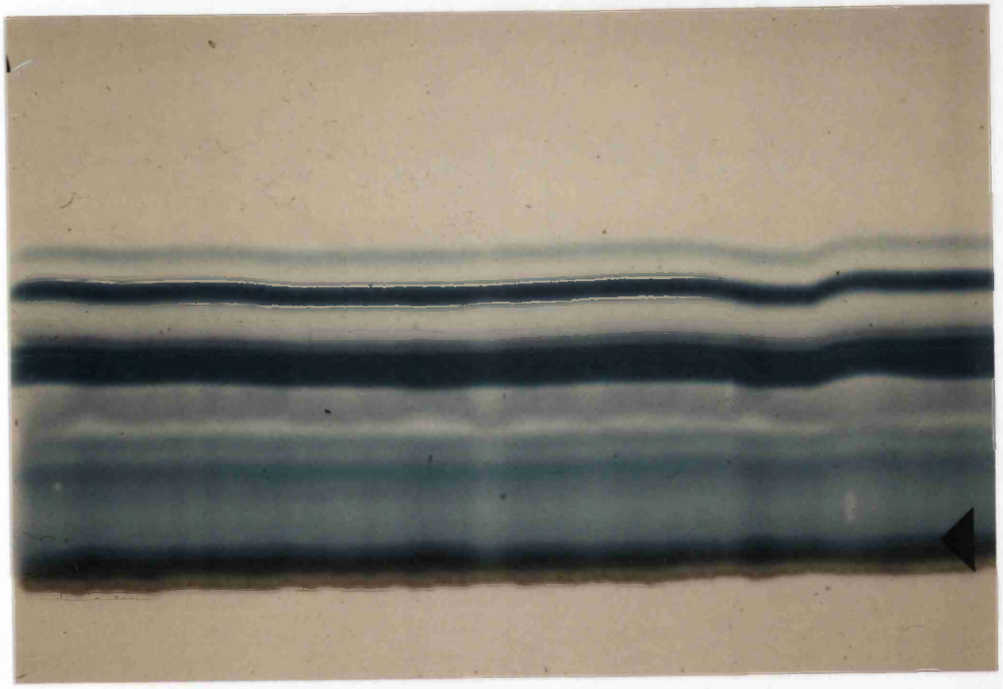
AlSPc powder (Ciba-Geigy) was dissolved in distilled water and passed into a column (1 x 25 cm) containing Sephadex LH-20 equilibrated with distilled water. AlSPc was eluted with distilled water, freeze dried to a powder and used to prepare liposomes. Many of the lipophilic components remained on the column.

B) PURIFICATION OF AlSPc BY TLC

AlSPc powder, dissolved in distilled water was streaked onto preparative TLC plates (20 x 20 cm 1000 μm Whatman PLK-5 silica gel plates) which were developed in a mixture of acetone, water, n-butanol and 5% ammonium hydroxide in the ratios 13:15:58:13. AlSPc as obtained from Ciba-Geigy was found to yield many fractions by TLC as shown in Figure 11, their positions depending on their degree of solubility. This was due to the presence of a mixture of a number of isomers of the mono-, di-, tri- and tetra-sulphonated forms. The most polar fraction, nearest the origin (Rf value = 0.13), arrowed in the TLC plate shown in Figure 11, was scraped off and eluted from the silica gel by shaking repeatedly with distilled water. The eluate was filtered and freeze dried. The purified sample was rechromatographed in the

FIGURE 11

THIN LAYER CHROMATOGRAPHY SEPARATION OF SULPHONATED ALUMINIUM PHTHALOCYANINE



LEGEND.

Separation of the differently sulphonated fractions of a solution of Ciba-Geigy AlSPc streaked onto preparative TLC plates was done by developing in a solvent system of acetone, water, n-butanol and 5% ammonium hydroxide in the ratio: (13:15:58:13).

The most polar fraction, closest to the origin (Rf value = 0.13), arrowed above, was scraped off the plate, eluted and used in experiments.

same system and yielded one spot.

High pressure liquid chromatography (HPLC) of the TLC purified fraction (by the method of Ali et al, 1988), showed the presence of several peaks and suggested that it was not a single compound but composed mainly of tetra- and tri-sulphonated AlPc, with a small amount of di-sulphonate contamination, (Figure 12). The resolution of the TLC was not sufficient to completely separate the tetra- and tri-sulphonated phthalocyanines, but the partial purification by TLC gave a more uniform and polar compound, which was more suitable for encapsulating in the aqueous compartment of liposomes than the original mixture.

Some HPLC purified fractions were used in several experiments (supplied by R. Svenson of the Royal Institution, London).

2.2.9. CYTOSPIN PREPARATION

Cytospins are made by centrifuging cell suspensions so that cells are thrown onto slides under centrifugal force (Chanarin, 1989). By adjusting the cell number in the suspension, suitable numbers can be adhered to slides without overcrowding.

Single cell suspensions were prepared at 1 x 10^6 cells/ml, stained appropriately and fixed in formaldehyde. One drop of cell suspension was added to each prepared funnel with labelled slide and filter attached, followed by a drop of PBS containing 5% FCS. The cytocentrifuge (Cytospin 2, Shannon) was spun at 700 rpm for

FIGURE 12

HIGH PRESSURE LIQUID CHROMATOGRAPHY OF UNPURIFIED AND TLC PURIFIED SULPHONATED ALUMINIUM PHTHALOCYANINE:

LEGEND

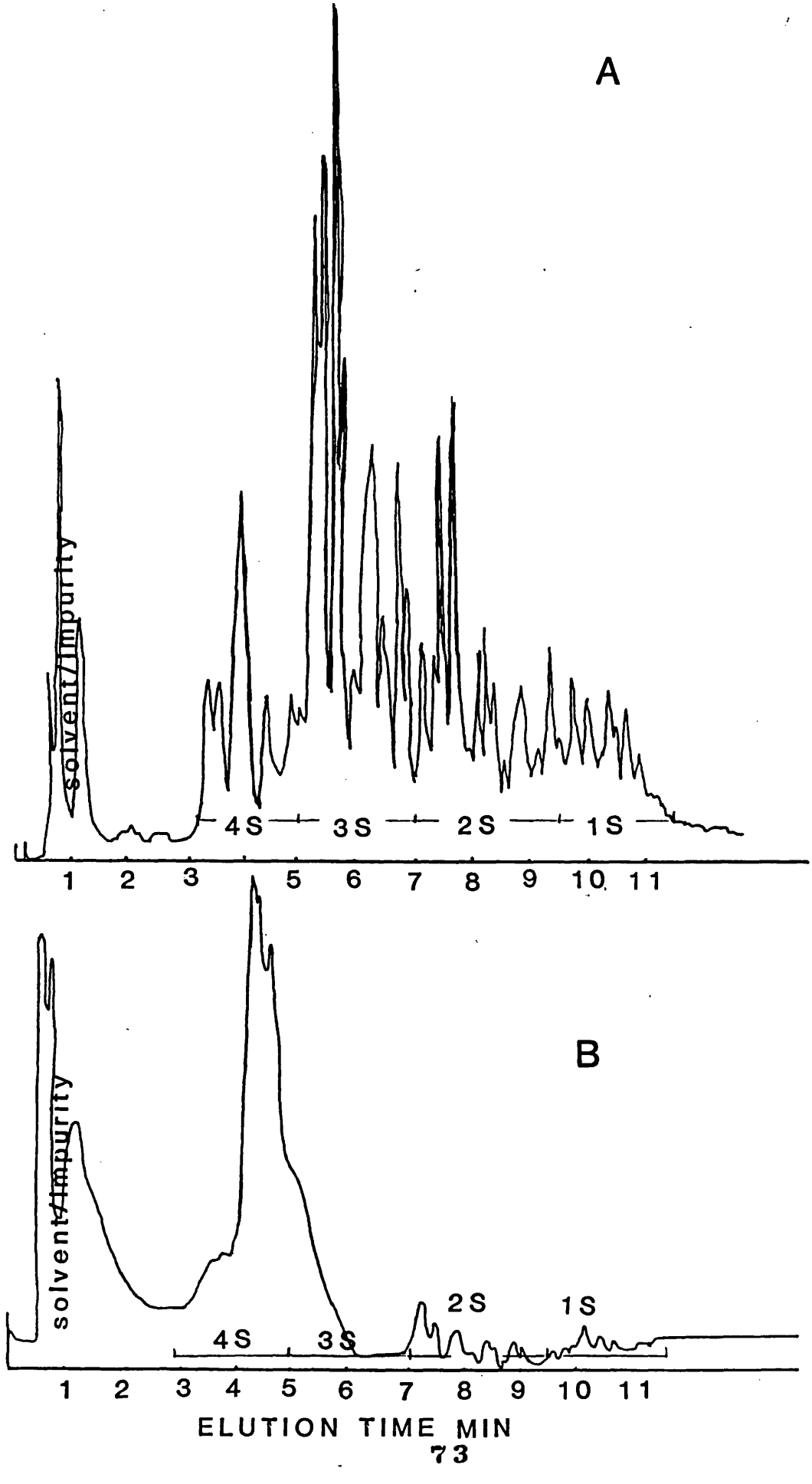
A) Ciba-Geigy mixture - unpurified

B) TLC purified fraction (Rf = 0.13)

The numbers denote the number of sulphonate groups per molecule:

1S = mono-sulphonate
2S = di-sulphonate
3S = tri-sulphonate
4S = tetra-sulphonate

FIGURE 12


A
solvent/impurity
4S
3S
2S
1S
1 2 3 5 6 7 8 9 10 11
B
solvent/impurity
4S
3S
2S
1S
1 2 3 4 5 6 7 8 9 10 11
ELUTION TIME MIN

10 minutes. Slides were removed, air dried and stored at 4°C in the dark until examined.

2.2.10. PREPARATION OF PROTEIN COUPLED LIPOSOMES

The method for preparing and coupling proteins to liposomes is summarised as a flow chart in Figure 13. The reactions occurring in the substituting, reducing and coupling reactions for both protein and lipid are shown in Figure 14.

FORMATION OF SMALL UNILAMELLAR VESICLES

Small unilamellar liposomes (or vesicles) (SUV) were prepared by probe ultrasonication according to the method of Barbet et al (1981).

SUV were composed of 66 mol % dipalmitoylphosphatidylcholine (DPPC), 33 mol% cholesterol (both from Sigma), and 1 mol% dipalmitoylphosphatidylethanolamine3-(2-pyridyldithio)propionate (DPPE-DTP). DPPE-DTP was synthesised by modification of DPPE (Calbiochem) with the heterobifunctional linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP), (Pharmacia). (Barbet et al, 1982), as described in Section 2.2.6. 40 μmol of total lipid was used for each 3 ml of aqueous medium.

a) The lipid, dissolved in chloroform, was placed in a glass test tube, and the solvent evaporated in a rotary evaporator. Final traces of solvent were removed by freeze drying for at least an hour.

b) Hepes-buffer at pH 7.4 (10 mM Hepes, 145 mM NaCl) was added to

FIGURE 13

SUMMARY OF METHOD FOR PREPARING

PROTEIN COUPLED LIPOSOMES

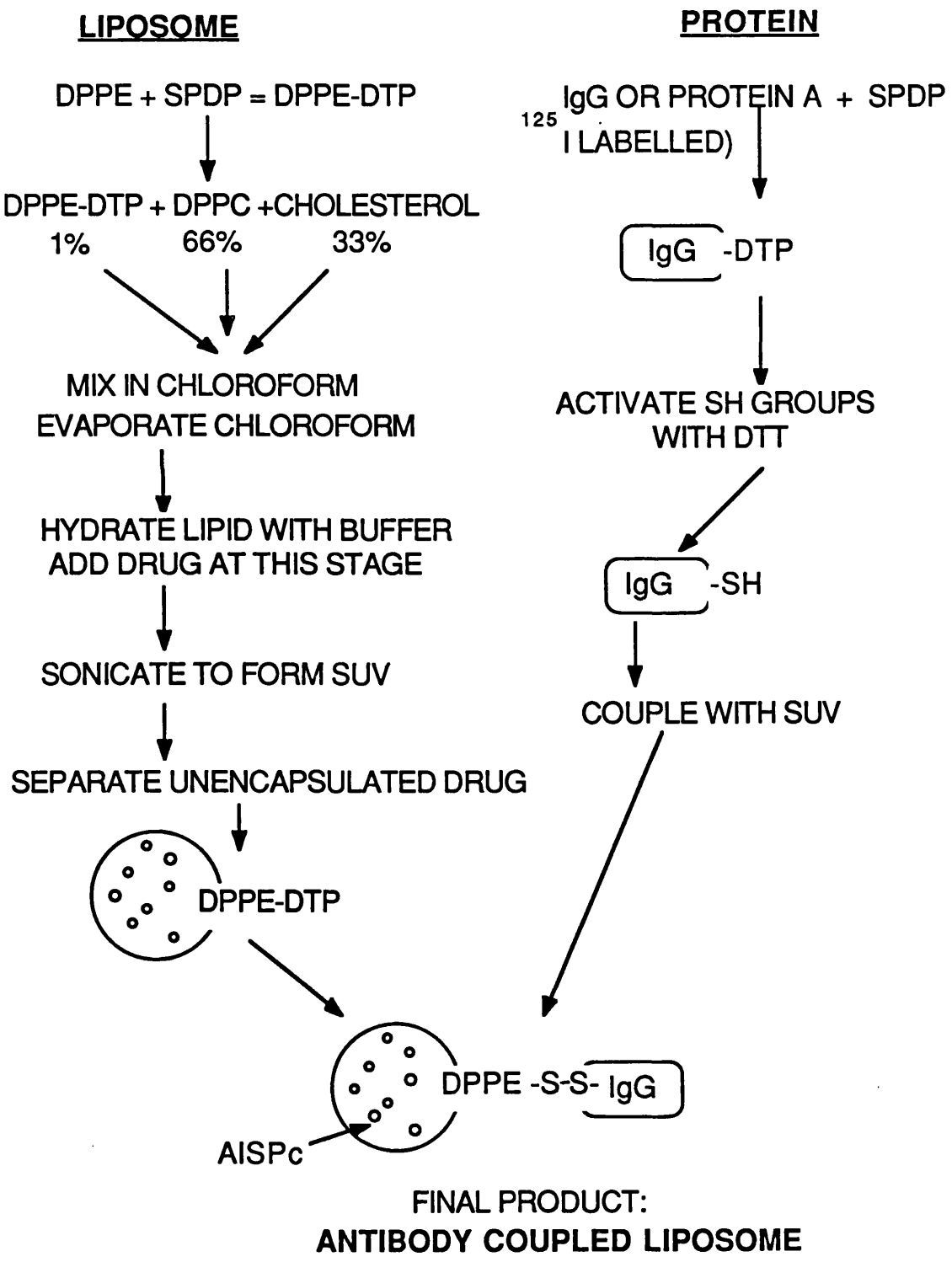
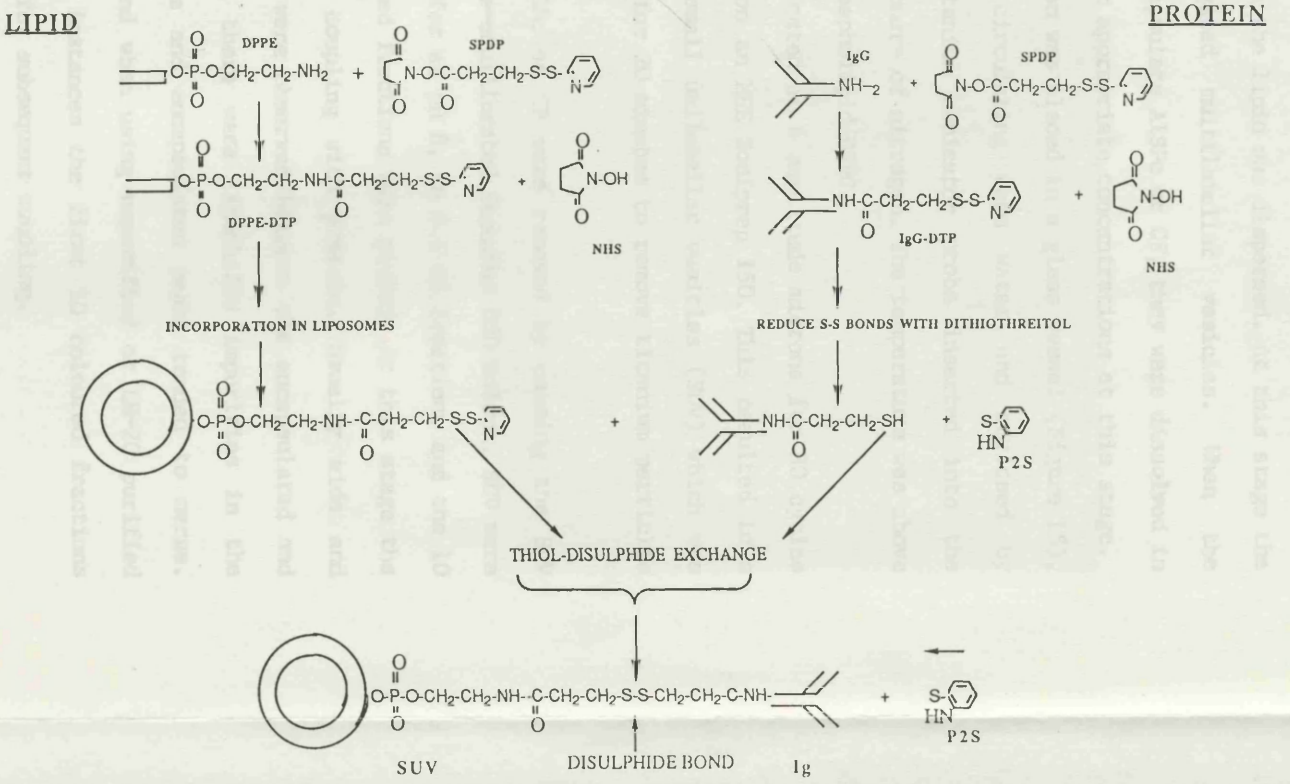


FIGURE 14 COUPLING REACTIONS OF LIPID AND PROTEIN



KEY TO FIGURE 14

DPPE - Dipalmitoyl phosphatidyl choline
SPDP - N- succinimidyl 3-(2-pyridyldithio)propionate
DTP - Di-thiopyridyl
NHS - N-hydroxy succinimide
P2S - Pyridyl -2 sulphide
SUV - Small unilamellar vesicles

the lipid film, incubated for a few minutes at 47°C and vortex mixed vigorously until the lipid was dispersed. At this stage the lipid formed aggregated multilamellar vesicles. When the liposomes were to encapsulate AlSPc or CF, they were dissolved in the buffer solution at appropriate concentrations at this stage.

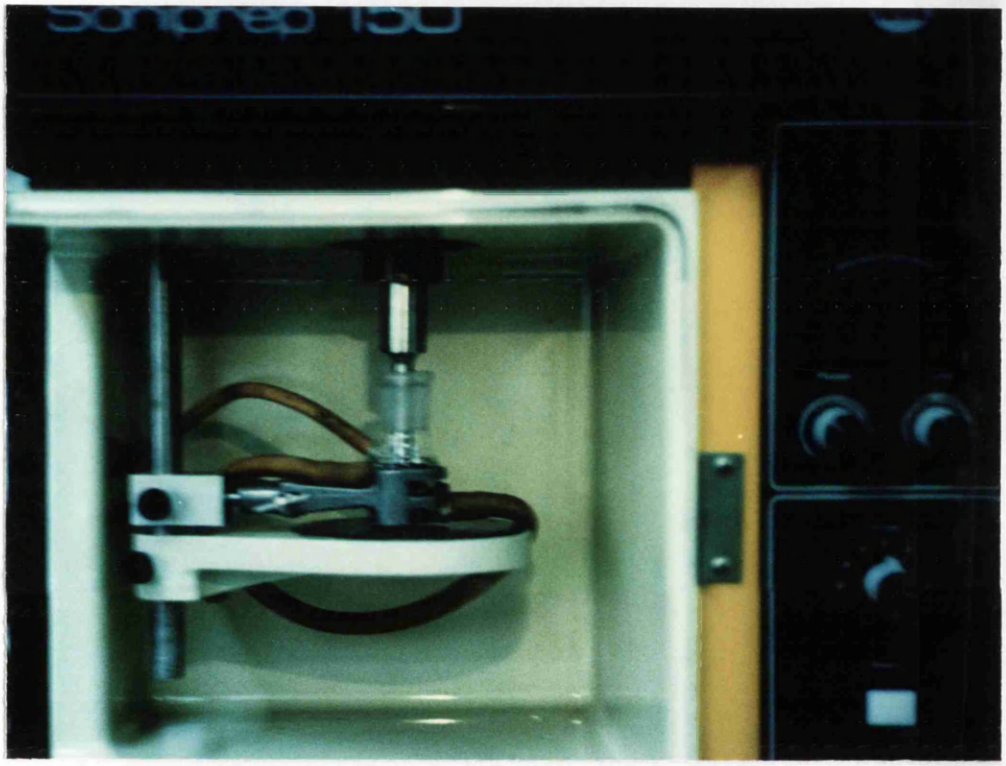
c) The lipid dispersion was placed in a glass vessel (Figure 15), heated to 47°C by circulating warm water and enclosed by parafilm, with a titanium sonicator probe inserted into the liquid under an atmosphere of nitrogen. The temperature was above the Tc of the main phospholipid DPPC.

d) The lipid was sonicated at 6 amplitude microns for 80 cycles of 30s on, 10s off, on an MSE Soniprep 150. This resulted in a clear suspension of small unilamellar vesicles (SUV) which was centrifuged at 1000g for 20 minutes to remove titanium particles and lipid aggregates.

e) Unencapsulated AlSPc or CF were removed by passing the SUV preparation down a pre-equilibrated Sephadex G50 column. SUV were eluted with Hepes-buffer at pH 8, in 0.5 ml fractions and the 10 most intensely coloured fractions were pooled. At this stage the SUV were ready for coupling with protein. Usually wide and distinct separations were observed between the encapsulated and free dyes, but when there were lipophilic impurities in the preparations the free and encapsulated peaks tended to merge. This was usually found when using unpurified or LH-20 purified AlSPc, and in these instances the first 10 coloured fractions were pooled and used for subsequent coupling.

FIGURE 15

PHOTOGRAPH OF SONICATOR SHOWING TITANIUM PROBE, GLASS VESSEL AND WATER CIRCULATING SYSTEM



PREPARATION OF PROTEIN

Iodination of protein

All proteins used for coupling with liposomes contained a trace of iodinated protein to follow the reactions and to allow the calculations of the degree of substitution in the protein molecules and the amount of protein coupled to liposomes. Iodination was performed by the Iodogen method (Pierce) with ^{125}I sodium iodide (Sigma) at 1 mCi/ml. For each preparation 500 μCi of ^{125}I were used per 200 μg of protein.

Modification of protein

Protein (containing a trace of ^{125}I labelled protein) was modified by SPDP at 10 times molar excess of protein (Barbet et al 1981), to introduce dithiopyridine (DTP) groups. The SPDP was freshly prepared as a 40 mM stock in methanol and a suitable volume added to the protein for the required molar excess. After incubating for 30 minutes (the optimum time) the protein-DTP was transferred to an acetate buffer (NaAc 0.1 M, NaCl 0.1 M) at pH 5 to protect native disulphide bonds by passing through a PD 10 column and collecting fractions. This also separated unreacted SPDP and unwanted reaction byproducts. The fractions were counted in a gamma counter and the 3 fractions with the highest counts were pooled. At this stage the derivatised protein could be safely stored at -20^{o}C if not immediately required for coupling.

The pooled fractions were reduced with dithiothreitol (DTT), (Sigma) at a final 50 mM concentration for 20 minutes. DTT was freshly prepared as a 500 mM stock in acetate buffer. The number of pyridyl disulphide residues introduced per molecule of

protein was 3-13 (depending on the protein) as calculated by the method of Carlsson et al (1978). The calculations are described in Appendix 2 and were checked by calculations based on the radioactive trace. After DTT reduction the modified antibody was transferred to Hepes-buffer at pH 8, by again passing down a pre-equilibrated PD 10 column. The fractions were counted, the 3 peak counts pooled and immediately mixed with the prepared liposomes. The mixture was incubated at room temperature for at least 20 hours, but up to 48 hours, for the coupling reaction to complete.

Conditions were modified slightly depending on the protein coupled. 791T/36 was coupled as described. S-MIg was modified by 15 times molar excess of SPDP. PA was reduced by DTT in Hepes buffer at pH 7.4 and the number of substitutions calculated by radioactive trace only since its concentration can not be calculated by absorption at 280 nm. Optimal protein concentrations were found to be 3 mg/ml for S-MIg, 1.5 mg/ml for 791T/36, and 2 mg/ml for PA.

Uncoupled protein was separated from protein bearing liposomes by passing down a Sepharose 4B column and eluting with Hepes-buffer at pH 7.4. Up to 85% of protein was eluted with the liposome fraction as measured by the ^{125}I trace. The liposomes were sterilised by filtering through a 0.45 or 0.2 μm Flowpore D unit and stored at 4^{o}C in the dark until used.

2.2.11. CHARACTERISATION OF LIPOSOMES

Measurements of phospholipid, protein and AlSPc concentrations and liposome size were made to determine whether the preparations were reproducible, stable and homogeneous.

For sheep anti-mouse coupled liposomes, the protein concentration measured on 9 preparations by radioactive trace was $1.3+/-0.02 \times 10^{-6}$ M (mean +/- S.E.M.) with a coefficient of variation of 24%.

The lipid concentration of DPPC, measured on 7 preparations was $3.4+/-0.18 \times 10^{-3}$ M (mean +/- S.E.M.) with a coefficient of variation of 13%.

The lipid to protein ratio was 2,615, which approximates to the number of phospholipid molecules in a liposome of this size (below), (Wilschit, 1982). Therefore on average each liposome has one immunoglobulin molecule attached.

LIPOSOME SIZE

Electron micrographs of liposomes were made and the diameters measured. In these preparations liposomes had an average diameter of 50nm. A typical example of the micrographs is shown in Figure 16.

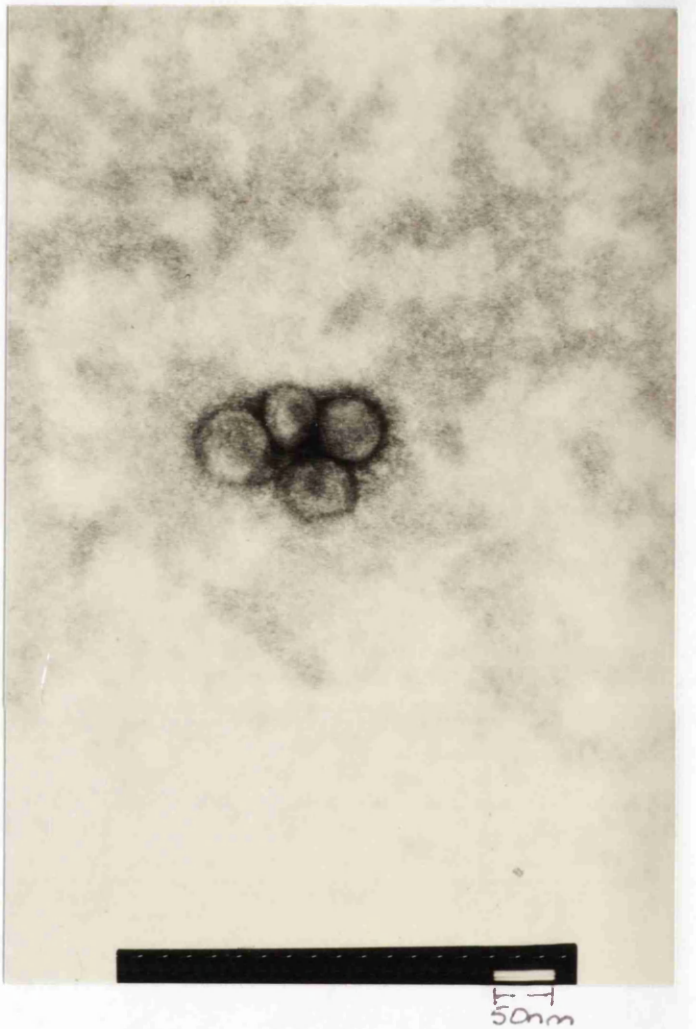
NUMBER OF AlSPc MOLECULES PER LIPOSOME

The number of AlSPc molecules per liposome can be calculated by 2 methods.

a) A calculation based on the volume of a sphere and the concentration of AlSPc used. Allowing for a bilayer width of 4 nm

FIGURE 16

ELECTRON MICROGRAPH OF LIPOSOMES



LEGEND

Typical e.m. of small unilamellar vesicles (SUV) prepared by ultrasonication.

Magnification = x 200,000

Bar = 50 nm

Liposomes were prepared by negative staining with 1% uranyl acetate on carbon coated grids. The electron microscope was a Jeol Model, JEM 1200 Ex. The e.m. were prepared by Graham Mcphail UCMSM.

and estimating 2680 phospholipid molecules per liposome of this size (Wilschut, 1982) after adjusting for cholesterol content, the approximate number of AlSPc molecules per liposome was calculated. For an encapsulated AlSPc concentration of 2.5 mM, liposomes contained an average number of 58 molecules of AlSPc. The calculations are shown below.

b) Measurements of AlSPc concentrations by spectrophotometry, (for AlSPc at 675 nm, $\epsilon = 1.6 \times 10^5$) and phospholipid concentrations, by a colorimetric assay (Section 2.2.7), of prepared liposomes gave a value of 53+-9(SEM) on three different liposome preparations (Table 1)

The average number of molecules per liposome at different concentrations of AlSPc was also measured and calculated, and summarised in Table 1. Both methods are in close agreement but values are slightly higher for the measured figures. This suggests that the AlSPc was mainly in the aqueous phase of the liposome interior, but with some extra also contained in the lipid bilayer, which accounts for the higher than expected measured values.

Theoretical calculation of the number of molecules encapsulated in an average liposome.

A liposome can be looked upon as a sphere containing an encapsulated compound. From the electron micrograph in Figure 16 it can be seen that the external diameter of a liposome (prepared by the present method) is about 50 nm. The width of the bilayer

is 4 nm (Wilschut, 1982) and therefore the internal radius of a liposome is 21 nm.

The internal volume (V) of a liposome assuming it is a sphere is:

$$V = 4/3 \times \pi \times r^3$$

$$V = 4/3 \times 3.142 \times (21)^3 \text{ nm}^3$$

Since 1 $nm^3 = (1 \times 10^{-9})^3 \text{ m}^3$

$$V = 3.88 \times 10^4 \times 10^{-27} \text{ m}^3$$

$$V = 3.88 \times 10^{-23} \text{ m3}$$

If the concentration of aluminium phthalocyanine encapsulated in a liposome is 2.5 mM then the number of molecules in its calculated volume may be determined as follows:

One mole of AlSPc contains Avogadro's number of molecules (6.02×10^{23}).

Therefore, 2.5 mM AlSPc contains:

$6.02 \times 10^{23} \times 2.5 \times 10^{-3}$ molecules per litre

$= 1.505 \times 10^{21}$ molecules per litre

Since 1 m^3 contains 1×10^3 litres

$= 1.505 \times 10^{21} \times 10^3$ molecules per m^3

$= 1.505 \times 10^{24}$ molecules per m^3

Therefore, a liposome (Vm^3) contains:

$1.505 \times 10^{24} \times 3.88 \times 10^{-23}$ molecules

$= 58$ **molecules**

TABLE 1

NO. OF MOLECULES OF AlSPc PER SUV AT DIFFERENT ENCAPSULATION CONCENTRATIONS: MEASURED AND CALCULATED VALUES.

Encaps. Conc. (mM)	DPPC Molar	AlSPc Molar	DPPC/AlSPc	AlSPc Mols/SUV	Calc. No. AlSPc Mols/SUV
0.05	2.5×10^{-3}	1.3×10^{-6}	1923	1.4	1.2
0.1	3.5×10^{-3}	5.1×10^{-6}	686	3.9	2.4
0.2	3.5×10^{-3}	9.9×10^{-6}	354	7.6	4.7
0.5	3.3×10^{-3}	2.1×10^{-5}	157	17	11.6
1.0	3.4×10^{-3}	3.6×10^{-5}	94	28	23
2.0	3.4×10^{-3}	6.2×10^{-5}	55	49	47
2.5				53 +/- 5	58

For the 2.5 mM encapsulation concentration the mean and standard error of the mean for 6 preparations is given for the measured values of AlSPc per SUV.

ASSESSMENT OF LIPOSOME STABILITY AFTER STORAGE

Liposomes containing 2.5 mM AlSPc (SUV(AlSPc)-S∝M) were stored for 4 months in the dark at 4°C, the usual storage conditions for all the liposome preparations. After this time the amount of free AlSPc released from the liposomes was determined, as a measure of their stability.

An aliquot (1.5 ml) of the stored SUV(AlSPc)-S∝M was passed into a Sephadex PD10 column and eluted with Hepes buffer (pH 7.4). Fractions of 1.5 ml were collected and their absorbance measured in a spectrophotometer at a wavelength of 675 nm, against a buffer blank. The absorbances are given in Table 2.

The total absorbance of the SUV(AlSPc)-S∝M which were eluted in the first 3 fractions, and the free AlSPc eluted in fractions 5 to 26, was calculated and the amount of free AlSPc as a proportion of total AlSPc determined as 24% in Table 2.

The SUV(AlSPc)-S∝M, after 4 months storage were equally phototoxic to cells as when they were freshly synthesised, which implies that the antibody was not appreciably affected by the storage conditions. In fact, SUV(AlSPc) with an IgM antibody raised against bladder carcinoma cells covalently attached by the method used here, were phototoxic to the bladder cells 8 months after their synthesis (H. Lottman, personal communication). This suggests that this type of liposome preparation is very stable to storage, and in spite of some loss of the contents, (24% over 4 months, which is about 0.2% per day), were still very effective. These results also suggest that 2.5 mM AlSPc is above the optimum

TABLE 2

ASSESSMENT OF LIPOSOMAL STABILITY AFTER STORAGE BY MEASURING LEAKED AlSPc.

ABSORBANCE OF FRACTIONS ELUTED FROM PD 10 COLUMN AFTER ADDING 1.5 ml SUV(AlSPc)

Fraction No.	A (675nm)	Fraction No.	A (675 nm)	Fraction No.	A (675nm)
1	0.464	10	0.011	19	0.009
2	0.016	11	0.011	20	0.007
3	0.004	12	0.013	21	0.005
4	0.000	13	0.010	22	0.004
5	0.005	14	0.009	23	0.006
6	0.011	15	0.009	24	0.005
7	0.008	16	0.008	25	0.009
8	0.009	17	0.008	26	0.002
9	0.009	18	0.008	27	0.000

Total free AlSPc Absorbance = 0.153
Total encapsulated AlSPc Absorbance = 0.484
Total free + encapsulated AlSPc Absorbance = 0.637

Therefore:

% free AlSPc = (0.153/0.637) x 100

= 24%

concentration since maximal toxicity was still produced after 24% loss of liposomal content.

2.2.12. EXPERIMENTAL METHOD FOR PHOTOTOXIC ASSAY OF 791T, C170, RC-BCL AND DW-BCL CELLS

OPTIMISING CONDITIONS FOR PHOTOTOXICITY OF 791T AND C170 TUMOUR CELL LINES

In order that the most suitable conditions were used when assessing the effect of AlSPc liposomes on the tumour cell lines used, some experiments were done to find the appropriate numbers of cells to plate, and the radiolabel which would most accurately assess the cell numbers present.

The main requirement of the assay was that a suitable time period should elapse after treatment, to allow:

a) recovery of cells from manipulations during treatment,
b) cells to become adherent,
c) phototoxic treatment to take effect,
d) control cells to reach their full growth potential and become confluent.

At seeding numbers of 2×10^5, 1×10^5 and 5×10^4, the cells were confluent within 24 hours. This meant that not all the viable cells were able to divide because of overcrowding (observed microscopically). Consequently, lower cell numbers were

used to correlate radiolabel uptake and cell number after growth, giving room for all viable cells to divide.

Procedure

Cells were added to triplicate flat bottomed wells in 96 well microtitre plates at 2×10^4, 1×10^4, 5×10^3 and 2.5×10^3 per well. Cells were grown at 37°C for 72 hours and either counted or pulsed with radioactive precursors, 0.5 μCi per well, for the last 12 hours of incubation. The pulse was added either:

a) directly into the incubation medium,

or

b) in fresh medium after washing the cells.

When ^{3}H leucine was used some of the cells were also pulsed in leucine free medium.

The precursors used were:

WASHED	A	+ leucine	+^{3}H leucine
	B	- leucine	+^{3}H leucine
	C		+^{3}H thymidine
	D		+^{3}H deoxyuridine
UNWASHED	E		+^{3}H leucine
	F		+^{3}H thymidine
	G		+^{3}H uridine

Harvesting the cells

a) Radio-labelled cells

After 12 hours incubation with the radiolabel, the supernatant was decanted, trypsin/EDTA solution added to the wells and incubated for a further 10 minutes at 37°C to detach cells. The cells were then harvested onto glass fibre filters on an Automash. The filters were dried and the discs containing the radiolabel were added to scintillation fluid and the radiation counted in a beta counter. Backgrounds (containing medium and radioisotope only) were subtracted and the means of the triplicates calculated.

b) Non radio-labelled cells

Unlabelled cells were trypsinised, the triplicates pooled and resuspended in 250 μl of medium and the viable cells counted by trypan blue exclusion. The mean cell numbers per well were calculated.

Results

The numbers of cells per well and the number of radioactive counts per well for both cell types are shown in Tables 3 (791T cells) and 4 (C170 cells). Seeding 1 x 10^4 cells per well resulted in confluence after 72 hours, lower numbers were sub confluent at this time. The correlation coefficient, r (see Appendix 3 for formula) was calculated for each radiolabel to show whether the counts correlated with the actual cell number as determined by trypan blue exclusion, Table 5.

In all cases except for ^{3}H uridine, r was very close to 1

TABLE 3

RELATIONSHIP BETWEEN CELL NUMBER AND UPTAKE OF RADIOACTIVE PRECURSORS FOR 791T OSTEOSARCOMA CELLS GROWN IN CULTURE.

	No. cells added/well	2.00×10^4	1.00×10^4	5.00×10^3	2.50×10^3
	No. cells counted/well	5.30×10^4	3.50×10^4	1.75×10^4	6.30×10^3
	cpm (mean of 3) ± SEM				
Washed	+ leu, $+^3$H leu	11,523 ± 260	7,352 ± 488	3,912 ± 321	2,892 ± 213
	- leu, $+^3$H leu	112,482 ± 6762	87,195 ± 3196	55,454 ± 4153	30,165 ± 870
	$+^3$H thy	5,306 ± 1022	3,715 ± 105	2,831 ± 166	2,608 ± 93
	$+^3$H durd	7,285 ± 1034	4,728 ± 667	3,295 ± 299	2,987 ± 599
Unwashed	$+^3$H leu	11,039 ± 1488	6,916 ± 422	3,906 ± 303	1,962 ± 119
	$+^3$H thy	3,931 ± 457	3,073 ± 120	2,068 ± 124	1,194 ± 103
	$+^3$H urd	2,768 ± 181	2,259 ± 174	1,091 ± 80	786 ± 38

TABLE 4

RELATIONSHIP BETWEEN CELL NUMBER ANDUPTAKE OF RADIOACTIVE PRECURSORS FOR C170 COLORECTAL CARCINAMA CELLS GROWN IN CULTURE

	No. cells added/well	2.00×10^4	1.00×10^4	5.00×10^3	2.50×10^3
	No. cells counted/welL	5.50×10^4	1.75×10^4	5.53×10^3	1.00×10^3
	cpm (mean of 3) ± SEM				
Washed	+ leu, $+^3H$ leu	10,275 ±685	6,260 ±405	2,319 ± 124	1,179 ±72
	- leu, $+^3H$ leu	81,267 ±9782	54,231 ±1828	24,450 ± 1873	12,590 ±651
	$+^3H$ thy	3,168 ±498	1,866 ± 215	1,106 ±7	793 ±90
	$+^3H$ durd	11,168 ±2312	7,326 ±731	3,415 ±340	2,003 ±153
Unwashed	$+^3H$ leu	15,642 ±633	7,543 ±526	3,544 ±205	1,939 ±59
	$+^3H$ thy	4,310 ±632	2,058 ±129	883 ±69	470 ±173

TABLE 5

CORRELATION COEFFICIENTS BETWEEN CELL NUMBER AND UPTAKE OF RADIOACTIVE PRECURSORS FOR 791T OSTESARCOMA CELLS AND C170 COLORECTAL CARCINAMA CELLS.

X= No. of viable cells counted by trypan blue exclusion for each point.
Y= No. of radioactive counts for each point for the various precursors.
r (of X and Y) = the correlation coefficient between the cell no. (by counting) and the radioactive counts (due to uptake of precursor).

	PRECURSOR	791T (r)	C170 (r)
Washed	+ leu + ^{3}H leu	0.99	0.96
	- leu + ^{3}H leu	0.91	0.95
	+ ^{3}H thy	0.97	0.99
	+ ^{3}H durd	0.97	0.96
Unwashed	+ ^{3}H leu	0.99	0.99
	+ ^{3}H thy	0.67	0.99
	+ ^{3}H urd	0.99	

signifying a good correlation. However, the highest counts were obtained for ^{3}H leucine in fresh leucine free medium, and this combination at a seeding level of 1 x 10^4 cells per well was chosen for subsequent assays since it would give the highest value for controls with which phototoxicity could be compared.

It is possible that using media free of some of the other precursors might have resulted in similar high counts, but using leucine free medium for pulsing was the simplest option.

THE PHOTOTOXICITY ASSAY

The following procedures were used unless otherwise indicated in the Results sections. The temperatures used for the incubations varied as indicated in the Results sections. The method is summarised in Figure 17.

a) For each experiment $1x10^4$ cells freshly harvested at confluence or log phase of growth were added to triplicate flat-bottomed wells in culture plates.

b) (i) **Direct targeting**

The cells were incubated at $4^{o}C$ with 50 μl of liposomes for 45 minutes and washed once.

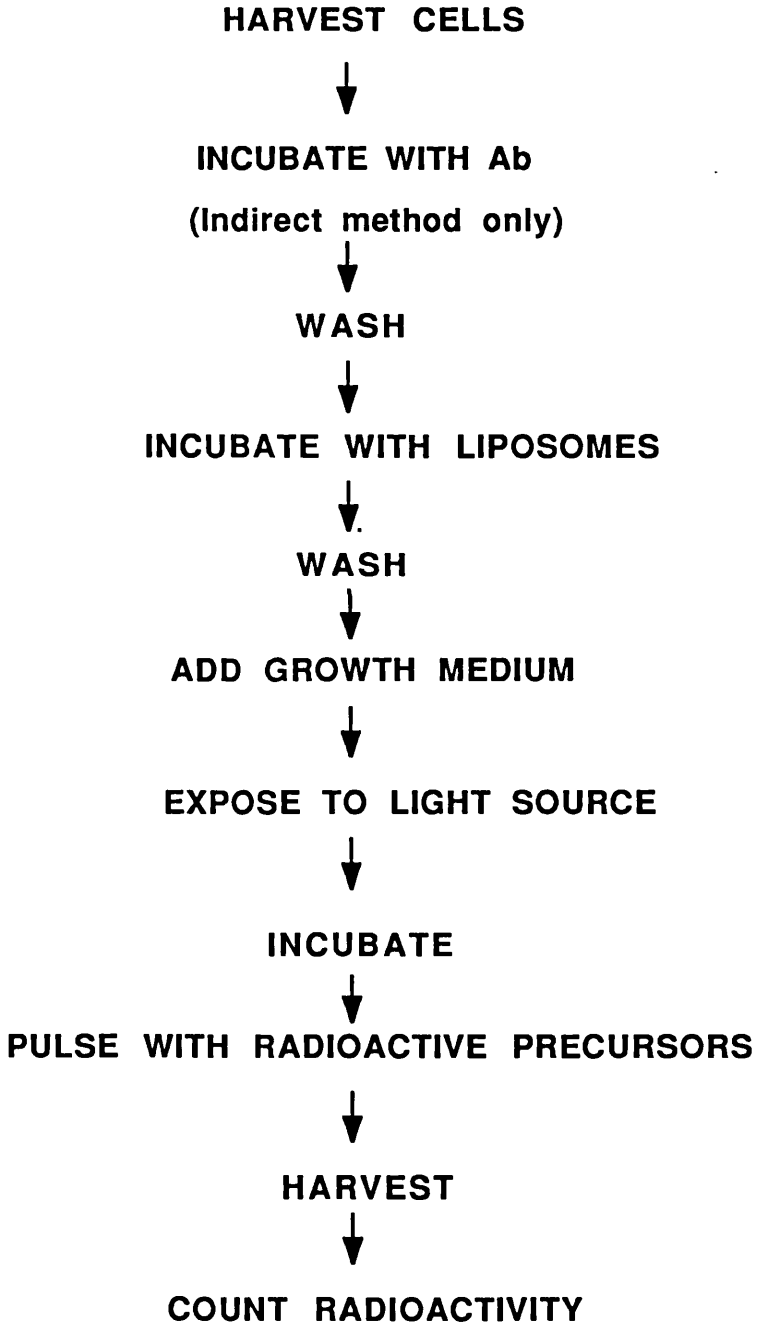
(ii) **Indirect targeting**

The cells were incubated with Ab followed by 30μl liposomes for 30 minutes each, with one wash each time.

c) Growth medium was added to the wells, and the plates exposed to red laser or fluorescent light for variable periods of time, or kept in the dark.

FIGURE 17

PROCEDURE FOR PHOTOTOXICITY ASSAY



d) Cells were then incubated in a humidified atmosphere with 5% CO_2 at 37°C for 72hr.

e) Cells were pulsed during the last 16 hours of culture with

(i) ^{3}H-leucine, (Amersham), in leucine free medium, for 791T and C170 cells, and

(ii) ^{3}H thymidine for PB[illegible], DW-BCL and RC-BCL.

f) Cells were harvested and counted as described before.

The experimental method for treatment of peripheral blood mononuclear cells (PBM) is described in the text of Chapter 3, Preliminary experiments, Section 3.2.1.

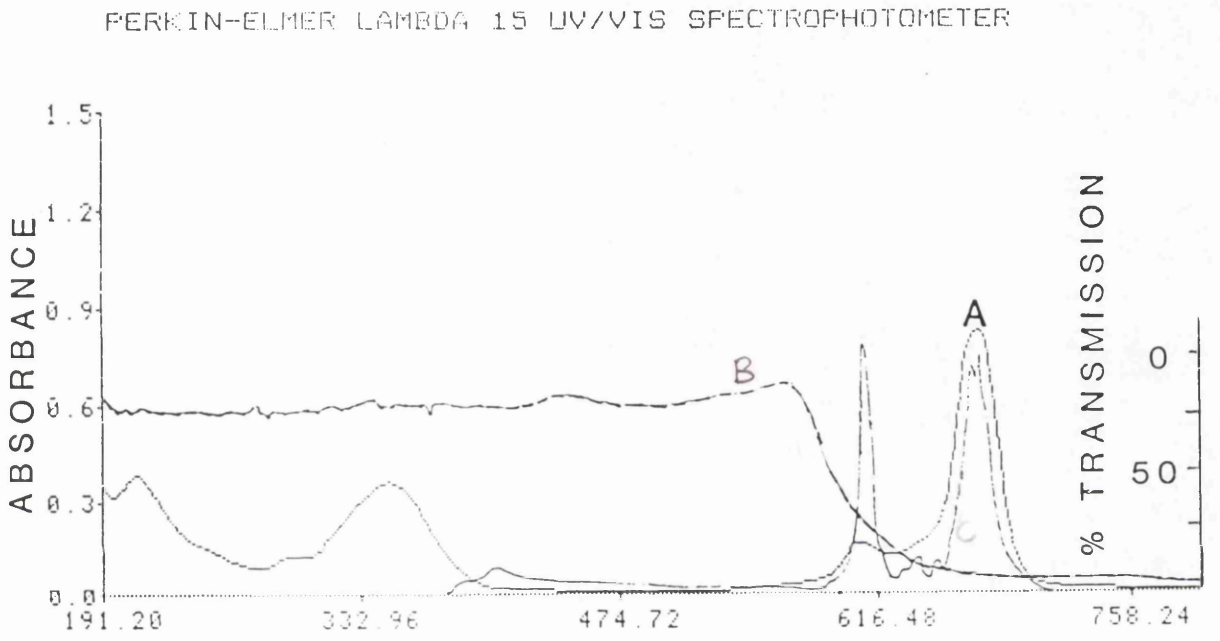
2.2.13. LIGHT SOURCES

FLUORESCENT RED LIGHT

The light source was two 15W Grolux fluorescent tubes (Thorn Lighting Ltd.) (Lidam Scientific) with a red gelatin filter, model 182 (Lee Filters Ltd.), fitted in a box with aluminium reflectors (as described by Chan et al, 1986) which gave peak emissions between 610 and 700 nm, corresponding to the the main absorption peaks of AlSPc in the visible part of the spectrum, Figure 18. The filter blocked more than 80% of light below 600 nm, and transmitted more than 83% of light above 600 nm as shown in Figure 18. Cells were irradiated at 4°C or room temperature as described in individual experiments, 7cm from the source, which gave a mean total light intensity (ie. over all the transmitted light wavelengths) of 1.7mW/cm^2 as measured by a Coherent 212 Power Meter.

FIGURE 18

RED LIGHT EMISSION SPECTRUM



LEGEND

The red light emission spectrum is compared with the absorption peaks of AlSPc and a blocking filter.

A - Absorption spectrum of AlSPc

B - % light transmission through a gelatin filter

C - Light emission of fluorescent red light

LASER LIGHT

The laser light source was an argon pumped dye laser (Aurora-Cooper Laseronics) which emitted light at 675 nm by using the dye DCM (4-dicyano-methylene-2-methyl-6-(p-dimethylaminostyryl)4-H pyran) dissolved in ethylene glycol and propylene carbonate (Bown et al, 1986). Light was delivered through a plastic coated quartz fibre (0.2 mm diameter) as described by Singer et al, 1987. Power was adjusted using a power meter to give 50 mW at the tip.

2.2.14. IMMUNOFLUORESCENCE ANALYSIS OF CELLS

Phenotyping of cells was done by the method of Linch et al, 1982. Antibody titrations were made for each antibody by logarithmic dilutions, to determine the saturating concentration of antibody for each cell type. Briefly, aliquots of 2 x 10^5 cells in suspension were incubated successively in flexible 96 well microtitre plates at 4^oC with saturating concentrations of monoclonal antibody for 45 minutes, and a second layer of fluorescent liposomes containing carboxy-fluorescein coupled to a polyclonal sheep anti-mouse antibody (Gray et al, 1989) for a further 45 minutes, washing thrice each time. The stained cells resuspended in medium were analysed on a Fluorescence Activated Cell Sorter (FACS 1V), (Beckton and Dickinson). For each analysis, irrelevant antibodies of the same subclass were included as controls, to determine non specific binding. Typical values of the fluorescence shown by cells binding antibodies used in this thesis are shown in Table 6.

TABLE 6

FLUORESCENCE ANALYSIS OF CELLS

CELL TYPE	ANTIBODY	PERCENTAGE POSITIVE	PEAK FLUORESCENCE
791T	NONE	1	43
791T	UCHT1 2b	2	44
791T	791T/36 (2.5µg/ml)	98	142
C170	,,	23	56
C170	791T/36 (40µg/ml)	49	71
C170	UCHT1 2b	2	45
C170	NONE	1	44
DW-BCL	NONE	2	47
DW-BCL	UCHT1 G1	2	46
DW-BCL	791T/36 (2.5µg/ml)	2	48
DW-BCL	8a (1/1000)	75	112
RC-BCL	,,	76	114
RC-BCL	UCHT1 G1	2	45

2.2.15. ISOLATION OF MONONUCLEAR CELLS FROM PERIPHERAL BLOOD AND BONE MARROW

Blood was taken from normal healthy donors, and bone marrow from patients harvested for transplantation, into preservative free heparin, diluted with an equal volume of RPMI, layered onto Ficol-Paque (Boyum, 1968) and centrifuged at room temperature for 20 minutes at 1000g. The cells at the interface which consisted mainly of mononuclear cells, were removed and washed twice in RPMI, centrifuging for 10 minutes at 500g for the first wash, and 350g for the second wash. The cells were then suspended in a suitable volume of medium and counted before use by mixing 50/50 (vol/vol) with white cell counting fluid (2% acetic acid), and by the trypan blue exclusion test to determine viability.

2.2.16. TRYPAN BLUE EXCLUSION TEST FOR CELL VIABILITY

Cells were mixed 50/50 (vol/vol) with 0.2% trypan blue solution, incubated at room temperature for 10 minutes to allow dye uptake and the viable cells were counted in a Neubauer chamber. Dead cells cannot exclude the dye and therefore appear blue.

2.2.17. MALONDIALDEHYDE ASSAY

Lipid peroxidation is mediated by free radicals, and the products are a mixture of peroxides which break down to give carbonyl compounds. One such compound is the three carbon malondialdehyde (MDA) which reacts with two molecules of thiobarbituric acid (TBA) to produce a characteristic pink

chromogen. Free MDA is only a small component of peroxidised lipid, but in the assay other peroxide precursors are broken down under the acidic conditions to produce MDA, so the assay reflects the total amount of peroxidation. The standard is also a precursor which breaks down to form MDA, and thus serves to demonstrate that the assay is functioning. The assay was adapted from that used by Gutteridge, 1981.

The stock standard was 100 μM tetramethoxypropane (TMP), (8.3 μl in 50 mls PBS). This was diluted 1 in 100 to give the top standard of 1000 nM. Dilutions of this gave 100, 200, 300, 400, 500, 600, and 700 nM standards.

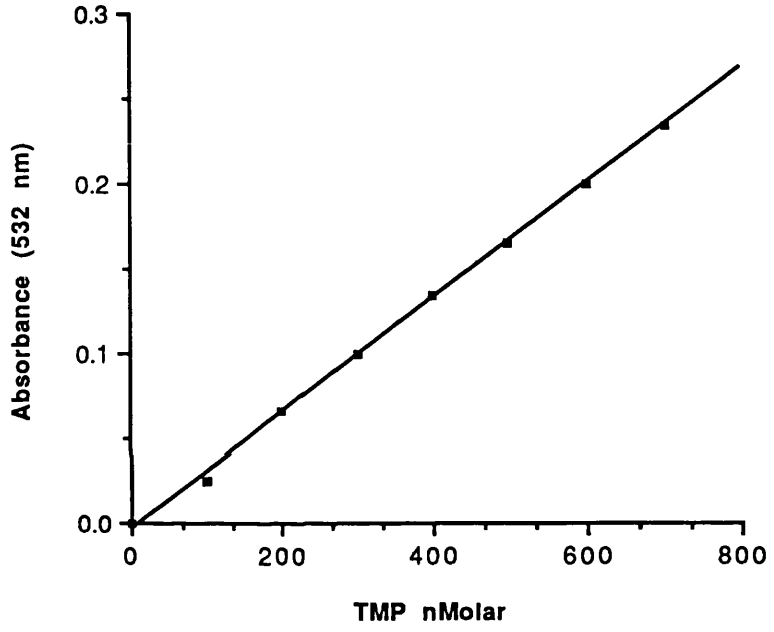
Thiobarbituric acid buffer (TBA): 800 mg TBA was dissolved in 10 ml NaOH (5 M) and 50 ml DW added. The solution was titrated to pH 7.4 with tricarboxylic acid (TCA), made up to 100 ml with DW and 50 ml 7% TCA (w/v) added.

METHOD

Equal volumes (eg. 1 ml each) of standard or sample, and TBA, were incubated at 80-90^{o}C for 30 minutes, cooled on ice, andcentrifuged at 700g for 10 minutes at 4^{o}C to remove debris which might otherwise interfere with the absorbance. The absorbance of the supernatant or standards was read at 532 nm. A standard curve, absorbance versus TMP concentration was plotted and the concentrations of the samples read from curve. The curve is linear over the concentrations used and a typical example is shown in Fig 19.

FIGURE 19

TYPICAL STANDARD CURVE FOR MDA ASSAY



LEGEND

The absorbance of thiobarbiturate reactive chromogen was plotted against the nMolar concentration of standard. Each mole of standard gives rise to 2 moles of MDA, but samples are expressed as nMolar equivalents of TMP read directly from the curve.

CHAPTER 3

PRELIMINARY EXPERIMENTS USING PROTEIN A TO TARGET LIPOSOMES TO CELLS

3.1. INTRODUCTION

These first experiments were designed to introduce AlSPc into the SUV-PA system described earlier (Section 1.5), and to find out what adaptations might be required to produce specific phototoxic killing of cells in a phototoxicity assay. Some of the initial factors to be considered were the incubation temperatures and the conditions for internalisation of the antibody-liposome complex and its contents.

3.2. DEVELOPMENT OF PHOTOTOXICITY ASSAYS

3.2.1. UNPURIFIED AlSPc

The first requirement was to find out if AlSPc, as supplied, could be successfully encapsulated in liposomes. The SUV(AlSPc) would then be coupled to PA and targeted to cells by monoclonal antibody to see if there was a phototoxic effect when they were exposed to red laser light.

Preparation of liposomes

An arbitrary concentration of AlSPc (10 mM) in Hepes buffer was encapsulated in liposomes for the preliminary experiments. During the preparation of the liposomes, as previously described in Section, 2.2.10, it was noted that the separations of encapsulated from free AlSPc on the G50 column, and of PA coupled liposomes from free PA on the S4B column, were not distinct. In each of the separations, the first 10 coloured fractions, which contained most of the liposomes, were collected,

pooled, and kept for the next stage, whilst later fractions which contained mainly free AlSPc or free PA were discarded.

Experimental procedure and results

The procedures were performed aseptically in a laminar flow hood with the internal lighting switched off. Light conditions were dim to help protect against accidental activation of AlSPc

a) Five million freshly prepared PBM per test sample, suspended in RPMI, were incubated in microtitre plates at room temperature with 100μl of UCHT1 2b monoclonal antibody at 10μg/ml for 45 minutes, and washed 3 times.
b) The cells with bound antibody were then incubated with 100 μl of liposomes for 45 minutes, washed 3 times and finally resuspended in RPMI containing 10% FCS.
c) All samples were incubated for 1.5 hours at 37^{o}C to allow some internalisation of the antibody-SUV complex.
d) The cells were transferred to Bijou bottles and either exposed to red laser light at 50 mW for 10 minutes per sample or kept in the dark.
e) After laser treatment the samples were divided into 2 portions and treated as follows:

(i) Cells were kept at 37^{o}C and their viability measured by the trypan blue exclusion test at intervals of 1,2,3 and 19 hours after laser treatment (Table 7).
(ii) Triplicates of 2 x 10^{5} cells per well from each sample

TABLE 7 PHOTOTOXICITY OF T-LYMPHOCYTES BY PROTEIN A AND ANTI-CD3 TARGETED AlSPc(UNPURIFIED) LIPOSOMES

			PERCENTAGE VIABILITY (means of 3 counts, SE <10%)				
			1 HR	2 HR	3 HR	19 HR	REGROWTH cpm means of triplicates ± SE
TREATMENT							
TEST							
MAb + SUV(AlSPc)-PA	+ LASER	**A**	72	59	37	11	1461 ± 397
CONTROLS							
MAb + SUV(AlSPc)	+ LASER	**B**	83	69	58	27	1423 ± 438
MAb + SUV	- LASER	**C**	99	98	98	97	39151 ± 5,392
SUV	+ LASER	**D**	97	97	96	96	91182 ± 9,908
SUV-PA	+ LASER	**E**	99	99	99	98	101565 ± 10,395

were placed in flat bottomed microtitre culture plates and PHA (a T lymphocyte mitogen, Nowell, 1960) growth medium added (which contained PHA at the lowest concentration (2 μg/ml) that produced a maximal proliferation response). The plates were incubated at 37^{o}C for 72 hours with a pulse of ^{3}Hthymidine added in the last 12 hours. Cells were harvested and radioactivity measured. The ^{3}Hthymidine uptake, as a measure of growth, is expressed as counts per minute (cpm) in Table 7.

The test samples and controls were treated with monoclonal antibody, liposomes and laser as shown in Table 7.

Dis cussion

Following incubation with AlSPc liposomes and laser treatment, T-cells failed to respond to mitogen whereas controls responded. The mechanism causing the lack of response was uncertain, but possibilities were; photodynamic damage to PHA binding sites on the cells or more widespread structural damage, resulting in an inability to respond. There was rapid cell killing in the first few hours after laser treatment as shown by the decreasing viability of the cells, but a lack of differential phototoxicity between targeted (PA) and untargeted (no PA) cells. This might be due to several factors. Firstly, the AlSPc concentration might have been too high, thus causing non specific toxicity. Secondly, the mixture of sulphonated phthalocyanines may have contained some components which were much more lipophilic than others, and these might have transferred from the

lipid bilayer of the liposome to the cell membrane. Other conditions which could be responsible for non specific toxicity were the temperature and the time of incubation of cells with liposomes. In subsequent experiments some of these factors were taken into consideration as the treatment conditions were developed.

Some further deductions were also made from the counts obtained after PHA stimulation for 72 hours (Table 7). Although the controls had high viability by trypan blue exclusion they displayed quite different growth capabilities as shown by ^{3}Hthymidine uptake.

Firstly, when PHA was used in the presence of the targeting antibody, much lower counts were obtained (C) than when PHA was used alone (D). This inhibitory effect has been observed before when lymphocytes were incubated simultaneously with PHA and CD3 antibody ((Van Wauwe et al, 1984; Valentine et al, 1985; Holter et al, 1986; personal observations (see appendix 4)). The degree of inhibition depended on the time period between addition of the two substances and the presence of accessory cells.

Secondly, when Protein A is present on the liposomes, a stimulatory effect above that of PHA alone was seen (E). This effect has been observed in this laboratory previously, but the degree of stimulation was variable and sometimes absent. Other authors have described a mitogenic response of T lymphocytes to protein A stimulation (Sakane and Green, 1978; Kasahara et al, 1980). When protein A is purified from Staphylococcus aureus (as in these experiments), the preparations sometimes contain small

amounts of toxin. Schrezenmeier and Fleischer (1987) have shown that the mitogenic effects of Protein A on T-lymphocytes are due to endotoxins A and B and are not observed when recombinant protein A (produced in E. coli) is used. The mitogenic effects seen here were therefore probably due to endotoxin being coupled to the liposomes at the same time as Protein A. To overcome these effects either recombinant Protein A would have to be used, or the Protein A carefully checked for the presence of endotoxin.

3.2.2. SEPHADEX LH-20 PURIFIED AlSPc

The PHA proliferation of T lymphocytes in the first experiment was repeated with some adaptations to the procedures.

PROCEDURE AND RESULTS

Liposomes were prepared with LH-20 purified AlSPc (Section 2.2.8) at 1 mg/ml (lower than the initial preparation to decrease non-specific toxicity due to high AlSPc concentrations), and coupled with PA. The LH-20 purification removed some of the hydrophobic contaminants, and decreased the likelihood of toxicity due to the possible transfer of these to cells. The incubation times were also decreased to 30 minutes, except for the internalisation step. UCHT1 G1 was used as a control antibody since at physiological pH it does not bind to PA. Other samples contained targeted and untargeted liposomes without AlSPc, which acted as 100% controls for those containing AlSPc. Some samples were not treated with PHA and acted as controls for background radioactivity.

Results were expressed as percentage growth of the 100% controls as assessed by incorporation of ^{3}Hthymidine after adjusting for background uptake, and are shown in Figure 20. Phototoxicity was observed in both targeted and untargeted cells, but not in dark controls. However, as before, the differential was not high, untargeted cells showed 61% toxicity, compared with 81% in targeted cells. The PHA only controls, show 150% growth compared to the dark controls. The decrease to 100% was again due to the inhibitory effect of the PHA/CD3 antibody combination.

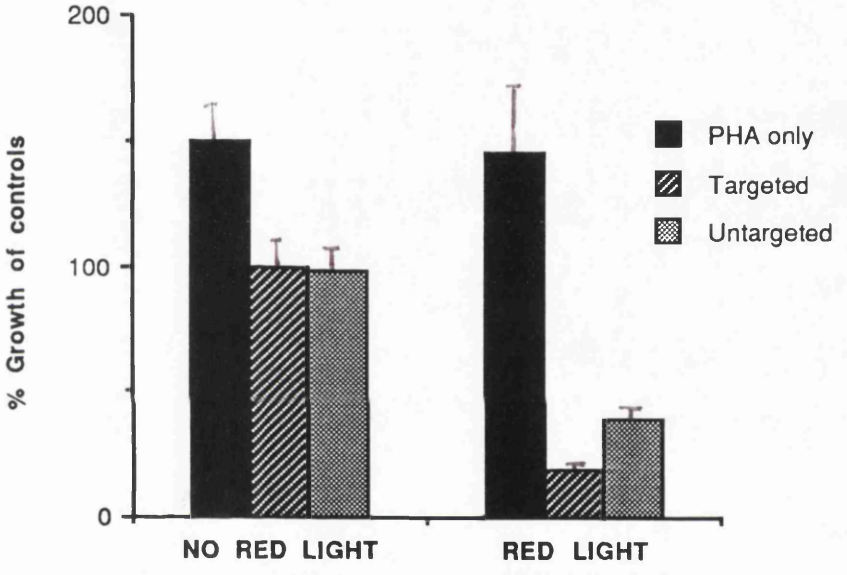
DISCUSSION

While preparing the liposomes with the LH-20 purified AlSPc, trailing was observed on the columns as with the unpurified AlSPc, though to a lesser extent. This implied that there were still lipophilic components present in the AlSPc which made the liposomes 'sticky'. The AlSPc bound to the lipid and destabilised the liposomes causing poor separation on both Sephadex G50 and Sepharose S4B-CL columns.

Lipophilic contaminants found in other compounds such as carboxy-fluorescein, have been found to concentrate in and perturb liposomal membranes and to transfer more rapidly to cells than purified carboxy-fluorescein (Ralston et al, 1981). In these experiments it was found that liposomes prepared with unfractionated AlSPc were very sticky, and bound to cells non specifically, thus decreasing the differential toxicity between targeted cells and untargeted controls. This effect was slightly reduced by first passing the dye down an LH 20 column, which

FIGURE 20

PHOTOTOXICITY OF T LYMPHOCYTES BY PROTEIN A AND ANTI-CD3 TARGETED ALSPC (LH-20 PURIFIED) LIPOSOMES



LEGEND

Cells were treated in triplicates as described in the text.

Bars are calculated from the means of the triplicates, and show the standard errors.

probably removed some of the most lipophilic components such as the di-, mono- and unsulphonated phthalocyanine molecules. It is likely that the non-specific toxicity was due to remaining lipophilic components transferring to the bilayer and cell membrane in the same way as carboxyfluorescein. For this reason further purification was thought necessary to increase differential phototoxicity, so for the next liposome preparation the most polar fraction of AlSPc separated into some of its components by TLC (Section 2.2.8.) was selected for encapsulation.

3.2.3. TLC PURIFIED AlSPc

Some further modifications to the phototoxicity assay were made to try and decrease nonspecific binding and thus increase the differential phototoxicity between targeted and untargeted cells. This experiment used liposomes encapsulating TLC purified AlSPc at 1 mg/ml. Separation of liposome encapsulated and free AlSPc on columns during preparation was complete, with no trailing. The final AlSPc concentration used in incubations (if the AlSPc was released into the incubation volume by lysis of the SUV) was 3 μg/ml.

PROCEDURE AND RESULTS

To eliminate the inhibitory effect of the PHA/CD3 mitogen/ antibody combination, UCHT1 IgG subclass 2a was used to target the SUV-PA. This antibody is mitogenic to T lymphocytes from most donors (the size of the response varying from one individual to

another) unlike UCHT1 2b or G1 which are only mitogenic in a proportion of individuals, (Smith et al 1986). This abrogates the need for PHA, since UCHT1 2a acts as both targeting antibody and mitogen.

Cells were treated in microtitre plates, and red light from fluorescent lamps (Section 2.2.13.) used to activate the AlSPc, with a one hour exposure time. (Red fluorescent light was used here and in most of the subsequent experiments because the laser was out of commission).

100% controls were SUV and SUV-PA without AlSPc. Incubations were done at 4°C and room temperature to test for any decrease of non specific toxicity at the lower temperature. Cells incubated at room temperature were kept at 37°C for 1 hour to allow internalisation of liposomes, while those incubated at 4°C were kept at 4°C.

Results are expressed as percentage growth of controls in Table 8.

DISCUSSION

The results demonstrate a poor differental of phototoxicity between targeted and untargeted liposomes when incubated at room temperature with internalisation, but at 4°C there is a very good differential. Moreover, at 4°C there was no internalisation step so it appears that toxicity can be achieved simply by targeting the liposome to cells and exposing to red light. AlSPc does not need to be internalised to be effective.

TABLE 8

PHOTOTOXICITY OF T-LYMPHOCYTES BY PROTEIN A AND ANTI-CD3 TARGETED AlSPc(TLC PURIFIED) LIPOSOMES

PERCENTAGE GROWTH OF CONTROLS

means ± SE of triplicates.

		INCUBATION TEMPERATURE	
		25°C	**4°C**
RED LIGHT	TARGETED	2.5±2	5.4±1
	UNTARGETED	19.6±5	94.0±15
NO RED LIGHT	TARGETED	82.0±1	83.5±10
	UNTARGETED	22.0±4	91.4±8

3.3. GENERAL DISCUSSION

The previous three experiments used T lymphocytes (in PBM) as target cells to show that AlSPc is phototoxic when encapsulated in liposomes and targeted to them with MAb and PA, and also to try to achieve specific targeting.

However, to demonstrate the cytotoxic effects, T lymphocytes need to be stimulated with a mitogen to show that proliferation decreased compared to controls. In the first 2 experiments this was done with PHA. However, PHA caused some degree of inhibition in combination with the CD3 targeting antibody. Using a mitogenic antibody by itself, as in the third experiment, provided more consistent controls, but the mitogenic response was lower.

Also present in the PBM samples were other cells, B lymphocytes which are known to proliferate in response to PA (Forsgren et al, 1976), and monocytes which are necessary as accessory cells for the proliferative response of T lymphoctes to both PHA and CD3 antibody. It was a possibility that the monocytes could have taken up AlSPc liposomes and been destroyed by light activation, their absence thus preventing proliferation, which was why there was no differential toxicity between targeted and untargeted cells. This was not so in the incubations at $4^{o}C$, but low temperatures would probably have lowered monocyte activity anyway. However, the viabilities in the first experiment show that larger numbers of cells than could be accounted for by monocytes alone, were unable to exclude trypan blue. Thus the phototoxicity was more likely to be due to non specific binding of lipophilic components in AlSPc and relatively high incubation

temperatures. This is borne out by the increased differentials found in the subsequent experiments which used less lipophilic AlSPc preparations and also, finally, low incubation temperatures.

Any effect due to monocyte phototoxicity and B lymphocyte proliferation could be controlled for more stringently, by producing a more pure T lymphocyte preparation (by rosetting with sheep red blood cells, Kaplan and Clark, 1974), and adding back monocytes as helper cells after liposome incubation.

Because of the complications described above, it was thought that a spontaneously proliferating cell line might provide a more homogeneous situation for better interpretation of results.

In the next section an osteosarcoma cell line 791T was used which had an associated antibody 791T/36, to target liposomes to an antigen on the surface of the cells. A preliminary experiment was done to see how well the targeted system in its present state of development would perform.

3.4. EXPERIMENTS WITH THE OSTEOSARCOMA CELL LINE 791T

PROCEDURE AND RESULTS

The phototoxicity assay for 791T cells was set up using the cell numbers and culture conditions described in Section 2.2.12. Liposomes encapsulating TLC purified AlSPc at 1 mg/ml and coupled to PA were used for targeting which gave a final (lysed) AlSPc concentration of 3 μg/ml in the incubation volume.

Successive incubations with antibody and liposomes were performed at room temperature, for 30 minutes with 2 washes each time. Cells were exposed to red light for variable periods of time: 0, 30, 45 and 60 minutes, and then grown as usual. Growth was assessed by ^{3}Hleucine uptake over the last 16 hours of incubation in leucine free medium as described in Section 2.2.12.

Cells were treated according to the following protocol:

A = Ab

B = Ab + SUV

C = Ab + SUV-PA

D = Ab + SUV(AlSPc)

E = Ab + SUV(AlSPc)-PA

The means of triplicates of the raw data (including controls) are given in Table 9A and the percentage growth of controls in Table 9B (which were calculated using the designated letters in the Tables).

DISCUSSION

The results were extremely variable which is why the original counts in Table 9A are shown as well as the percentage growth of controls in Table 9B. The latter give the impression of no toxicity by targeted AlSPc liposomes (E/C) except after long light exposures. Together they give a better interpretation of the results.

The main problem was that SUV-PA alone had considerable

TABLE 9

PHOTOTOXICITY OF 791T CELLS BY PROTEIN A AND 791T/36 TARGETED AlSPc LIPOSOMES

A **EFFECT OF SUV(AlSPc)-PA ON 791T CELLS**

	TIME (minutes)			
PROTOCOL	**0**	**30**	**45**	**60**
	cpm (mean of triplicates) ± SEM			
A Ab	52,560 ±8861	68,848 ±8919	74,568 ±10,859	72689 ±2327
B Ab + SUV	49,290 ±13.124	83,572 ±2328	70,965 ±3210	83,546 ±5779
C Ab +SUV-PA	14,749 ±2375	14,619 ±1289	19,475 ±4349	32,377 ±4389
D Ab +SUV(AlSPc)	24,273 ± 728	28,447 ±2900	29,251 ±2050	12,937 ±2156
E Ab + SUV(AlSPc)-PA	19,616 ±3756	18,646 ±1964	15,996 ±4763	24,036 ±3115

B **PERCENTAGE GROWTH OF CONTROLS**

Calculated from means in Table 9A

		TIME (minutes)			
		0	**30**	**45**	**60**
PROTOCOL					
Ab + SUV-PA	(C/A)	28	21	26	45
Ab + SUV(AlSPc)	(D/B)	49	34	41	15
Ab + SUV(AlSPC)-PA	(E/C)	103	127	82	74

TABLE 9 (continued)

C **EFFECT OF SUV-PA ON CELL GROWTH**

		TIME (minutes)			
		0	25	40	60
Ab + SUV-PA	(C/B)	75	70	50	79

toxicity on 791T cells (C), which was not increased by the presence of AlSPc (E), except possibly at the longer light exposures. Untargeted liposomes (without PA) containing AlSPc were also toxic (D), but ordinary liposomes had no effect on growth (B).

Toxicity by untargeted SUV(AlSPc) was probably due to non specific binding during the incubation at room temperature, although this would not account for inhibition at zero light exposure. Possibly insufficient care was taken to minimise exposure to room light.

Inhibition of growth in the presence of protein A might be due to a toxic effect of the protein on this particular cell line, or possibly the presence of endotoxin in the protein A preparation as described earlier had a toxic effect rather than a stimulatory one as on the T lymphocytes.

The inhibitory effect of a different batch of SUV-PA (without AlSPc) was tested again under similar conditions (Table 9C) and again found to be inhibitory by 20-50% over cells incubated with SUV without protein A.

3.5. CONCLUSIONS

(a) To be sure that any toxicity seen is due to factors other than AlSPc activation, great care must be taken to keep dark controls dark

(b) Incubations should be done at $4^{o}C$ rather than at room temperature to decrease non specific binding of liposomes to cells (as was observed with lymphocytes).

(c) It was decided that due to the variable effects of protein A on both 791T cells and lymphocytes, liposomes should be targeted using a different system.

Two methods appeared possible:

(i) Direct targeting using a monoclonal antibody coupled to the liposome.

(ii) Indirect targeting using a polyclonal anti-species immunoglobulin coupled to the liposome. This would bind to monoclonal antibodies bound to the cells in a sandwich technique in the same manner as SUV-PA.

Both of these methods were tried in the next series' of experiments (Chapter 4).

CHAPTER 4

PHOTOTOXICITY EXPERIMENTS USING DIRECT AND INDIRECT ANTIBODY TARGETING OF LIPOSOMES

4.1. EXPERIMENTS WITH DIRECTLY TARGETED AlSPc LIPOSOMES

4.1.1. INTRODUCTION

The monoclonal antibody 791T/36, to be used in these experiments coupled readily to SUV as described in Section 2.2.10. These liposomes can be incubated with cells directly without the necessity of a pre-incubation with antibody.

Preliminary experiments looked very promising, so a series of experiments were performed in which the phototoxicity of SUV(AlSPC)-791T/36 was tested against two cell lines, both of which expressed an epitope which bound 791T/36 antibody; 791T, an osteosarcoma; and C170, a colorectal carcinoma.

4.1.2. PHOTOTOXICITY ASSAY WITH DIRECTLY TARGETED AlSPc-LIPOSOMES

PROCEDURE

The liposome/cell combinations for the tests and controls for all cell types as shown in Table 10 were incubated with 791T, C170 and DW BCL cells according to the procedure in Section 2.2.12. They were exposed to red light at $4^{o}C$ for 25 minutes or kept in the dark. Cell samples were incubated with AlSPc containing liposomes coupled with 791T/36 antibody for targeted tests, and without coupled antibody for untargeted controls. The 100% controls were cells incubated with liposomes containing no AlSPc, but with or without free 791T/36 at 10 μg/ml (the same amount as coupled to the liposomes), for targeted and untargeted controls respectively.

The amount of liposomes added in each experiment corresponded to a final AlSPc concentration of 2.5μg/ml (which is the amount of AlSPc which would be present in the incubation volume if the liposomes were lysed), and a final antibody concentration present on liposomes of 10μg/ml. One series of experiments done with C170 cells (D8 in Table 10) contained a more concentrated liposome solution, with AlSPc at 4.5 μg/ml and antibody at 18 μg/ml. In this case the 100% controls contained the appropriate amounts of liposomes and free 791T/36 antibody.

Several controls were included. These were control cells DW-BCL which did not bind 791T/36 (A1); AlSPc liposomes coupled to an irrelevant antibody (S-MIg) and incubated with 791T cells (B2) and free AlSPc (TLC purified) at 2 different concentrations (2.5 and 25 μg/ml) in the presence of buffer containing liposomes, also incubated with 791T cells (C5 and C6 respectively), (Table 10).

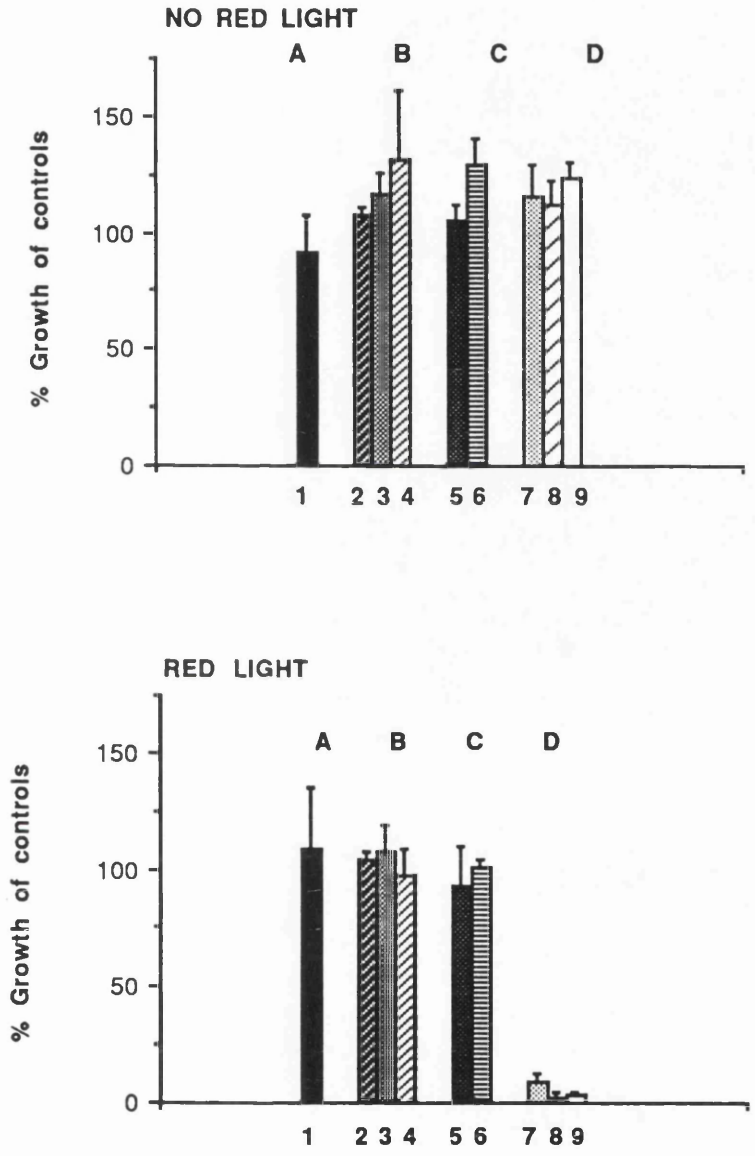
RESULTS

The results of the full complement of controls and target cells as per Table 10 are summarised in Figure 21. They were expressed as the percentage growth of controls 72 hours after light treatment. Significant differences were determined by Students t tests (Appendix 3). Bars show SEM. of n experiments.

AlSPc photosensitised liposomes were only toxic to 791T and C170 cancer cell lines when both targeted by antibody and exposed to red light, (7,8,9). Targeted AlSPc liposomes at 2.5μg/ml were less toxic for C170 cells than for 791T cells,

FIGURE 21

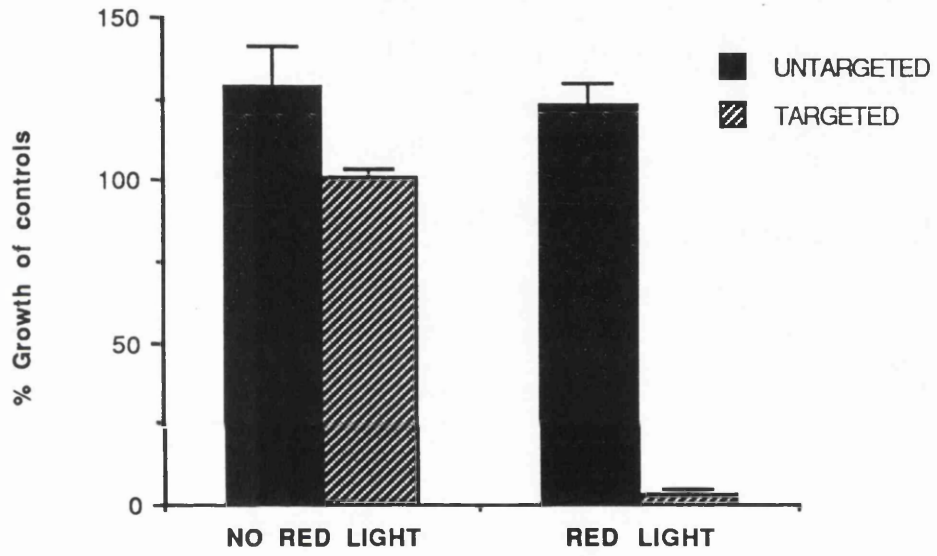
PHOTOTOXICITY OF SEVERAL CELL TYPES BY DIRECTLY TARGETED AlSPc and FREE AlSPc



FOR LEGEND: SEE TABLE 10

FIGURE 22

PHOTOTOXICITY OF 791T CELLS BY DIRECTLY TARGETED AlSPc LIPOSOMES



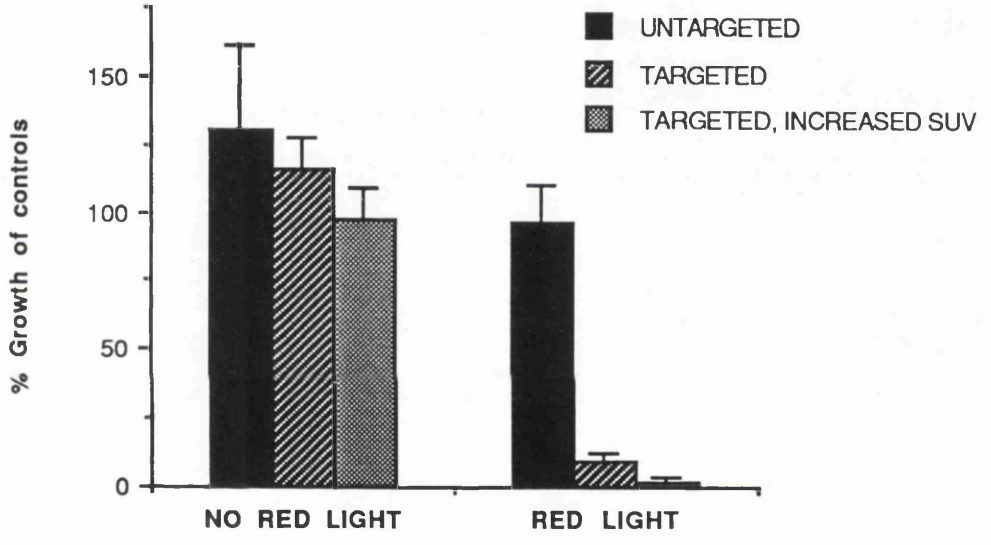
LEGEND.

Cells were treated as described in the text for Figure 21 and according to Table 10.

Bars are means +/- S.E.M. of 5 separate experiments with triplicates at each point.

FIGURE 23

PHOTOTOXICITY OF C170 CELLS BY DIRECTLY TARGETED AlSPc LIPOSOMES



LEGEND.

Cells were treated as described in the text for Figure 21 and according to Table 10.

Bars are means +/- S.E.M. of three separate experiments with triplicates at each point.

TABLE 10

Liposome and antibody combinations for the directly targeted phototoxicity assay with 791T, C170 and control cells, DW BCL..

Cells were treated in triplicates as described in the text and according to the following protocol:

(n = number of triplicate experiments per group)
Results are means ± S.E.M.

All samples were treated with and without light

CONTROLS

A) <u>Cells not carrying the appropriate surface antigen</u>

1) Targeted AlSPc-SUV with 791T/36 MAb attached + DW-BCL (n=3)

B) <u>Untargeted AlSPc-liposomes</u>

2) Untargeted AlSPc-SUV with irrelevant Ab attached + 791T (n=5)

3) Untargeted AlSPc-SUV with no attached Ab + C170 (n=3)

4) Untargeted AlSPc-SUV with no attached Ab + 791T (n=5)

C) <u>Free AlSPc</u>

5) Free AlSPc (2.5μg/ml) + buffer-SUV + 791T (n=3)

6) Free AlSPc (25μg/ml) + buffer-SUV + 791T (n=3)

TESTS

D) <u>Targeted AlSPc-liposomes</u>

7) Targeted AlSPc-SUV (2.5μg/ml) with 791T/36 Ab attached + C170 (n=3)

8) Targeted AlSPc-SUV (4.5μg/ml) with 791T/36 Ab attached + C170 (n=3)

9) Targeted AlSPc-SUV (2.5μg/ml) with 791T/36 Ab attached + 791T (n=5)

(7,9), ($p < 0.005$), but at 4.25μg/ml (8,9) were equally toxic, (no significant difference). Free AlSPc at concentrations of 2.5 and 25μg/ml, and all controls, showed no significant toxicity whether irradiated with red light or not. For easier observation of the test results the phototoxicity is shown in Figure 22 for 791T cells, and Figure 23 for C170 cells.

DISCUSSION

Immunofluorescence analysis of C170 cells showed a lower expression (23% positive) than 791T cells (98% positive) of the antigen that bound 791T/36 monoclonal antibody at a concentration of 2.5μg/ml (Table 6). For maximum fluorescence of C170 cells a concentration of 40μg/ml was required, giving 49% positive cells. The antigen expression reflects the lower toxicity of C170 cells observed with the liposomes containing 2.5μg/ml AlSPc and 10μg/ml antibody. However, using more concentrated liposomes with a greater amount of antibody available for binding (18μg/ml), similar toxicity to 791T cells was achieved, even though the antibody concentration was less than that required for maximum binding to C170 cells.

Antigen on C170 cells appears to have a lower affinity for 791T/36 than antigen on 791T cells thus requiring the presence of more antibody for equivalent toxic effects. The reasons for this are uncertain but could be due to slight differences in the epitope that binds the antibody, or different physical expression of the antigen on the cell surface. Possibly the antigen is less exposed on C170 cells, thus making it less accessible for

antibody binding. Alternatively the liposome itself could contribute to obstruction of binding on C170 cells, in which case indirect targeting, with the addition of an extra antibody to provide a spacer, might prove more toxic. Nevertheless, despite these apparent differences, maximal toxicity under these conditions was attained with both cell types.

4.1.3. CONDITIONS FOR OPTIMAL PHOTOTOXICITY

The next steps were to determine whether the phototoxic effect depended on the amount of incident light energy received by the cells, and the concentration of photosensitiser present.

A) DOSE RESPONSE TO LIGHT EXPOSURE

791T cells were set up with targeted and untargeted liposomes, and controls as previously described, but the red light irradiation varied from 0 to 25 minutes.

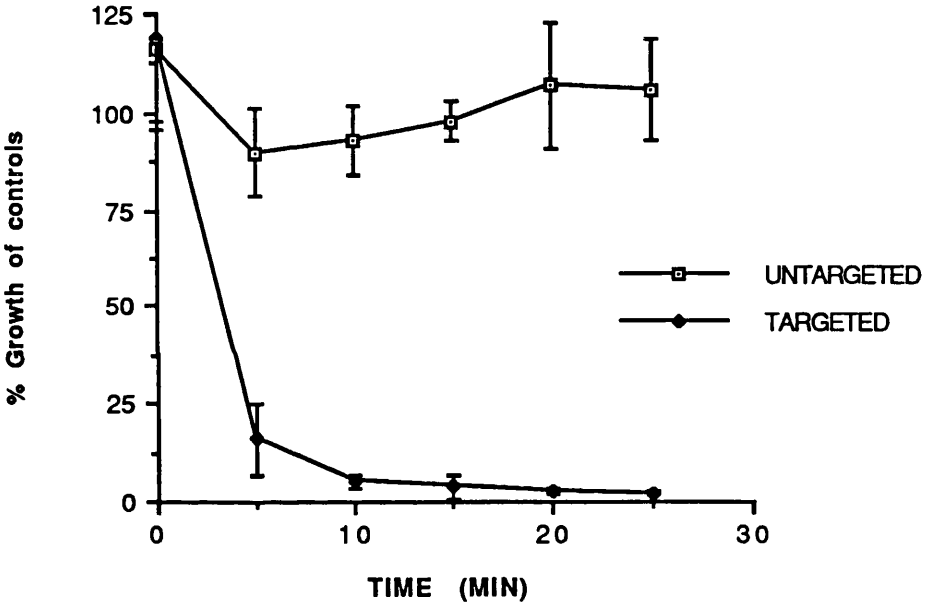
Figure 24 shows a typical dose-response curve in which increasing exposure time to red light caused increasing toxicity up to a maximum at 25 minutes. There was no significant effect on untargeted controls.

B) DOSE RESPONSE TO LIPOSOME DILUTION

Further dose response curves were set up, but this time the variable was a reduction in the numbers of liposomes by serial dilution for each time point. For each dilution, both the amount of antibody and AlSPc change proportionately, since they are directly dependent on each other and the number of liposomes. A

FIGURE 24

EFFECT OF EXPOSURE TIME ON PHOTOTOXICITY OF 791T CELLS DIRECTLY TARGETED BY AlSPc LIPOSOMES



LEGEND.

Cells were treated as described in the text.

Points represent means +/- S.E.M. of 3 experiments with triplicates at each time point.

control was included using an irrelevant antibody bound to the liposomes at the highest liposome concentration.

The dose response curves in Figure 25 show that increasing dilution of liposomes results in decreasing phototoxicity for a fixed time exposure.

DISCUSSION

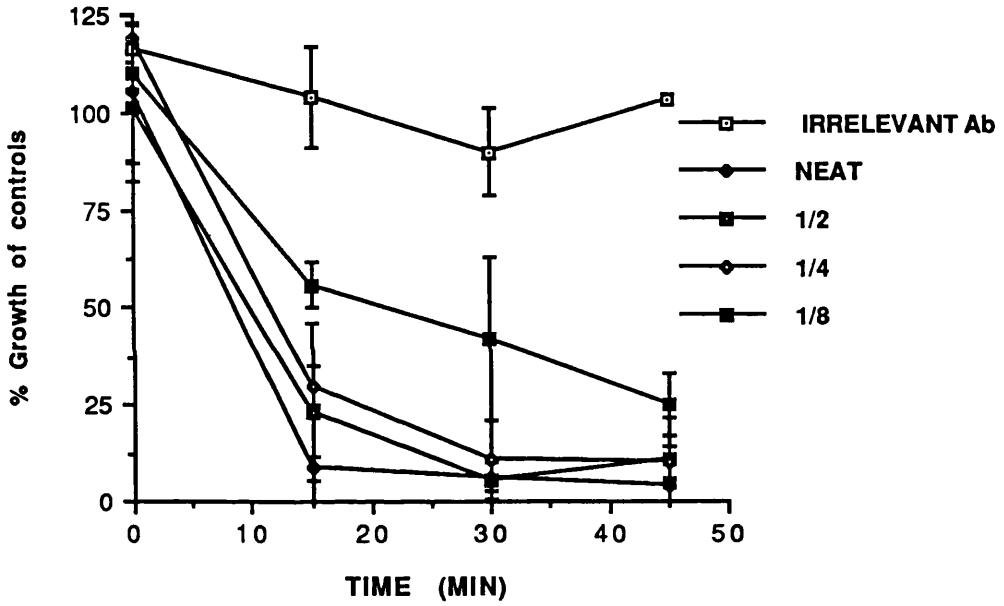
The results of these experiments show that AlSPc can be successfully encapsulated in liposomes, and targeted against cells without non-specific phototoxicity; and that the degree of toxicity depends on the dose of both light and photosensitiser. It also depends on the amount of antibody, since it is this that determines to a large extent how much AlSPc becomes bound to the cells. The effect of different AlSPc concentrations with a fixed antibody and lipid dose is discussed later in Section 4.3.

The treatment is rapid, requiring only 45 minutes for antibody binding, plus up to 25 minutes for light treatment.

In any targeting system there is the possibility of unwanted non-specific effects. These have been minimised in the present system by a number of factors. Firstly the liposomes are reasonably stable (24% leakage of contents after 4 months storage at $4^{o}C$ see Section 2.2.11), so there is little leakage into the medium. Secondly, once the light source is removed, there is no further activation of the dye, and any cell damage will be due to the effects of the toxic species already generated, and the reactions that proceed from them. Thirdly, even if some dye does escape from liposomes, this fraction of AlSPc which has been

FIGURE 25

DOSE RESPONSE EFFECTS OF LIPOSOME DILUTION ON PHOTOTOXICITY OF 791T CELLS BY DIRECTLY TARGETED AlSPc LIPOSOMES



LEGEND.

Cells were treated as described in the text.

Points represent means +/- S.E.M. of 3 separate experiments with triplicates at each time and dilution point.

selected, is not easily taken up into cells due to its polar nature. No light mediated toxicity was produced in 791T cells by free AlSPc, even at tenfold the concentration used in targeted liposomes (Fig. 21).

4.2. EXPERIMENTS WITH INDIRECTLY TARGETED AlSPc LIPOSOMES

4.2.1. INTRODUCTION

In these experiments, liposomes with covalently coupled sheep anti-mouse immunoglobulin containing AlSPc (SUV(AlSPc)-S∝M) were indirectly bound to cells via antibody attached to antigen on the cell surface. The experimental procedures were similar to those with direct targeting, but with an extra incubation step for binding monoclonal antibody to the cell (as for SUV-PA targeting). Two different cell types and antibodies were used to show the technique is not restricted. Incubations were all done at 4°C and according to the procedure described in Section 2.2.12.

4.2.2. PHOTOTOXICITY ASSAY FOR INDIRECT TARGETING

A) 791T CELLS

The targeting antibody was 791T/36 at 2.5 μg/ml and an irrelevant antibody at the same concentration, and of the same subclass (UCHT1 2b) was used for controls. SUV(AlSPc)-S∝M contained 6 μg/ml AlSPc (concentration of AlSPc in the incubation volume released by lysis) and 130 μg/ml of S∝MIg coupled to the

surface. 100% controls were incubated with antibodies and SUV-S∝M containing buffer only. Cells were exposed to red light for 25 minutes after the incubation steps, and cultured as usual.

B) RC-BCL CELLS

The cells, RC-BCL, were incubated with an anti-B-cell monoclonal antibody 8A (purified ascites at 1:000 dilution), to target liposomes, or UCHT1 G1 as an irrelevant ntibody. Different liposome dilutions were used, and the cells were exposed to red light for variable time periods (0, 15, 30 and 45 minutes), to give a series of dose response curves. The neat liposomes contained 6 μg/ml AlSPc and doubling dilutions of this were made for the remainder, giving 3, 1.5 and 0.75 μg/ml. 130 μg/ml of S∝MIg were coupled to the neat liposomes. Only the highest AlSPc concentration (neat) was used with the irrelevant antibody. 100% controls were incubated with antibody and buffer containing SUV-S∝M, at similar dilutions to those with AlSPc.

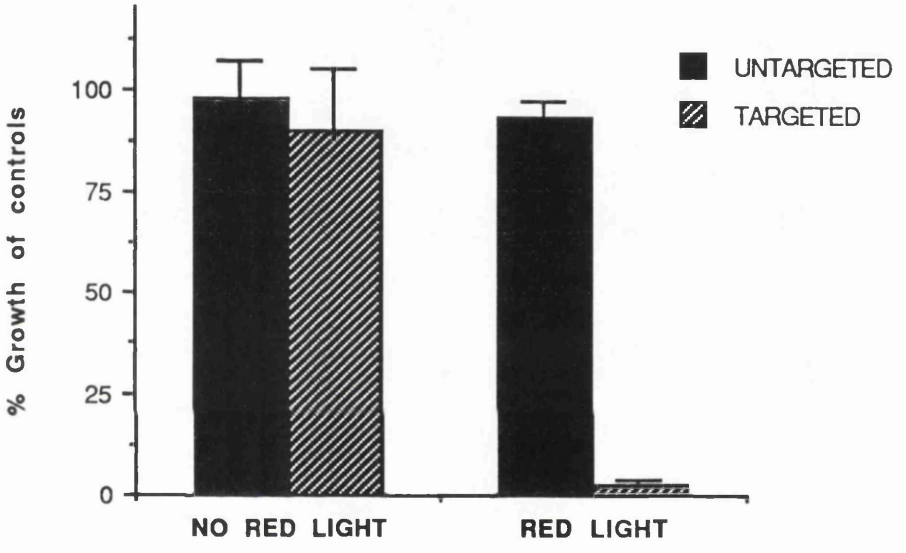
RESULTS

As shown in Figure 26, indirect targeting was as effective s direct targeting with 791T/36 Ab on 791T cells (Fig 22). Targeted liposomes in the presence of light were highly phototoxic and inhibited ^{3}Hleucine uptake, whereas in the absence of light or targeting antibody, growth was normal.

Typical dose response type curves were obtained for the B-cells (Figure 27), similar to those previously obtained for directly targeted 791T cells (Fig 25), showing decreasing

FIGURE 26

PHOTOTOXICITY OF 791T CELLS BY INDIRECTLY TARGETED AlSPc LIPOSOMES



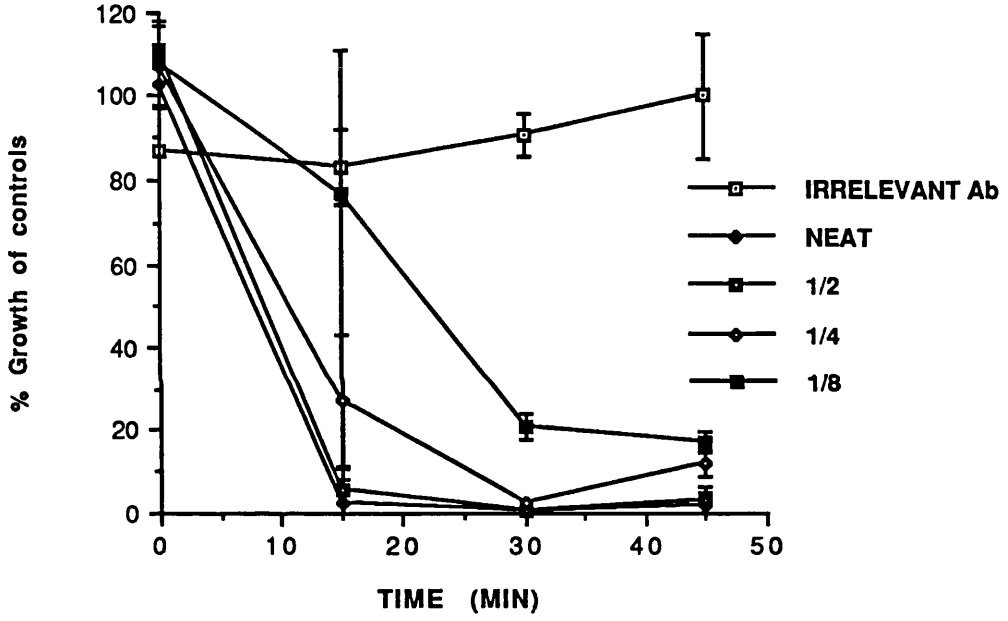
LEGEND.

Cells were treated as described in the text.

Bars are means +/- S.E.M. of 3 separate experiments with triplicates at each point.

FIGURE 27

DOSE-RESPONSE EFFECTS OF LIPOSOME DILUTION ON PHOTOTOXICITY OF RC-B-CELL LINE BY INDIRECTLY TARGETED AlSPc LIPOSOMES



LEGEND.

Cells were treated as described in the text.

Points represent means +/- S.E.M. of 3 experiments with triplicates at each time and dilution point.

inhibition of growth for increasing liposome dilutions (and hence less AlSPc bound), and greater inhibition of growth as light exposure was lengthened, for any liposome dilution.

DISCUSSION

Despite the increased distance between the cell and the AlSPc in the liposome conferred by the extra antibody, phototoxicity by indirect targeting worked as well as by direct targeting. Indirect and direct targeting are not strictly directly comparable because different amounts of monoclonal antibody were used, though in both cases there was excess present in the wells. However, for the direct targeting method, the antibody was coupled to the liposome, and although there was five times the amount present compared with the indirect experiments, not all of it was available for binding, because some liposomes will carry more than one antibody molecule. Similarly, by introducing the S-MIg, in indirect targeting, another factor affects the kinetics of liposome binding to cells. However, under the conditions used the same maximum toxicity was obtained in both cases.

A disadvantage with the indirect method is the extra incubation step required with free monoclonal antibody. This could be overcome by preincubating S-M liposomes with the antibody and removing any excess unbound so it will not compete for antigen sites on the cell.

Advantages of the indirect method are many:

a) less antibody is required for saturating sites on the

cell than would be needed for coupling to the liposomes. This is of prime importance for some monoclonal antibodies which are in short supply, or very expensive.

b) one batch of SUV-S≪M can be targeted to any mouse antibody bound to any cell, whereas direct targeting restricts a particular batch to a particular cell type, (or a limited number of cell types where the antibody is less specific).

c) SUV-S≪M can also be targeted to a 'cocktail' of antibodies of the same species, which is useful when a cell expresses a number of antigens to which there are antibodies available, particularly when they are only present in low numbers.

d) similarly, when a tumour consists of a heterogeneous population of cells, for example at different stages of maturation, use of a 'cocktail' of antibodies might be more useful at destroying the majority of the tumour than a single antibody effective against only a proportion of cells.

4.3. EXPERIMENTS WITH LIPOSOMES CONTAINING DIFFERENT CONCENTRATIONS OF AlSPC

4.3.1. COMPARISON OF PHOTOTOXICITY AND FLUORESCENCE QUENCHING

Previous dose response curves dealt only with variations in dose of light, and dilution of liposomes (Sections 4.1 and 4.2).

However, when liposomes are diluted, apart from decreasing overall AlSPc concentrations, there are also decreasing concentrations of lipids, and of the antibodies covalently bound to the surface. To gain a better idea of which AlSPc concentrations **in liposomes** are most effective as photosensitisers a whole array of liposomes containing different AlSPc concentrations (0, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mM AlSPc) was prepared. These were all similar in levels of lipid and antibody content within the standard errors of liposome preparation (Section 2.2.11).

Phototoxicity assay

A dose response curve was prepared using this array at a fixed dose of 25 minutes of red light, and the same protocol as before for both 791T and C170 cells (Section 2.2.12). The only difference was that during irradiation, the cells were suspended in PBS without FCS and growth medium was added after irradiation.

Results

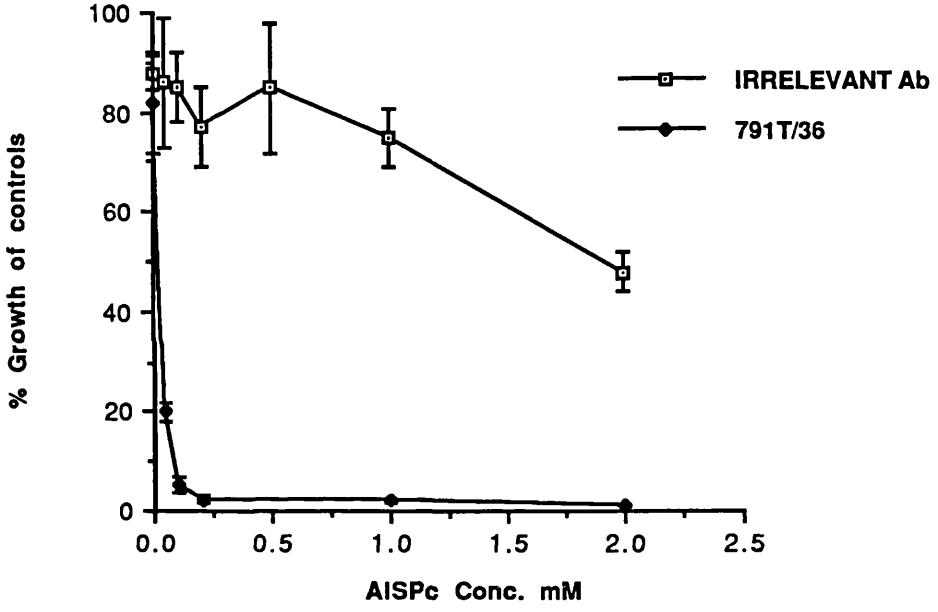
The dose response phototoxicity assays are shown in Figure 28 for 791T cells, and Figure 29 for C170 cells. They indicate that maximum phototoxicity was reached at an internal liposome AlSPc concentration of 0.2 mM for this light dose, for both cell types, though the true concentration may be between this and 0.01 mM (point not done).

Fluorimetric analysis

The liposome array was examined fluorimetrically to

FIGURE 28

EFFECT OF DIFFERENT CONCENTRATIONS OF AlSPc ENCAPSULATED IN LIPOSOMES ON PHOTOTOXICITY OF INDIRECTLY TARGETED 791T CELLS



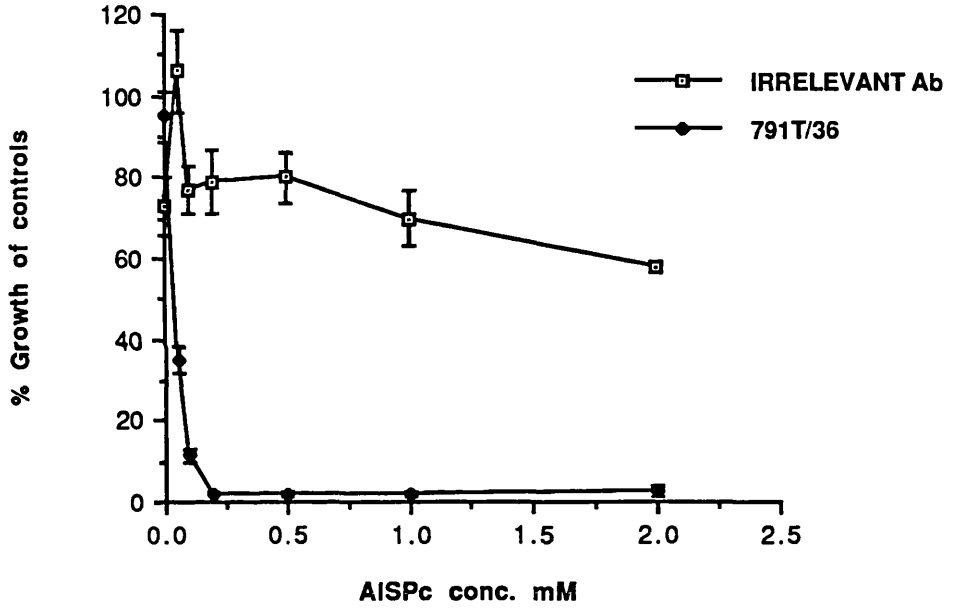
LEGEND.

Cells were treated as described in the text.

Points represent means +/- S.E.M. of 3 experiments with triplicates at each point.

FIGURE 29

EFFECT OF DIFFERENT CONCENTRATIONS OF AlSPc ENCAPSULATED IN LIPOSOMES ON PHOTOTOXICITY OF INDIRECTLY TARGETED C170 CELLS



LEGEND.

Cells were treated as described in the text.

Points represent means +/- S.E.M. of 3 experiments with triplicates at each point.

investigate quenching.

A small aliquot of liposomes was diluted 100 fold in PBS and the fluorescence scanned on a Perkin Elmer fluorimeter from 650 - 720 nm emission, using an excitation wavelenghth of 605 nm, Because the excitation and emission wavelengths were so close, a 3 mm filter, (Schot RG 645) was used to help prevent light scatter. The liposomes were then lysed by addition of 10 μl of detergent (Nonidet P- 40) to release the sequestered AlSPc, and scanned again. The sensitivity was adjusted depending on the AlSPc concentration (since higher concentrations release more AlSPc from the quenched to the unquenched form on lysis), and an allowance was made for this in subsequent calculations. For example, if for liposomes containing 2 mM AlSPc the sensitivity had to be decreased by a factor of 2, to keep the trace on scale, the fluorescence maximum would be multiplied by 2.

Results

The fluorimetric scans are shown in Figure 30. They show that the peak fluorescence is at 683 nm, and the amount of quenching (Q) at this wavelength was calculated for each AlSPc concentration according to the equation:

$$Q = 100 - \left(\frac{\text{Fluorescence before addition of NP-40}}{\text{Fluorescence after addition of NP-40}} \times 100 \right)$$

The quenching coefficients (Table 11) show that there is quenching at all concentrations of AlSPc except the lowest

FIGURE 30

FLUORIMETRIC SCANS OF AN ARRAY OF LIPOSOMES ENCAPSULATING VARIOUS CONCENTRATIONS OF AlSPc BEFORE AND AFTER DETERGENT LYSIS (A and B respectively)

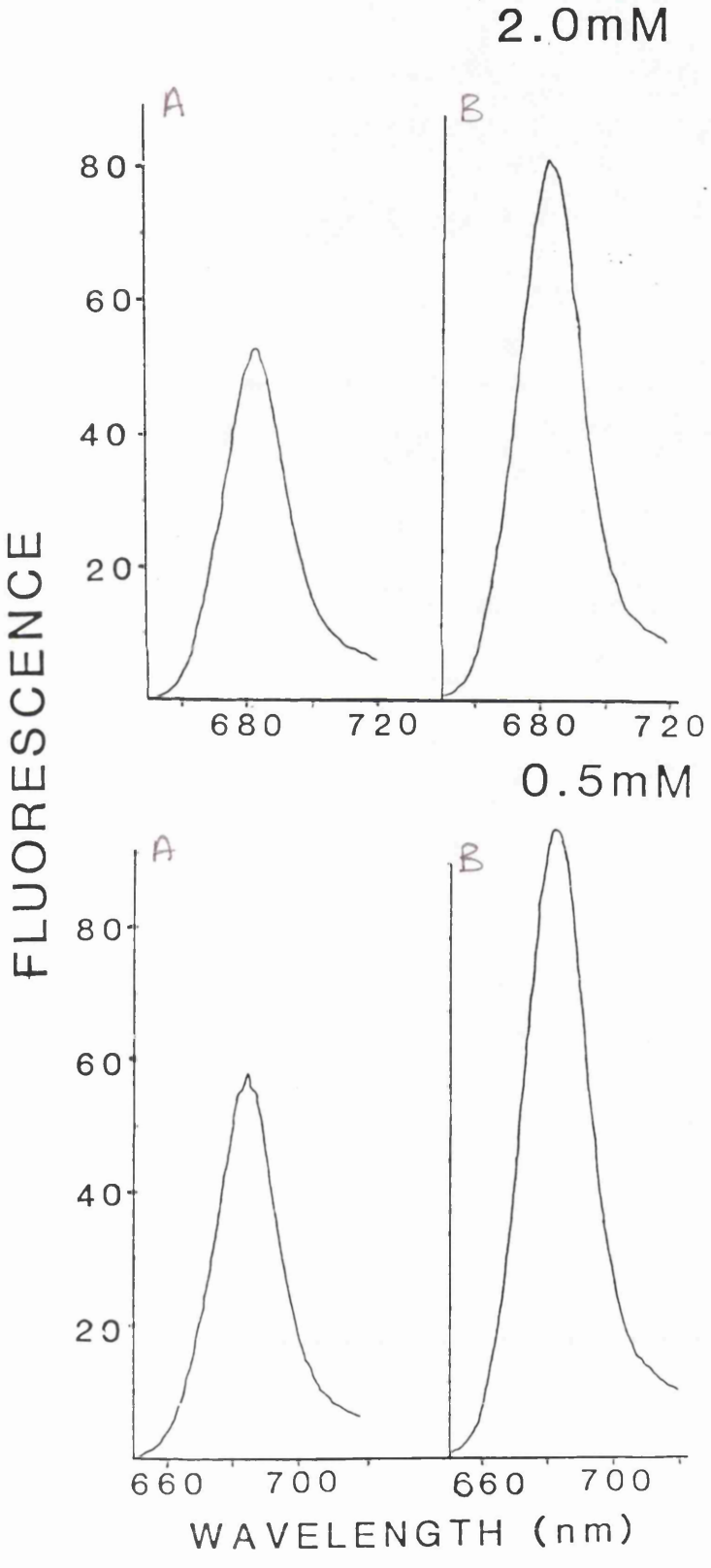


FIGURE 30 (continued)

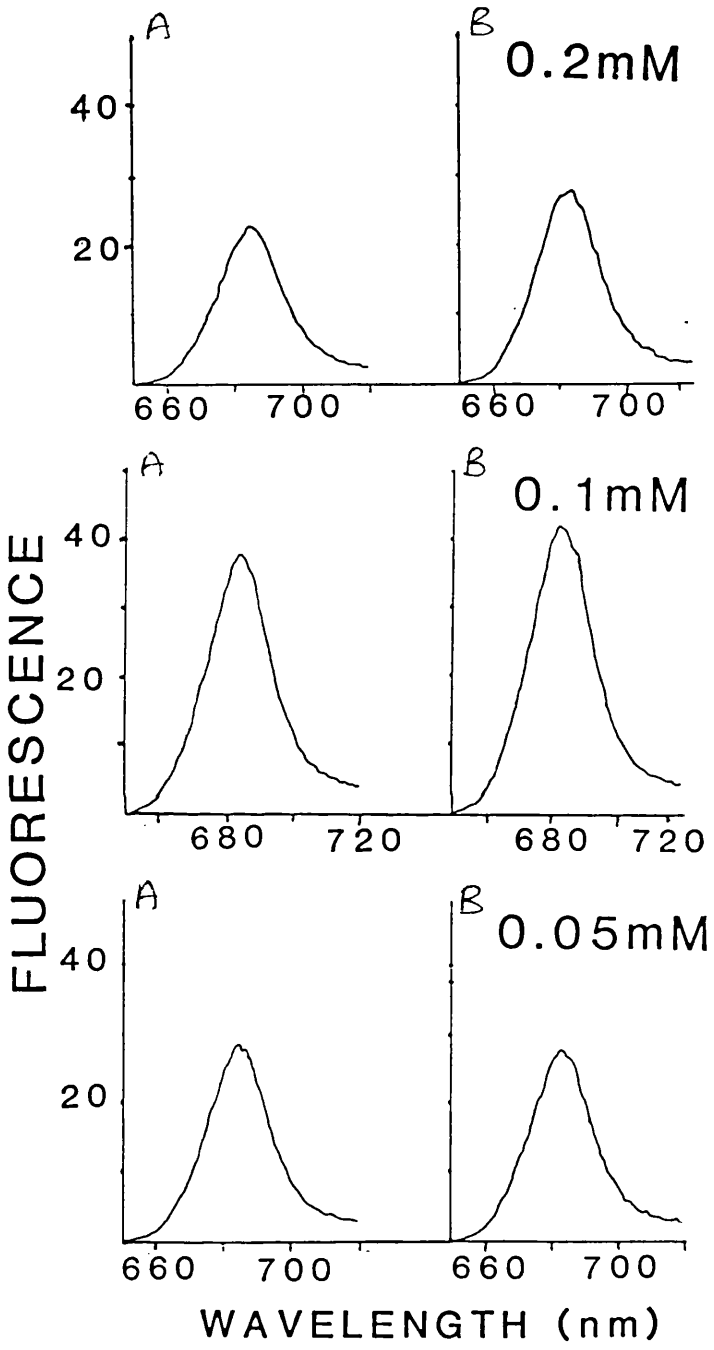


TABLE 11

FLUORESCENCE QUENCHING OF AlSPc AT DIFFERENT ENCAPSULATION CONCENTRATIONS IN LIPOSOMES

Conc. AlSPc in SUV (mM)	Relative fluorescence		% Quenching
	Before NP 40	After NP 40	
2.0	8025	24600	67
0.5	5850	9450	38
0.2	2300	2850	19
0.1	1925	2150	10
0.05	957	941	0

(0.05 mM). Despite increased quenching, fluorescence still increases with AlSPc concentration even up to the highest used here (2 mM).

Discussion

The results of the phototoxicity assay and the degree of quenching were compared to see if there was a relationship between the amount of quenching and the level of toxicity.

It was observed that there was a broad correlation between the degree of quenching and the AlSPc concentration. The maximal toxicity was achieved at a fairly low AlSPc concentration, and increasing the concentration did not increase the toxicity of cells in the assay used. This was probably because the optimal toxicity had already been reached. Increased quenching meant that some of the extra AlSPc was not available for the photosensitising reaction. It is possible that if AlSPc concentrations within the liposome were much higher than used here, the phototoxicity might have actually decreased, instead of remaining at the maximum level. This would be because the amount of quenching would eventually be greater than any increased fluorescence, thus decreasing the efficiency of the photosensitising reaction. Possibly, a more sensitive assay (such as counting colonies formed by surviving cells) would show greater toxicity at the higher AlSPc concentrations, because although there is greater quenching there is also higher fluorescence which reflects the degree of photoactivity possible (Berg et al, 1989).

An important observation was that non-specific toxicity using an irrelevant antibody was more significant ($p < 0.025$) for 791T cells and ($p < 0.005$) for C170 cells at the highest AlSPc concentration (2 mM) in these experiments than in previous ones, for example, Section 4.1.2. (when it was not significant). The lower AlSPc concentrations were not significantly toxic. The reason for this is that during irradiation there was no protective effect from the presence of FCS which has been shown to quench singlet oxygen produced by other photosensitisers (Parker and Stanbro, 1984). Also, in previous experiments, media contained phenol red as an indicator, which is also a quencher of singlet oxygen and free radicals (Foote, 1981).

Figures 28 and 29 show that maximal targeted toxicity for both 791T and C170 cells was reached at the same concentration (0.2 mM) even though the antibody concentration (2.5 μg/ml) used was not enough to saturate the antigen binding sites of the C170 cells (Table 6). It seems that without the protective quenching effects of singlet oxygen by components in the irradiation medium, more cells are killed efficiently at low concentrations of AlSPc even when they have low antigen expression, but at at high AlSPc concentrations there is more non-specific toxicity. This is not surprising since the same amount of liposomes are likely to bind non specifically to cells regardless of the concentration of their contents, but the higher the concentration of the AlSPc inside the liposome, the more AlSPc is bound non specifically.

It is interesting to compare the AlSPc concentrations which

are able to cause toxicity with the measured and calculated numbers of AlSPc molecules per liposome (Table 1). It seems that around 8 AlSPc molecules (measured value) per liposome are enough for maximum toxicity and to use more might not be necessary. However, at lower AlSPc concentrations the effect of photobleaching (the photodestruction of molecules so that they are no longer available to produce active species), might be of greater importance (Mang et al, 1987; Tralau et al, 1989; Roberts et al, 1989; Spikes and Bommer, 1989). It would be necessary to balance the effects of photobleaching against quenching. From these results it seems that although the quenched AlSPc is less photoactively efficient there is a very broad window 0.2 - 2.0 mM over which maximum toxicity of cells can be produced. It would probably be better to err on the side of a higher AlSPc concentration, albeit more quenched, than to risk photobleaching at borderline AlSPc levels.

These experiments were done at only one time point. The more subtle nuances of quenching, bleaching and phototoxicity might be better adressed by using both shorter and more prolonged light exposure times. Since there is little phototoxicity due to non specific binding except at the highest AlSPc concentrations (for the time exposure used here), there is plenty of leeway for experimentation.

4.4. PHOTOTOXIC EFFECT OF DIFFERENTIALLY SULPHONATED FRACTIONS OF AlSPc

4.4.1. INTRODUCTION

In vitro experiments with free AlSPc fractions in which the AlPc is sulphonated to a different degree, show that the phototoxic effect on cells differs, depending on the number of sulphonate groups per molecule (Paquette et al, 1988; Berg et al, 1989). Since the polarity of the molecule increases with the number of sulphonate groups, and the lipid solubility by their position on the AlPc molecule, this is bound to affect their uptake into cells. The phototoxicity is therefore influenced to some extent by the amount of photosensitiser taken up by and retained by the cell.

However, when liposomes are used, the photosensitiser remains outside the cell, so if the same concentration of the different fractions is used in liposomes, similar toxic effects might be expected regardless of the degree of sulphonation.

PREPARATION OF LIPOSOMES

In these experiments, HPLC purified fractions of tetra; tri- and di-sulphonated AlSPc plus the TLC purified fraction used in previous experiments were encapsulated in liposomes to give the same final AlSPc concentration per mole of lipid. To achieve the same AlSPc:lipid ratio, 1mM AlSPc was used for tetra- and tri-sulphonated and TLC purified AlSPc, and 0.3 mM for the di-sulphonated fraction. This latter, lower concentration was

because the di-sulphonated AlPc was taken into the lipid bilayer, as well as the aqueous compartment of the liposomes. SUV were then coupled to S MIg to give similar protein concentrations (1.3+/-0.09 x 10^{-6} molar) on the surface for all the preparations. The lipid, protein and AlSPc concentrations were therefore all constant and the only variable was the number of sulphonate groups. Any influence on the phototoxicity should therefore be due to factors caused by the effect of the sulphonate groups.

4.4.2. PHOTOTOXICITY ASSAY USING DIFFERENTIALLY SULPHONATED AlSPc FRACTIONS ENCAPSULATED IN TARGETED LIPOSOMES

791T and C170 cells were incubated as previously described (Section 2.2.12), using 791T/36 as the targeting antibody and UCHT1 2b as an irrelevant antibody (both at 2.5 μg/ml). 100% controls contained antibody only. Neat and 1 in 4 dilutions of the liposome preparations containing differentialy sulphonated AlSPc at a concentration of 1 mM were used. Neat liposomes only were used with the irrelevant antibody. Cells were treated with red light for 15 minutes, then grown, pulsed, and harvested as usual.

Neat liposomes were used to give optimal conditions to establish the efficacy of the assay, and a 1 in 4 dilution for sub optimal conditions so that any differences could be observed. It was previously shown that a 1 in 4 dilution did not produce maximal cell killing with the TLC purified fraction (though at a

higher AlSPc concentration and in the presence of FCS and phenol), after 15 minutes light exposure (Figure 25, Section 4.1.5).

RESULTS

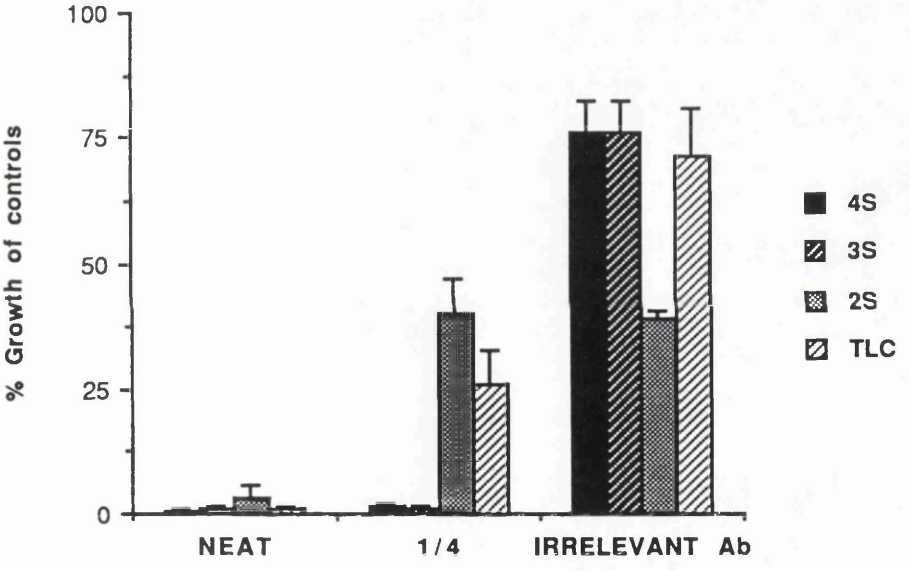
Figures 31 and 32 show the results expressed as the percentage growth of dark controls, treated in the same way except for red light exposure, for 791T and C170 cells respectively.

The neat liposomes caused maximal toxicity of 791T cells for all fractions, while for the 1 in 4 dilution, maximal toxicity was observed only with the tetra- and tri-sulphonted fractions. Toxicity was inhibited when using the 1 in 4 dilution of the TLC and di-sulphonated fractions, with the greatest effect in the latter. For the samples containing the irrelevant antibody, non specific toxicity was significant only for the di-sulphonated fraction ($p < 0.01$). Non specific toxicity was equivalent to that obtained with the 1 in 4 dilution of the di-sulphonated fraction.

Little toxicity of C170 cells was observed, even using neat liposomes. This was probably because the light exposure time of 15 minutes was too short. Previously, high toxicity of C170 cells was observed using liposomes containing this internal concentration of AlSPc (1 mM), with similar amounts of antibody attached (Section 4.3), but the light treatment was 30 minutes, twice the time used here.

FIGURE 31

EFFECT OF DIFFERENTLY SULPHONATED FRACTIONS OF AlSPc ENCAPSULATED IN LIPOSOMES ON THE PHOTOTOXICITY OF INDIRECTLY TARGETED 791T CELLS



LEGEND.

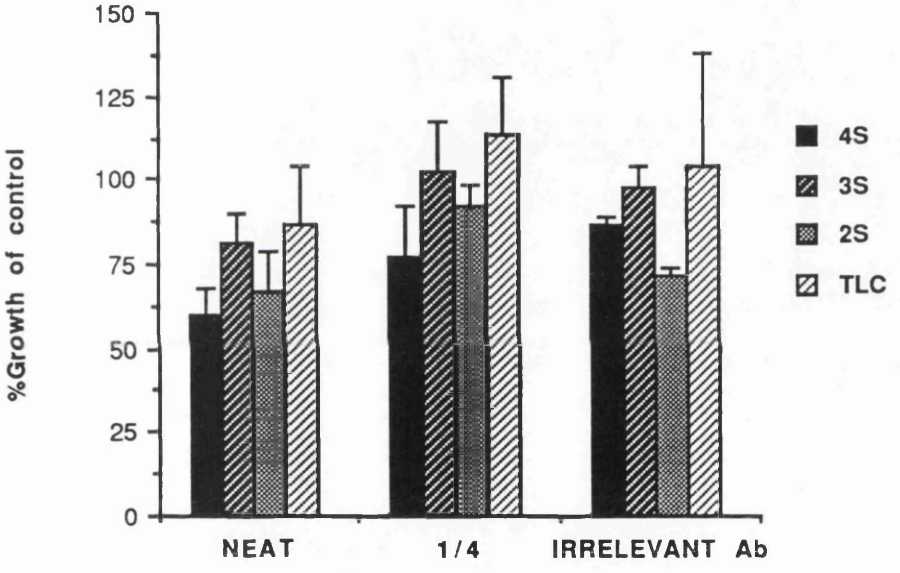
Cells were treated as described in the text.

Bars are means +/- S.E.M. of 3 experiments with triplicates at each point.

The code for the different AlSPc fractions is:
4S = tetra-sulphonated AlSPc
3S = tri-sulphonated AlSPc
2S = di-sulphonated AlSPc
TLC = Thin layer chromatography purified fraction of AlSPc

FIGURE 32

EFFECT OF DIFFERENTLY SULPHONATED FRACTIONS OF AlSPc ENCAPSULATED IN LIPOSOMES ON THE PHOTOTOXICITY OF INDIRECTLY TARGETED C170 CELLS



LEGEND.

Cells were treated as described in the text.

Bars are means +/- S.E.M. of 3 experiments with triplicates at each point.

The code for the different AlSPc fractions is:
4S = tetra-sulphonated AlSPc
3S = tri-sulphonated AlSPc
2S = di-sulphonated AlSPc
TLC = Thin layer chromatography purified fraction of AlSPc

These differential effects can partly be explained by the effect sulphonation has on aggregation of AlSPc molecules. The absorption spectra in Fig 5 (Section 1.4.1.) show the scans for tetra-, tri- and di-sulphonated AlSPc when encapsulated in liposomes. Slight shifts in the absorption peaks are due to aggregates and there is a distinct peak (signifying aggregation) at 640 nm for the di-sulphonated fraction, a shoulder for the tetra-sulphonated fraction, and no peak for the tri-sulphonated fraction. Increased absorption above 700 nm also indicates aggregation, which is again more evident for the di-sulphonated fraction. Since aggregates are not photoactive this might account for the decreased phototoxicity of di-sulphonated AlSPc liposomes at the 1 in 4 dilution. At the higher liposome concentration this is not evident because there are probably more liposomes bound to the cell, containing overall a larger total amount of unaggregated AlSPc.

Decreased toxicity for the TLC purified fraction at the 1 in 4 dilution can also be accounted for by the presence of some di-sulphonate in the partly purified mixture as shown in the HPLC trace (Fig 12), and hence increased aggregation.

Non specific phototoxicity of the untargeted liposomes (by irrelevant antibody) can be explained by the lipophilicity of the encapsulated fraction. The greatest non specific phototoxicity is seen with the most lipophilic fraction, the di-sulphonate and indeed is as great as that with the targeted 1 in 4 dilution. Non specific toxicity was not significant for the other fractions.

Previously it was shown (Sections 3.2.1 and 3.2.2) that lipophilic components present in liposomes made with unpurified AlSPc caused non specific toxicity and the use of the di-sulphonated fraction tends to bear this out. The molecules probably intercalate with the lipid bilayer and become exposed to the lipids of the cell membrane and surface proteins. This non specific binding might mask the true specific toxicity of the neat liposomes which could be much lower.

The most suitable fraction for encapsulating in liposomes seems to be either of the tri- or tetra-sulphonated components. The latter has increased aggregation at 1 mM AlSPc which is five times higher than that needed for maximum specific toxicity (Section 4.3.3). At lower concentrations this disadvantage would probably be diminished although it did not appear to have any significant affect under the conditions used here.

4.5. TREATMENT OF BONE MARROW WITH TARGETED AlSPc LIPOSOMES

To determine whether untargeted cells in cell mixtures were affected by AlSPc liposomes targeted with monoclonal antibody to a population of cells in the mixture, bone marrow was treated and the recovery of colony forming units of granulocytes and macrophages (CFU-GM colonies) was compared with untreated controls.

4.5.1. PROTEIN A TARGETED AlSPc LIPOSOMES

METHOD

The target cells in the bone marrow were T lymphocytes and the targeting antibody UCHT1 (anti CD3).

Mononuclear cells were obtained from normal bone marrow from donors (Section 2.2.16). One million cells were incubated successively at 4°C in the dark with UCHT1 2a antibody (2.5μg/ml), and AlSPc liposomes with PA attached (1 mM internal AlSPc concentration), for 30 minutes each, washing twice after each incubation.

The following antibody and liposome combinations were used:

A) No treatment

B) Ab + SUV(AlSPc)-PA

C) Ab + SUV(AlSPc)

D) Ab + SUV-PA

E) Ab + SUV

Cells were then treated with laser for 15 minutes at 40 mW or kept in the dark and added to soft agar to give a final number of 2 x 10^5 per 30 mm plate (triplicates) in 1 ml of medium. The growth medium consisted of IMDM (double strength), FCS and 0.9% agar in the ratio 3:2.5:3, and contained 10% GCT (Giant cell tumour) conditioned medium, which contained factors necessary for colony growth. When the agar had set, the plates were incubated in a humidified atmosphere at 37°C in 4% CO_2 for 14 days and the

CFU-GM colonies were counted. A colony was scored when it contained more than 50 cells.

RESULTS

Figure 33 shows the colony recovery of both laser treated marrow and dark controls as a percentage of untreated cells. (Means of 2 experiments). The results are quite variable, but it is clear that cells treated with targeting antibody and SUV(ALSPc)-PA to bind to the antibody, plus laser light, did not produce fewer colonies than the untreated controls. It therefore appears that the AlSPc liposomes were not toxic to progenitor cells present in the bone marrow

DISCUSSION

Only 2 experiments were done with the PA liposomes, which probably explains the high variability. However, it was decided for reasons stated earlier (Section 3) that PA was not the ideal protein to use for targeting to antibodies on cells. A further series of experiments on colony recovery from AlSPc liposome treated bone marrow, using S×MIg as the binding protein gives a more statistically valid result. (Section 4.5.2.).

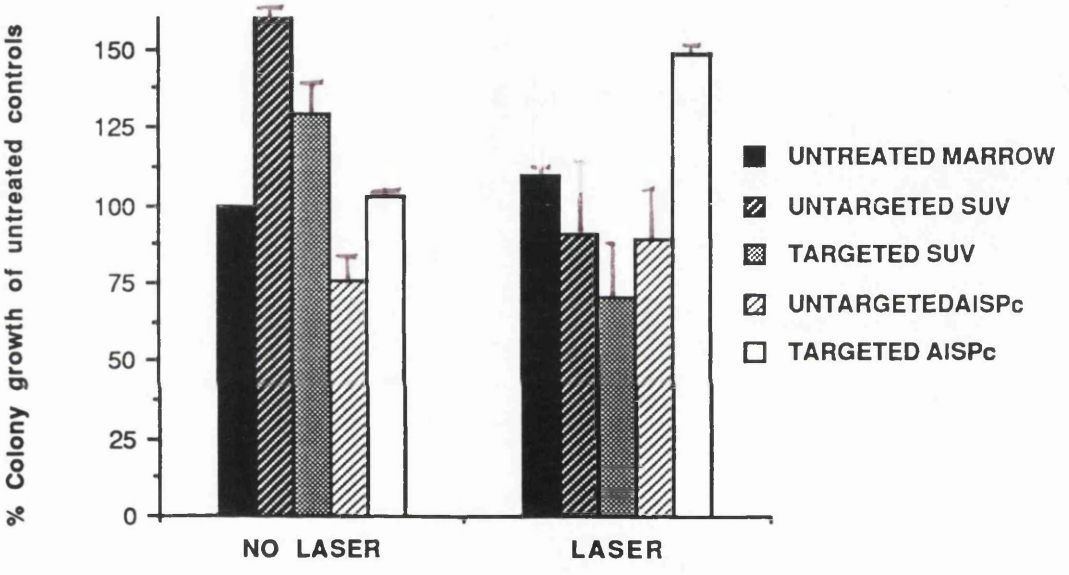
4.5.2. S×MIg TARGETED AlSPc LIPOSOMES

METHOD

Bone marrow harvested from patients with non Hodgkins lymphoma in remission were used in these experiments. The target

FIGURE 33

COLONY GROWTH OFCFU-GM AFTER PHOTODYNAMIC TREATMENT OF NORMAL BONE MARROW WITH ANTI-CD3 ANTIBODY. PROTEIN A TARGETED AISPc LIPOSOMES. AND LASER LIGHT



LEGEND.

T lymphocytes in normal bone marrow were targeted indirectly with anti-CD3 antibody, and Protein A coupled liposomes with and without AISPc. After laser irradiation the cells were cultured and CFU-GM colonies counted to determine the effect of treatment on the non targeted progenitor cells.

Bars are means of 2 separate experiments with triplicates at each point, and show S.D.

cells were T lymphocytes and B cells, and the targeting antibodies UCHT1 G1 (2.5 μg/ml) and 8A (1 in 1000 dilution of ascites) respectively.

One million mononuclear bone marrow cells (Section 2.2.16) were incubated successively at 4°C with antibody and AlSPc liposomes (1 mM internal AlSPc concentration) with S∝MIg attached, for 30 minutes each, washing twice each time.

The following antibody and liposome combinations were used:

A) No treatment

B) Ab only (UCHT1 or 8A)

C) SUV(AlSPc)-S∝MIg only

D) Ab (UCHT1 or 8A) + SUV(AlSPc)-S∝MIg

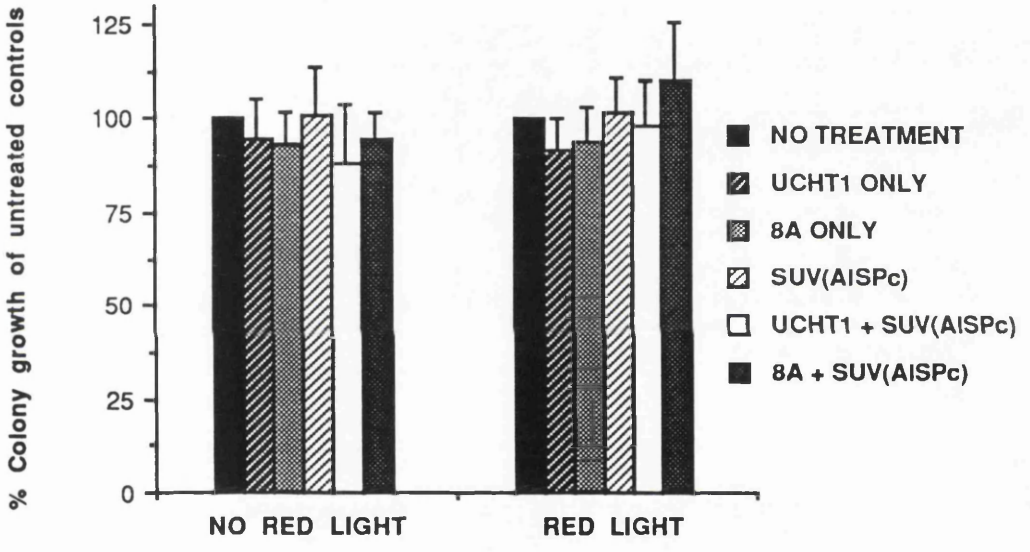
The bone marrow cells were then exposed to fluorescent red light for 30 minutes or kept dark, plated in soft agar in triplicates and grown as described in Section 4.5.1. Growth factors were supplied by addition of 10% 5637 bladder carcinama conditioned medium. CFU-GM colonies were counted after 14 days.

RESULTS

Figure 34 shows the recovery of light and dark treated bone marrow as the means+/-S.E.M of four experiments on four different marrows with triplicates at each point. There was no significant difference (Student's t-test) between any of the treatments with or without light exposure, or targeted or untargeted with either antibody.

FIGURE 34

COLONY GROWTH OF CFU-GM AFTER PHOTODYNAMIC TREATMENT OF BONE MARROW CELLS WITH ANTI-CD3 OR ANTI-B-CELL ANTIBODY. ANTI-MOUSE AISPc LIPOSOMES. AND RED FLUORESCENT LIGHT



LEGEND.

Subpopulations of T lymphocytes and B-cells in bone marrow were targeted with AISPc liposomes using the specific antibodies as shown in the key above. The cells were then treated with red light and cultured to observe the effects on the non targeted CFU-GM progenitor cells.

Bars are means +/- S.E.M of 4 separate experiments with triplicates at each point.

DISCUSSION

It was previously shown that AlSPc liposomes could cause specific toxicity when targeted to T lymphocytes (Section 3.2.3.), and B cells (Section 4.2.2). In this experiment AlSPc liposomes were targeted to these same cell types, but in the prescence of non-target cells (the other bone marrow components), and exposed to red light. This treatment had no effect on the survival of the untargeted progenitor cells which produce CFU-GM. However, this experiment did not actually show that any SUV(AlSPc)-S-MIg reached their target or indeed, killed any of the target cells. It is possible that in cell mixtures such as bone marrow the influence of non-target cells might affect phototoxicity, due to effects on the distribution of liposomes, antibody, or light penetration. This type of protective effect by other cells has been found for chemical agents (Hervé et al, 1983), but provided sufficient liposomes are able to reach the target cells, should not be a great problem with photodynamic treatment of bone marrow.

Specific targeting in both cell mixtures and single cell suspensions is examined by a fluorescence technique in Chapter 5.

4.6. INVESTIGATION OF LIPID DAMAGE CAUSED BY PHOTOACTIVATED AlSPc LIPOSOMES

INTRODUCTION

That light mediated toxicity occurs in the presence of

targeted AlSPc liposomes has been shown, but the mechanism whereby the cells are lysed and killed is uncertain. Membrane peroxidation is one way in which cells can be lysed and to find out if it plays a role in photosensitised toxicity, cells were examined for evidence of lipid peroxidation after photodynamic treatment in the presence of AlSPc liposomes.

METHOD

2.5 million cells (791T and C170) in triplicate for each sample were incubated with antibody and liposomes in the same way as for a phototoxicity assay. Cells alone, and with targeted or untargeted AlSPc liposomes and AlSPc liposomes alone (100 μl) were illuminated with red fluorescent light for 45 minutes, then cultured for 24 hours in RPMI without phenol red and with 10% FCS. The samples (cells plus supernatant) were made up to 1 ml with PBS and assayed for thiobarbiturate reactive chromogen as described in the MDA assay section 2.2.17.

RESULTS

Results were expressed as n molar TMP per 2.5 million cells as shown in Table 12. Levels of thiobarbiturate reactive chromogen were low for all samples and no significant difference was observed between cells alone and either untargeted or targeted light treated samples. Liposomes alone also showed very low levels of chromogen.

TABLE 12

PEROXIDATION OF LIPIDS AFTER PHOTODYNAMIC TREATMENT OF CELLS TARGETED WITH AlSPc LIPOSOMES

Treatment	nM TMP No Red Light	nM TMP Red Light
791T cells		
Cells alone	10.5+/- 2.5	11.6+/-5.1
Targeted	13.3+/- 6.5	8.2+/-2.9
Untargeted	4.7+/- 3.8	7.0+/-2.5
C170 cells		
Cells alone	23.4+/-14.9	8.2+/-3.7
Targeted	8.2+/- 3.7	5.8+/-5.6
Untargeted	7.0+/- 2.5	5.9+/-1.4
Liposomes Only	5.9+/- 3.8	5.9+/-1.4

Results are means +/- S.E.M. of triplicates in 3 separate experiments.
The results are expressed for 2.5 million cells per sample.
Liposomes only results were for 0.1 ml of liposome preparation per sample, which were approximately 2 mM with respect to phospholipid content.

DISCUSSION

There was no evidence of lipid peroxidation greater than that of untreated cells for photosensitised samples. There was also no evidence of peroxidation in the liposome only samples, but this is not surprising since they are composed mainly of saturated phospholipids which are not susceptible to oxidation by singlet oxygen. Typical figures measured by the same assay for peroxidation initiated by addition of ferric ions (ferric ammonium citrate) to rat liver cells, were at least more than five times the highest figure obtained in these experiments and for only one million cells (Porter et al, 1986).

These results suggest that cell toxicity is not mediated by lipid peroxidation. A more likely mechanism is crosslinking of membrane proteins on the cell surface which would be easily accessible to singlet oxygen produced in the liposomes. Crosslinking of proteins would cause functional inactivation, resulting in a perturbation of the normal homeostatic mechanisms of the cell. This would eventually lead to cell death if sufficiently severe.

CHAPTER 5

DETECTING AlSPc FLUORESCENCE

5.1. DETECTION OF AlSPc FLUORESCENCE

Fluorescence from light activated AlSPc bound to cells in antibody targeted liposomes can be shown using a sensitive photometric detector, a cooled charge couple device (CCD) camera, as described by Barr et al 1988. It was used here with only slight modifications. Briefly, an inverted microscope (Nikon Diaphot) with epifluorescence and phase contrast attachments was coupled with a cooled CCD camera (Wright Instruments, model 1, resolution 600 x 400 pixels). The CCD camera is a slow-scanning device which time-integrates the fluorescent signal received. Cooling with liquid nitrogen reduces background noise in order that long scanning times can be used, resulting in high sensitivity. The light source for excitation was a 1 mW helium-neon laser, tuned to 632.8 nm. AlSPc fluorescence between 665 and 700 nm was detected by a photomultiplier, using suitable band pass filters.

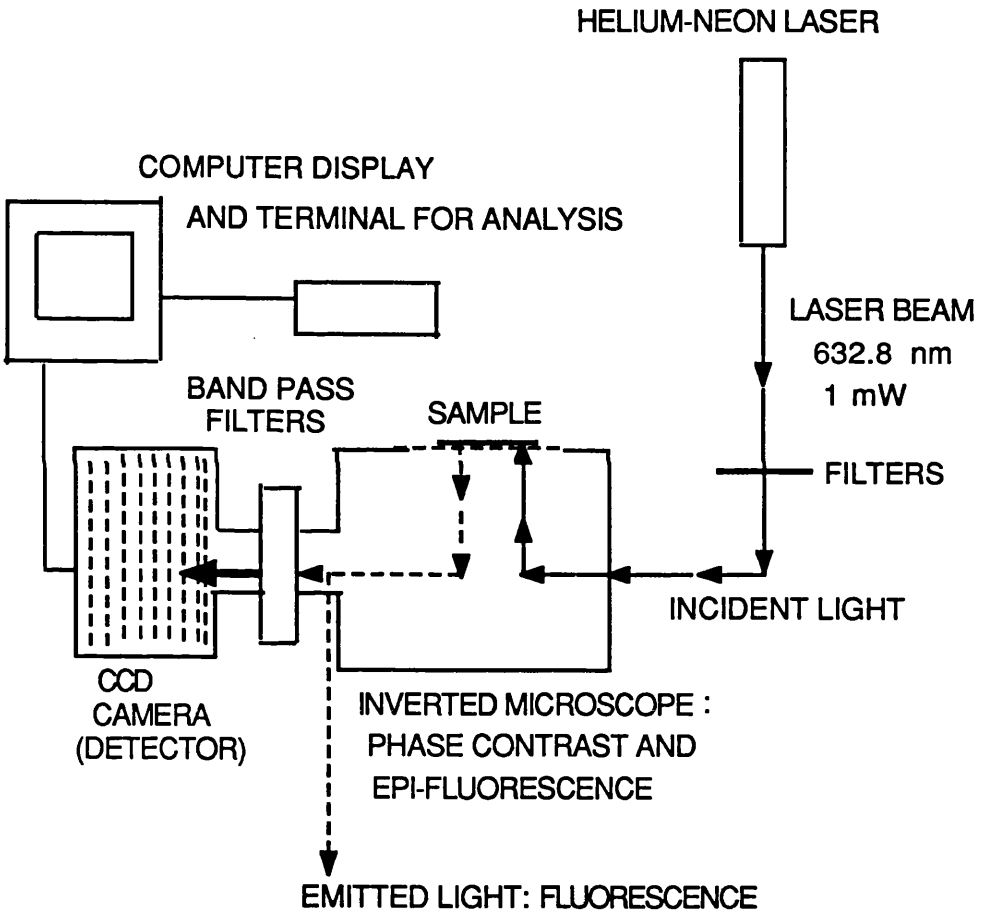
The laser beam was focused through the objective and a glass slide placed upside down on the stand, onto the cell sample underneath. Fluorescence was detected by the CCD camera, and processed to produce a digital image by an IBM PC, which also controlled the camera operation. The layout is shown in Fig. 35.

5.2. DEMONSTRATION OF SPECIFIC TARGETING TO CELLS IN CELL MIXTURES

Specific phototoxicity of AlSPc in liposomes targeted to cells by antibody has been demonstrated in single cell populations in previous sections. Because of difficulties in

FIGURE 35

DIAGRAMMATIC REPRESENTATION OF THE LAYOUT OF CCD CAMERA, COMPUTER, LASER AND MICROSCOPE FOR ANALYSIS OF AlSPc FLUORESCENCE



growing up cells from mixtures separately, this has not been done in the present work (although CFU-GM have been successfully grown from treated bone marrow, section 4.5.). However, specific targeting in cell mixtures can be shown by fluorescent microscopy using the CCD camera.

CELL SAMPLES

These were cytospins prepared from cell mixtures of 791T and DW-BCL in equal numbers, incubated with 791T/36 or irrelevant antibody (UCHT1 2b), and SUV (AlSPc)-S∝M under the same conditions as the phototoxicity studies (Section 2.2.12.).

METHOD

Using the CCD camera, phase contrast and AlSPc fluorescence pictures were recorded, with a 500 ms exposure time, from the same field of the slide (with a 40 X objective), and photographs taken of the processed digital images displayed on the computer screen, using Kodak Ektachrome positive film, ASA 400.

RESULTS

Figure 36 shows the the phase contrast and corresponding fluorescent images of the cell mixtures. The phase contrast picture (A) shows 791T cells, the larger, arrowed cells, surrounded by smaller DW-BCL cells. In the fluorescent picture (B), only 791T cells displayed fluorescence. DW-BCL cells did not bind 791T/36 and therefore could not bind SUV(AlSPc)-S∝M and hence show no fluorescence.

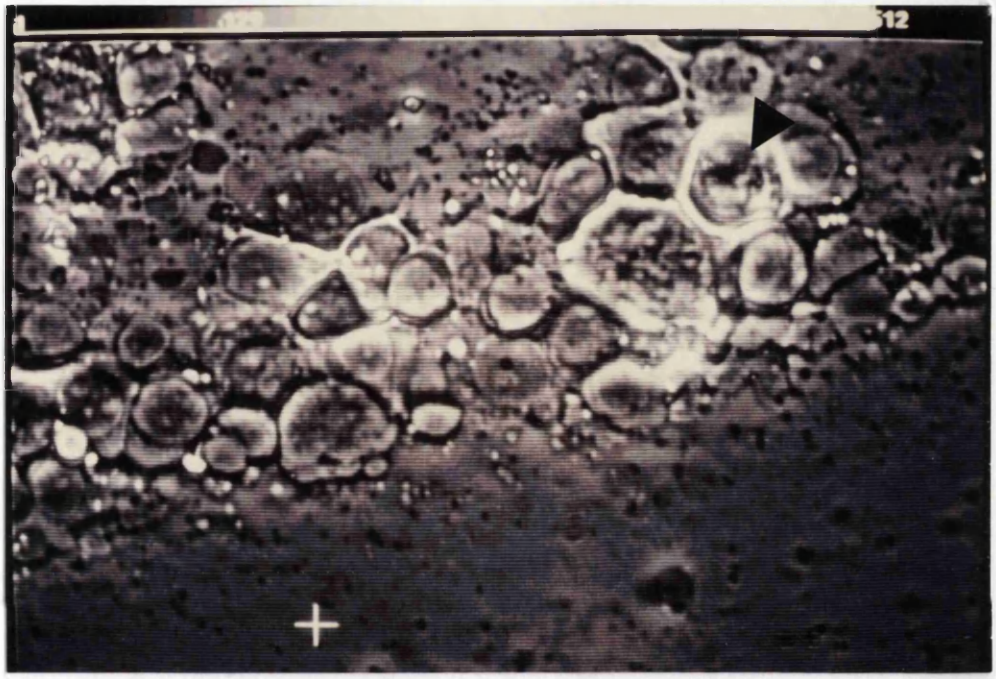
LEGEND TO FIGURE 36

The phase contrast figure (I) shows a cluster of large 791T cells, arrowed, surrounded by smaller B-cells.
The fluorescence figure (II) shows fluorescence only on the targeted 791T cells. there is no fluorescence on the untargeted B-cells

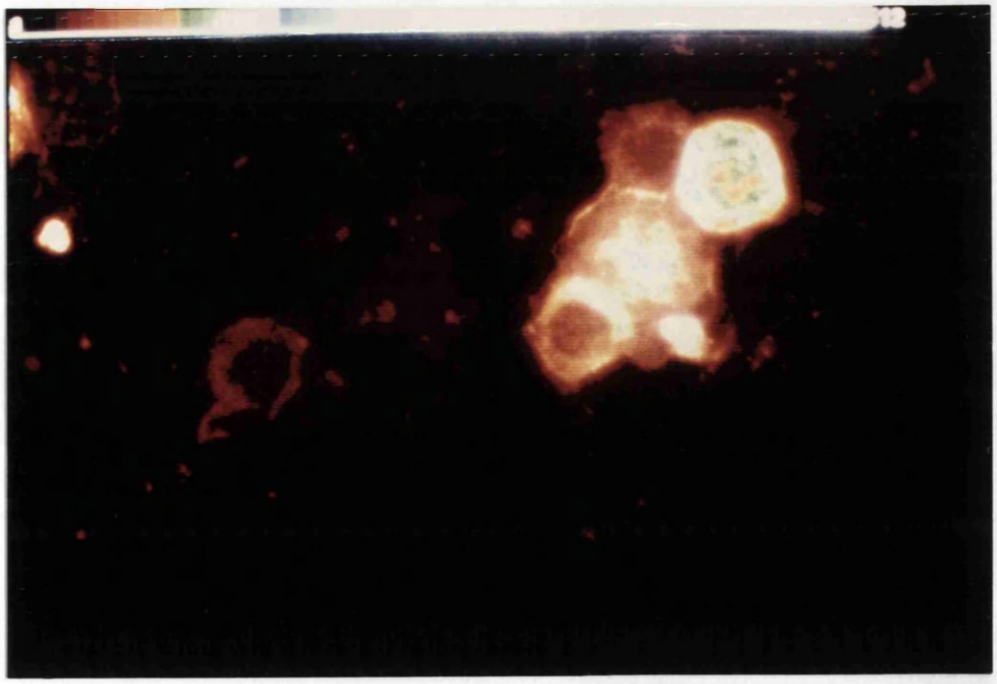
FIGURE 36

PHASE CONTRAST AND FLUORESCENT IMAGES OF A CELL MIXTURE TARGETED INDIRECTLY BY AlSPc LIPOSOMES AND RECORDED BY CCD CAMERA

I) PHASE CONTRAST



II) FLUORESCENCE



The fluorescence intensity is indicated by the scale at the top of the photograph, increasing from left to right, and from red, the lowest to white, the highest.

DISCUSSION

The photographs of the cell mixtures indicate that AlSPc in liposomes can be successfully targeted to specific cells in heterogeneous populations by using appropriate antibodies.

The pattern of fluorescence observed on most targeted cells showed greatest intensity at the perimiter, which is typical of the 'rim' of fluorescence seen in ordinary cell surface immuno-phenotyping by fluorescence microscopy. Single line and box profiles of fluorescence emitted from single cells, Figures 37A and 37B respectively, were measured on the computer by plotting the intensity detected in each pixel, giving semi-quantitative data. The profile shapes also indicate that the fluorescence is probably due to surface binding. Large variations in fluorescence can be seeen in the photographs, a reflection of the numbers of antigens expressed on the cells.

With this microscope and system, only two-dimensional scans can be done, so the signal is the total fluorescence detected at the focal point of the microscope, and cannot differentiate between external and internal AlSPc. A confocal microscope with the technology to build up a three-dimensional scan would be necessary to determine whether or not AlSPc had entered the cell. Instead, to demonstrate that phototoxicity was due to externally

LEGEND TO FIGURE 37

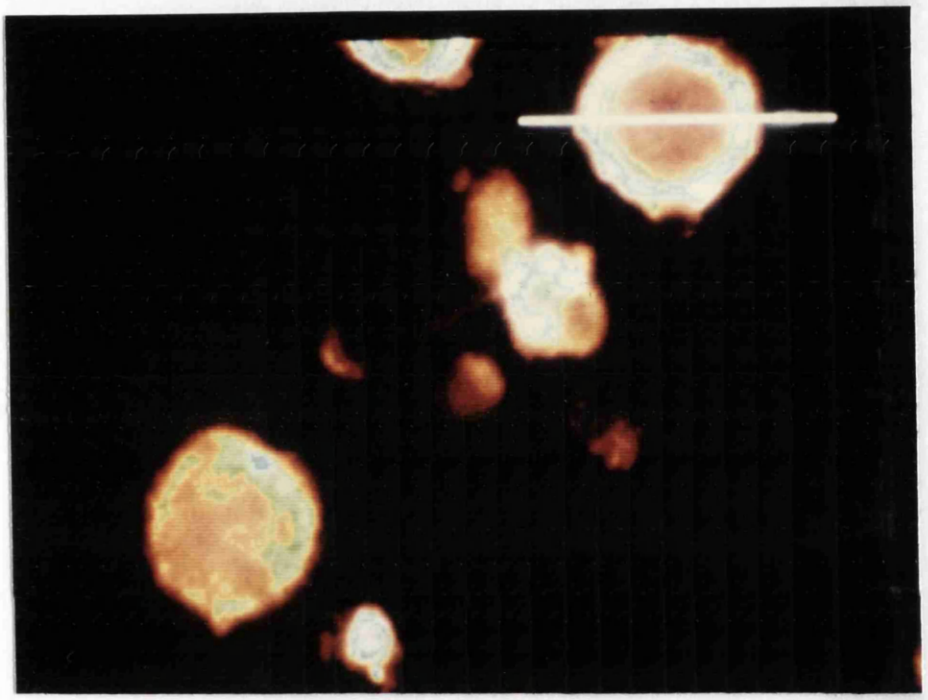
The upper photographs in both A and B show the line or area delineated for building up the profile shown in the lower photographs.
The intensity of fluorescence in each pixel covered by the defining line or box is plotted to give arbitrary figures which are proportional to the length of time the field was scanned.

FIGURE 37

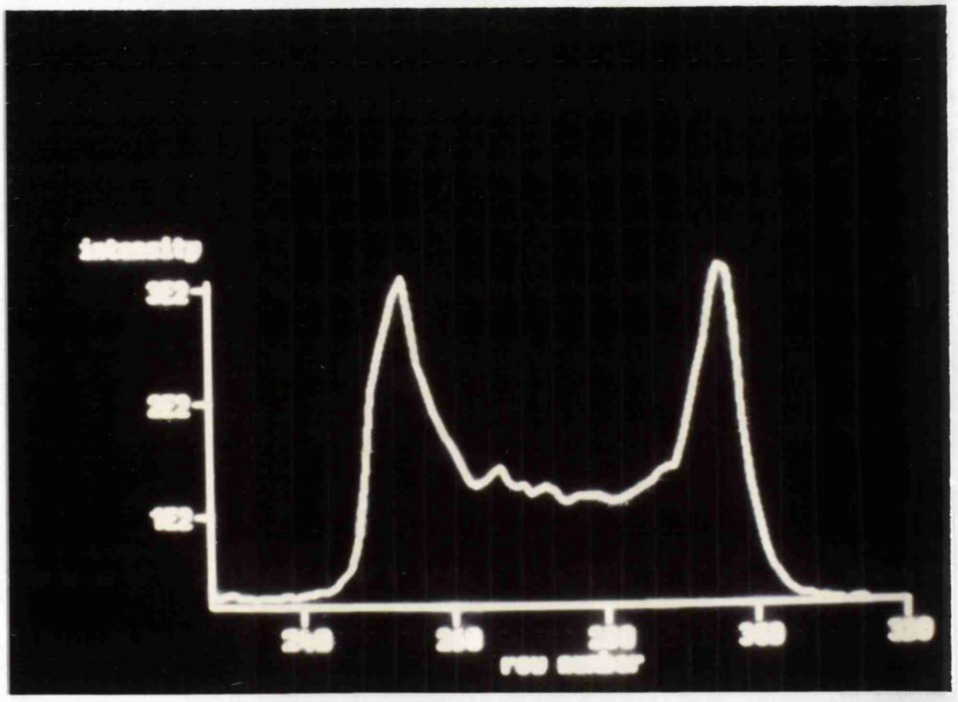
SINGLE LINE AND BOX PROFILES OF CCD CAMERA RECORDED FLUORESCENT IMAGES FROM CELLS INDIRECTLY TARGETED WITH AlSPc LIPOSOMES

A) SINGLE LINE PROFILE

I)

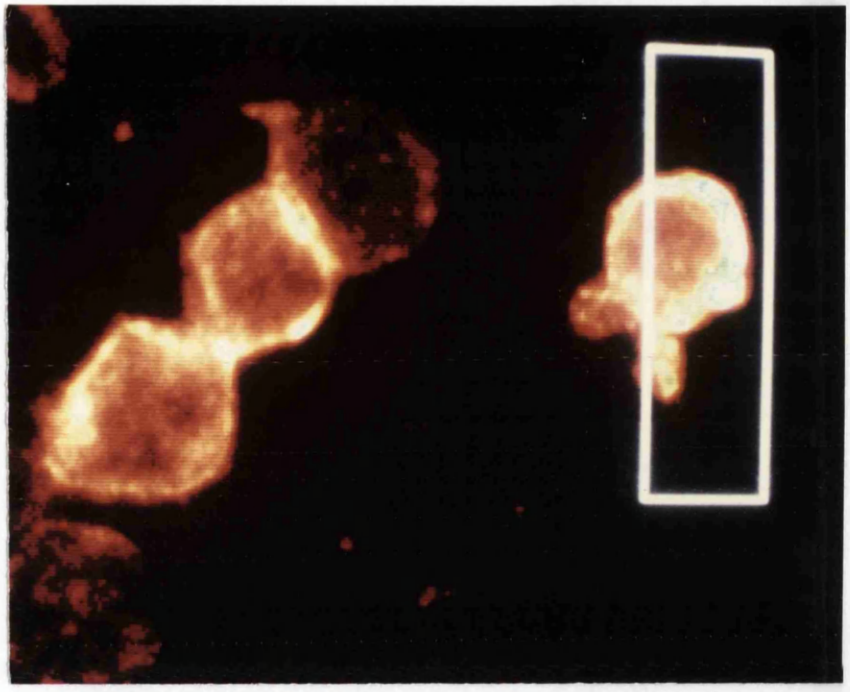


II)

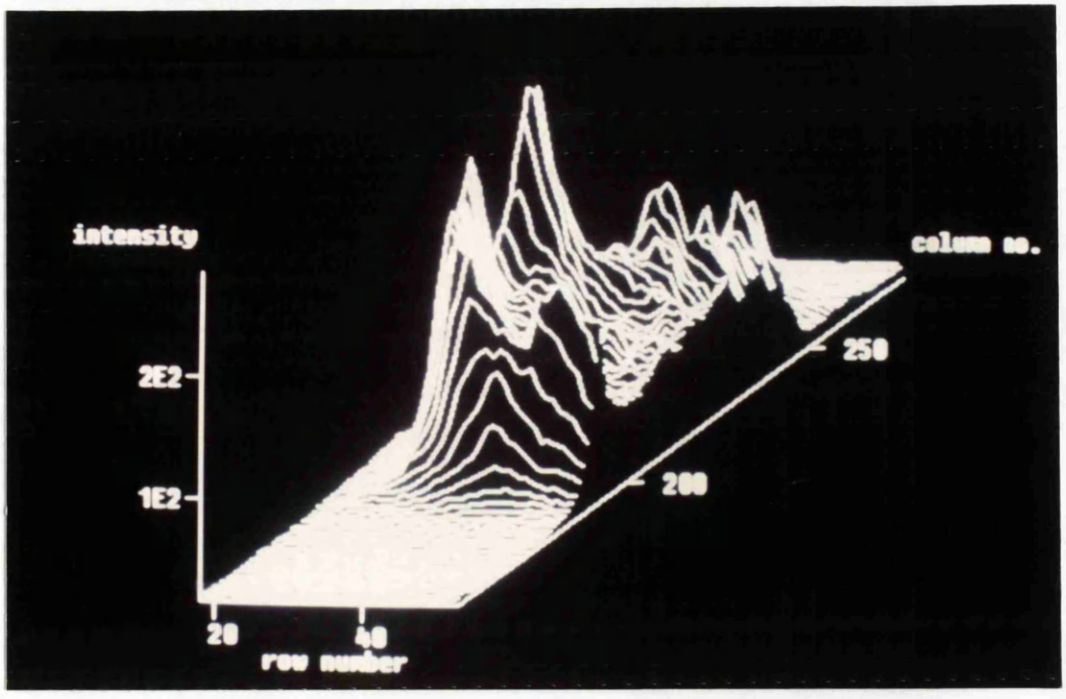


B) BOX PROFILE

I)



II)



bound liposomes, a technique using enzyme to strip off antibody bound liposomes was used.

5.3. EVIDENCE SHOWING THAT PHOTOTOXICITY IS DUE TO AlSPc IN LIPOSOMES BOUND TO THE SURFACE OF CELLS AND NOT INTERNALISED AlSPC

Both specific and non specific or background SUV(AlSPc)-S∝M binding to cells can be demonstrated by capturing and processing fluorescent images of treated cells by the CCD camera technique. Background fluorescence due to non specific binding is very low and has been shown to have no phototoxic effect experimentally (see previous sections). However, it has been suggested that phototoxicity is caused by bound SUV(AlSPc)-S∝M being taken up into the cell and activated, and not by external AlSPc. To demonstrate that phototoxicity is not due to AlSPc taken up into the cell, cells were incubated with antibody and SUV(AlSPc)-S∝M, then treated with ZZAP, a reagent containing 0.1% cysteine-activated papain and 0.1M dithiothreitol (a modified version of the ZZAP method (Branch and Petz, 1982)) to strip off liposomes that were bound to the cell only by antibody. Any liposomes or AlSPc that had bound to the lipid membrane or had been taken into the cell would not be removed.

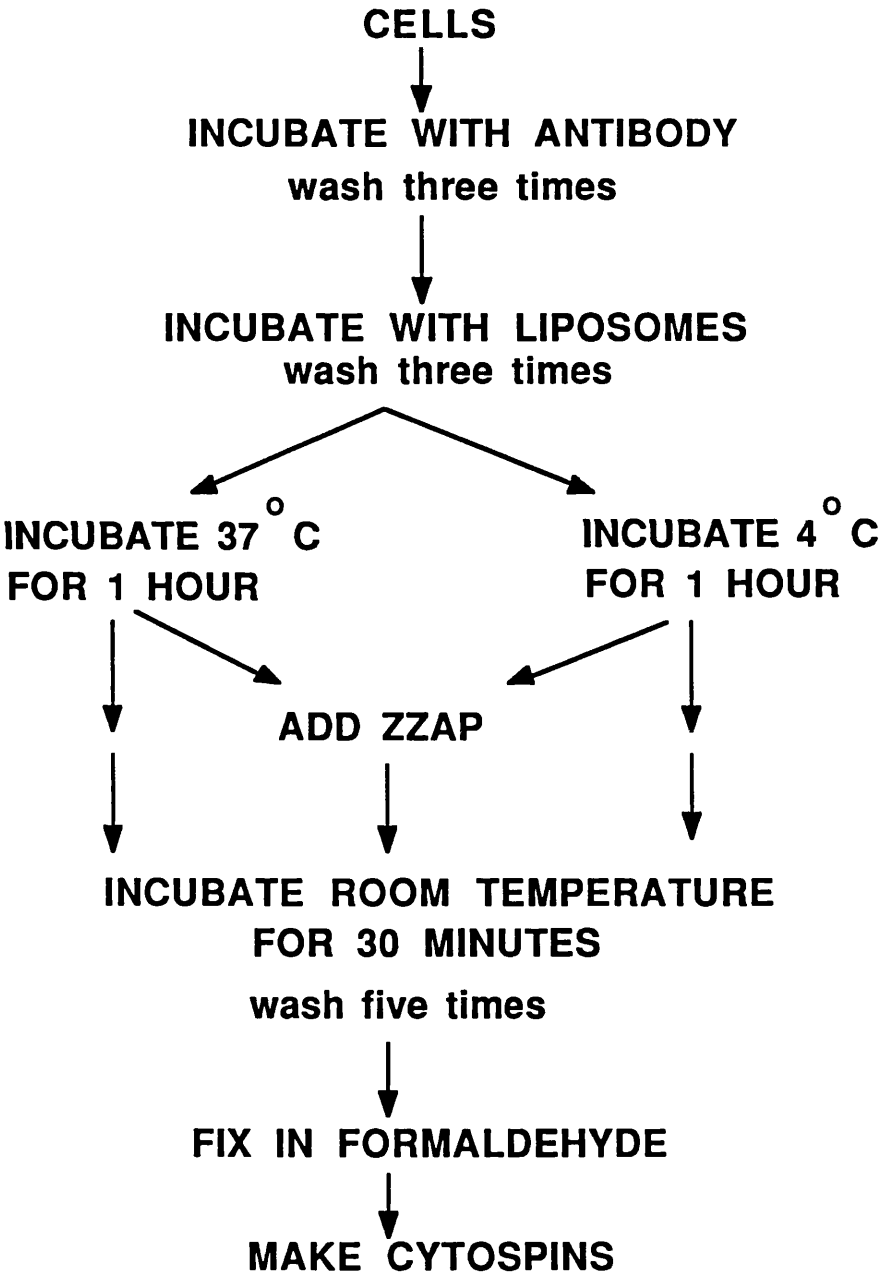
5.3.1. PAPAIN TREATMENT OF TARGETED CELLS

METHOD

Cells were incubated at 4°C with 791T/36 or irrelevant antibody UCHT1 2b, and then SUV(AlSPc)-S∝M (containing 2 mM

FIGURE 38

PAPAIN TREATMENT OF CELLS TARGETED BY ANTIBODY AND AlSPc LIPOSOMES



AlSPc), for 30 minutes each followed each time by 3 washes. The following combinations, each designated a letter for easy identification were used:

A) Cells + UCHT1 + SUV(AlSPc)-S∝M

B) Cells + 791T/36 + SUV(AlSPc)-S∝M (2 sets)

C) Cells + 791T/36 + SUV(AlSPc)-S∝M + ZZAP (2 sets)

Some cells (one each of B and C) were then incubated at 37°C for one hour to give better conditions for uptake of bound liposomes. The remainder were kept at 4°C. ZZAP reagent was then added to both sets of C, and all the cells except those previously incubated with irrelevant antibody (A), were incubated at room temperature for 30 minutes. Cells were then washed five times, and finally resuspended in formaldehyde fixative. The treatment procedure is summarised in Figure 38.

The incubation at 37°C might allow some of the liposomes to internalise, and the cells then compared with those kept at 4°C, when no internalisation could occur.

Cytospins were made of these cell preparations and the cells examined under the microscope with the CCD camera.

RESULTS

Figure 39 shows the phase contrast and fluorescence pictures of the treated cells. The results show that the cells with specifically bound liposomes fluoresce brightly (Figure 39A). Results were similar for cells kept at both 4°C and 37°C) Non

LEGEND TO FIGURE 39

A) Bright fluorescence due to AlSPc specifically bound by antibody at 4°C.

B) Background fluorescence due to non specifically bound AlSPc liposomes at 4°C.

C) Fluorescence of ZZAP stripped cells due to any AlSPc liposomes that is not specifically bound by antibody at 4°C, (similar to background, B).

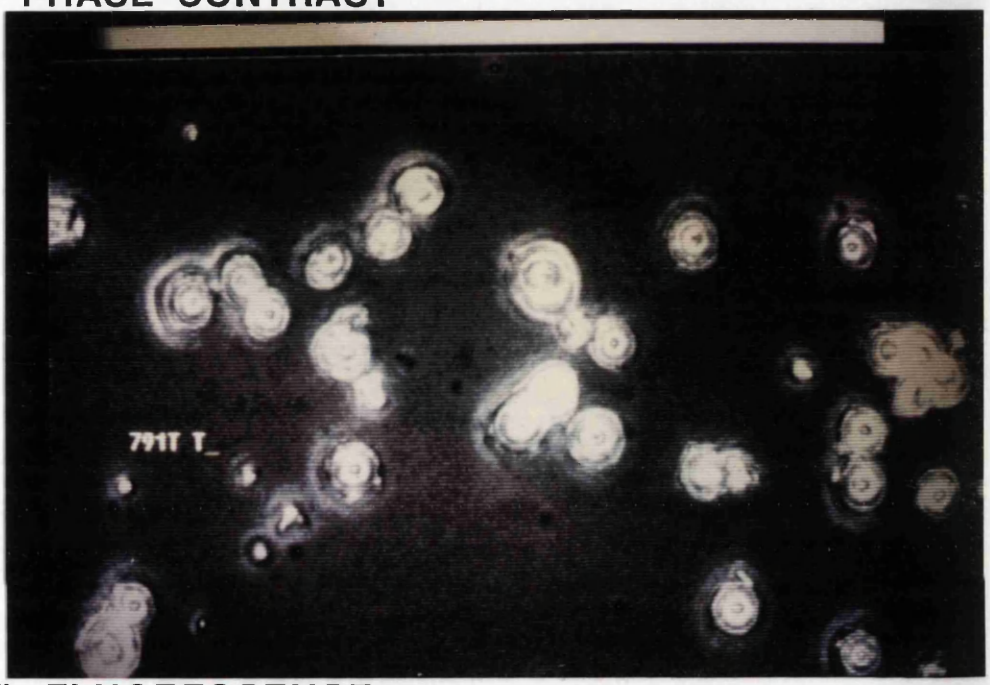
D) Fluorescence of ZZAP stripped cells due to any AlSPc that had been internalised at 37°C (similar to background, B).

FIGURE 39

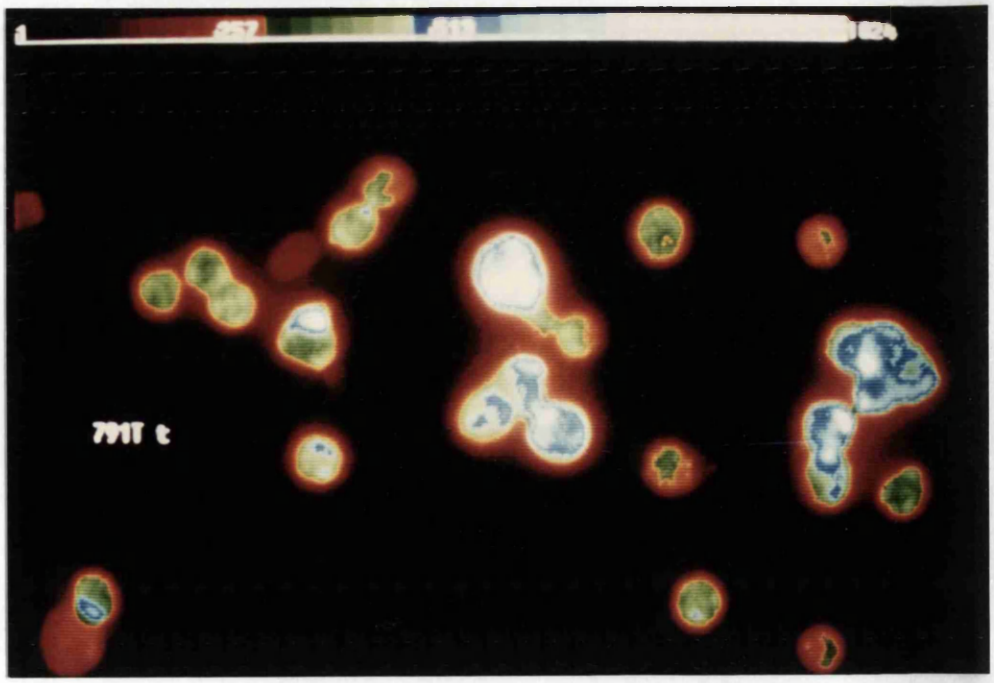
PHASE CONTRAST AND FLUORESCENT IMAGES OF CELLS TARGETED INDIRECTLY BY ANTIBODY AND AlSPc LIPOSOMES BEFORE AND AFTER PAPAIN TREATMENT

A

I) PHASE CONTRAST

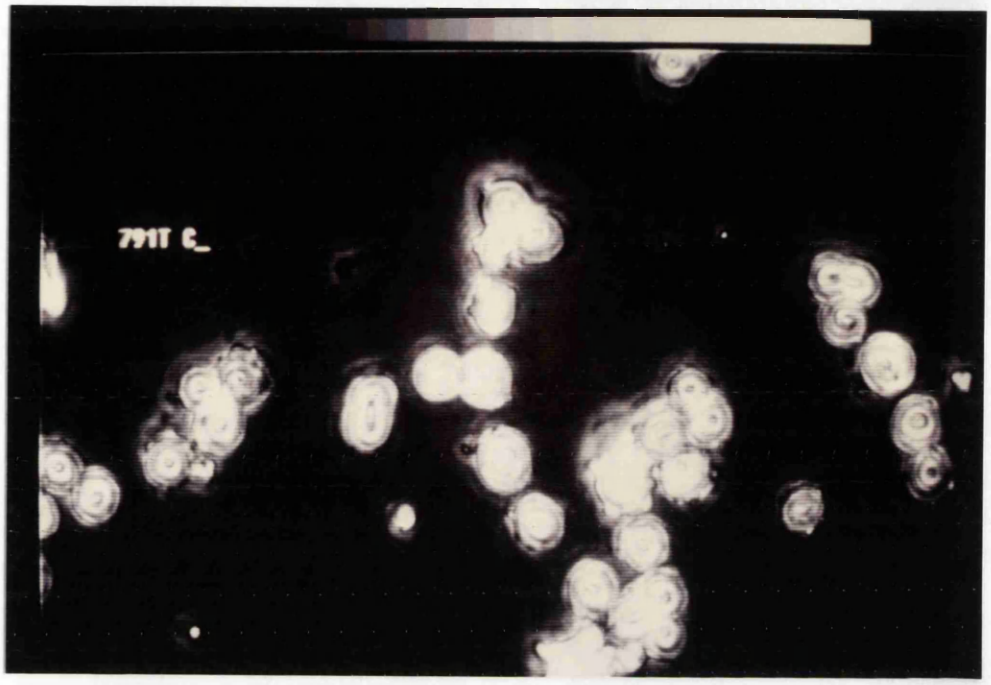


II) FLUORESCENCE

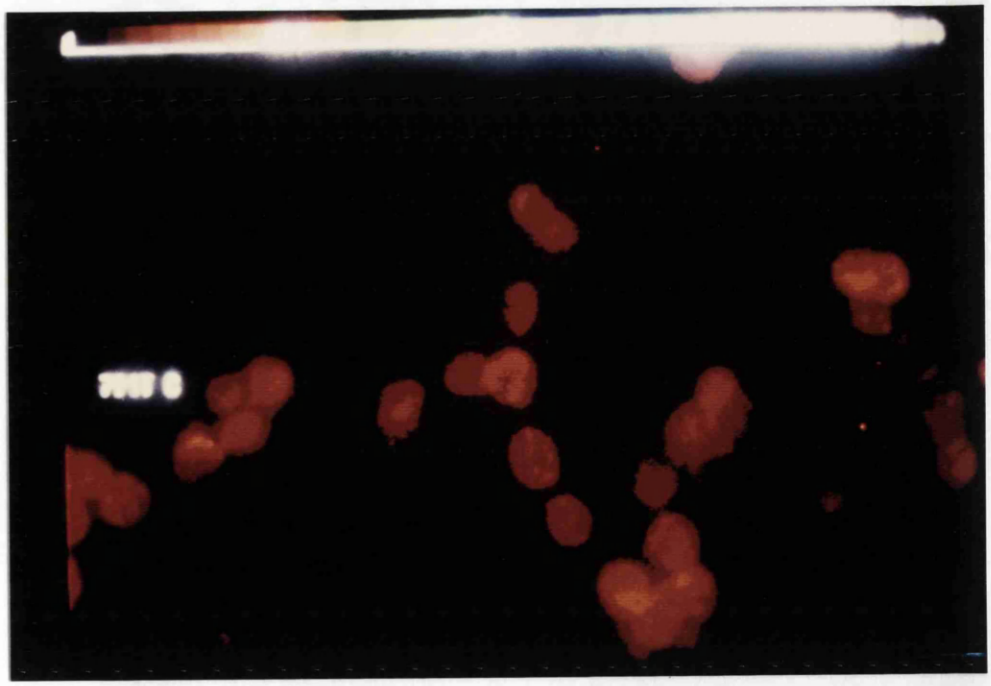


B

I) PHASE CONTRAST

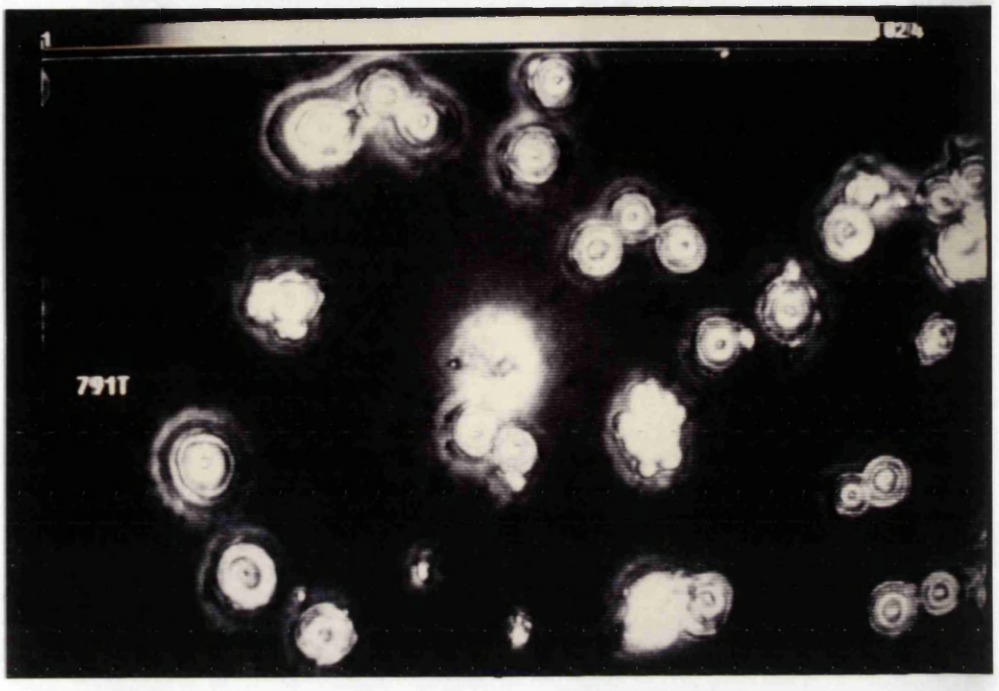


II) FLUORESCENCE

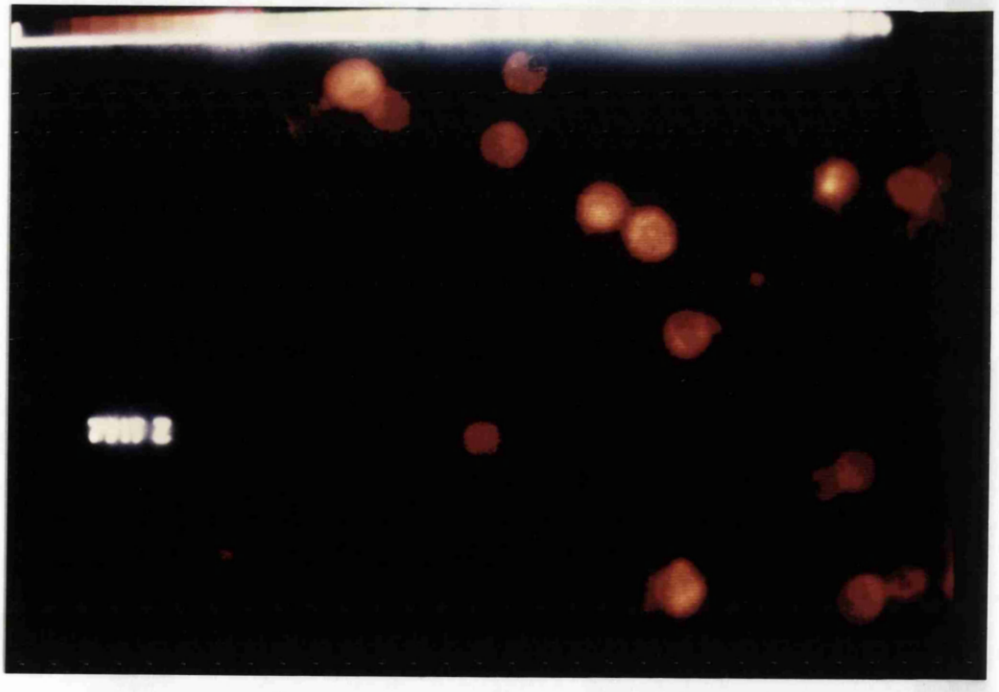


C

I) PHASE CONTRAST

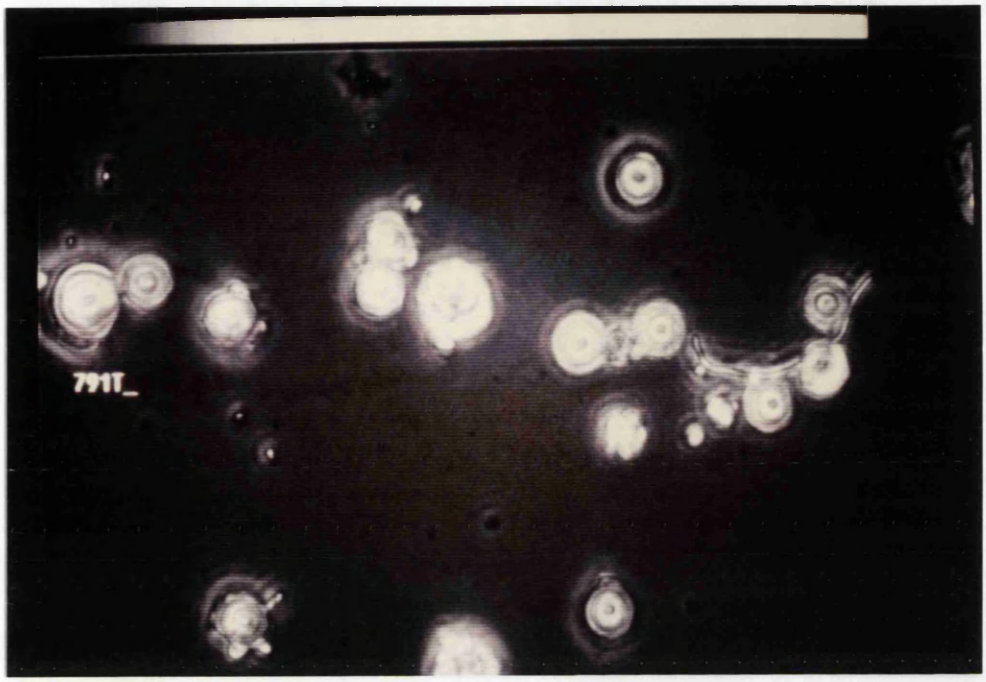


II) FLUORESCENCE

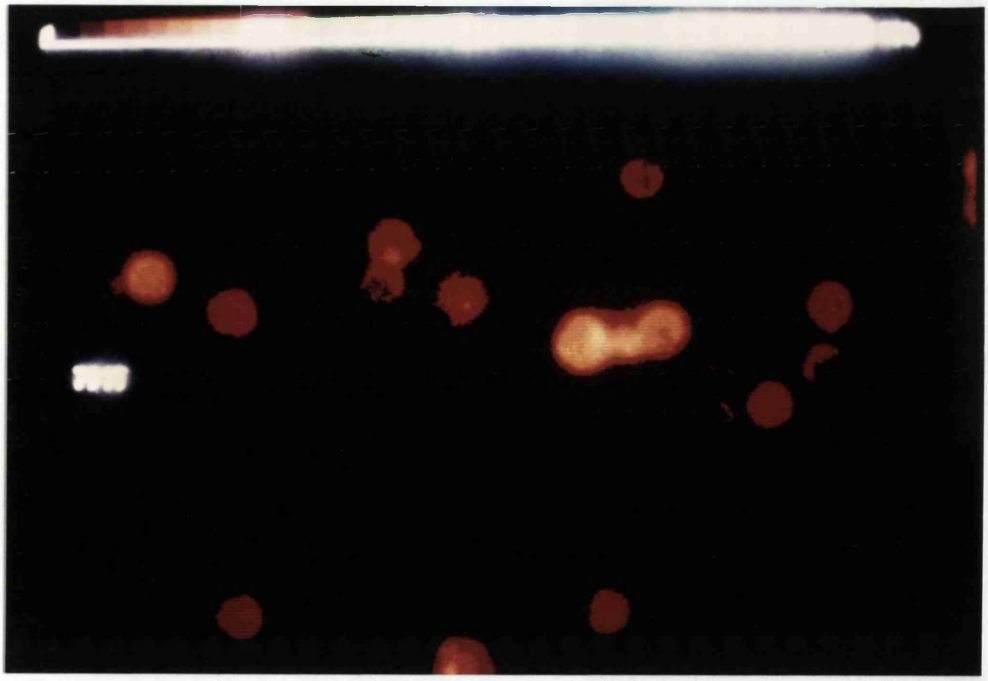


D

I) PHASE CONTRAST



II) FLUORESCENCE



specific background fluorescence was low (Figure 39B). Stripping the cells with ZAPP reagent removed all fluorescence except that due to non specific binding, (Figure 39C) showing that no liposomes had internalised (Figure 39D).

DISCUSSION

Despite incubating one set of cells with attached SUV(AlSPc)-S∝M at 37°C for an hour, the amount of AlSPc that had been internalised, and could not therefore be stripped off by ZZAP was negligible It was also similar to that of cells kept at 4°C and stripped by ZZAP, and cells which had only bound AlSPc non specifically, which did not cause phototoxicity. The incubation at room temperature (for more efficient activity of ZZAP), or even at 37°C did not cause increased uptake over background. It is therefore very unlikely that there is any uptake of liposomes above background in experiments done at 4°C (as were most of the phototoxicity experiments), when even after cells had been illuminated for one hour under red light the temperature rose only by a maximum of 6°C, to 10°C, far below room temperature.

5.4. FLUORESCENCE OF CELLS TARGETED BY ANTIBODY AND AlSPc LIPOSOMES IN BONE MARROW SAMPLES

In Section 4.5.2. treatment of bone marrow samples with red light after targeting with CD3 or anti-B-cell antibody and AlSPc

liposomes, had no detrimental effect on the recovery of CFU-GM colonies compared with untreated controls. To show that there was specific targeting in these experiments, similar samples were examined by the CCD camera.

METHOD

Bone marrow samples (from the same source as those in Section 4.5.2), were incubated with antibody (UCHT1 or 8A) and AlSPc liposomes, as before; or AlSPc liposomes only, for background controls. Cytospins were made of these samples, examined with the CCD camera, and photographs taken of the phase contrast and fluorescent images.

RESULTS

Figure 40 shows typical examples of bone marrow targeted with UCHT1 and AlSPc liposomes (A), 8A and AlSPc liposomes (B), and AlSPc liposomes only (C). Intense fluorescence staining was observed on some of the cells in the antibody targeted samples. The fluorescent cells were presumably T lymphocytes (40A) and B-cells (40B). The remainder of the cells showed only low background fluorescence. The samples incubated with AlSPc liposomes and no antibody also showed intense fluorescence on a few cells, but the majority gave background fluorescence.

DISCUSSION

Some specificity of targeting was observed with the bone marrrow using antibodies to target the AlSPc liposomes. Some of

LEGEND TO FIGURE 40

Photographs show phase contrast (I) and fluorescence (II) images of bone marrow cells that have been incubated with antibody and liposomes.

The fluorescence images show that subpopulations of cells have been targeted by the AlSPc liposomes depending on the antibody used, thus:

A - shows T lymphocytes fluorescing

B - shows cells of the B lineage fluorescing

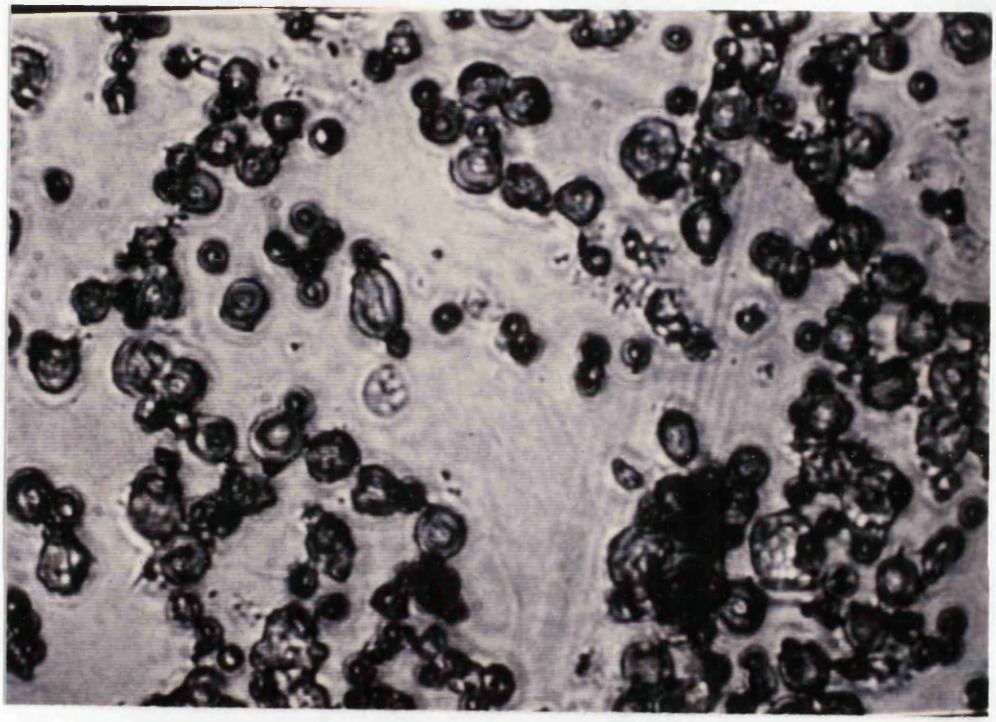
C - shows intense non specific fluorescence in a few cells (Boxed) which are probably of the monocyte lineage and have engulfed the 'particulate' liposomes.

FIGURE 40

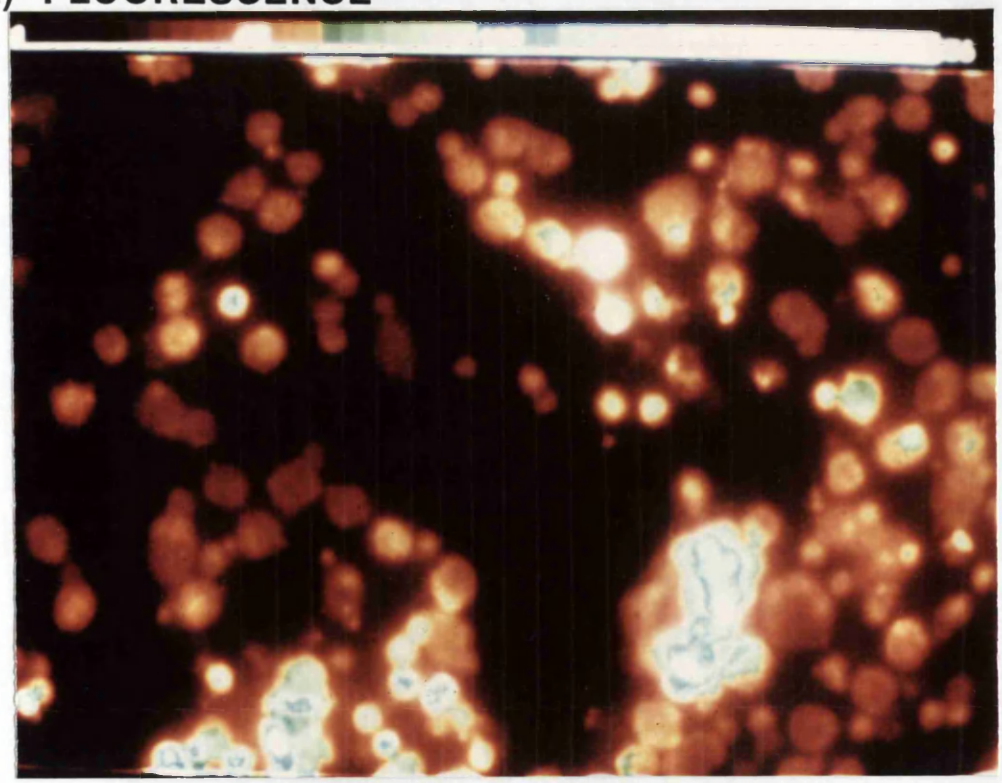
PHASE CONTRAST AND FLUORESCENT PICTURES OF BONE MARROW MONONUCLEAR CELLS TARGETED AND UNTARGETED BY AlSPc LIPOSOMES

A UCHT1 TARGETED

I) PHASE CONTRAST

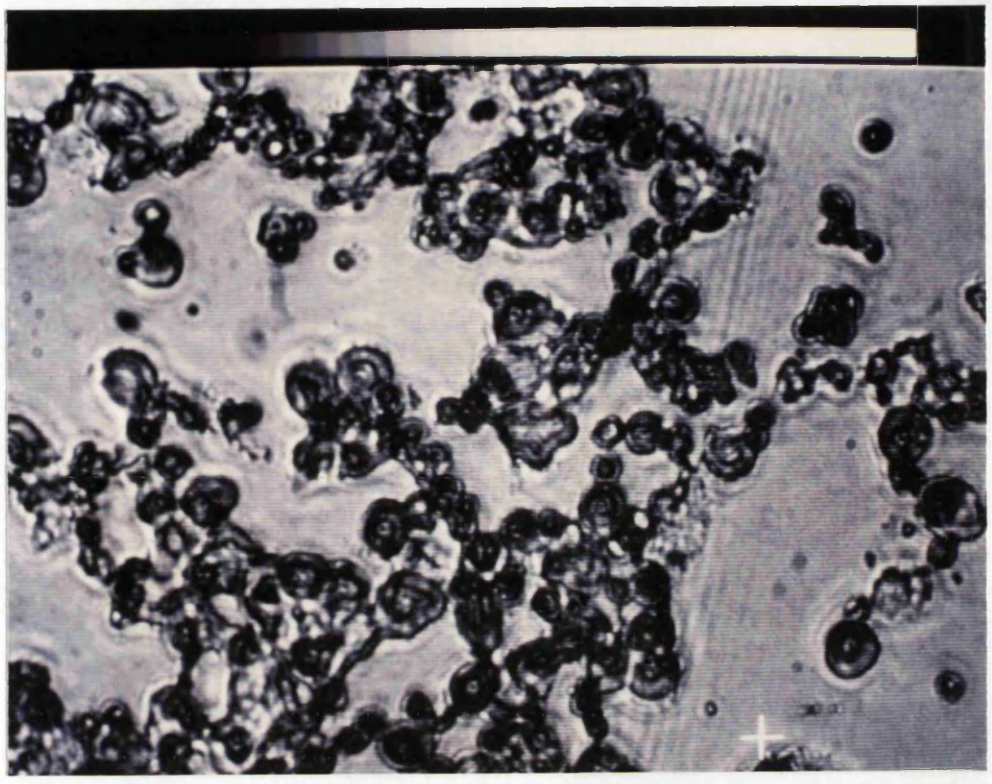


II) FLUORESCENCE

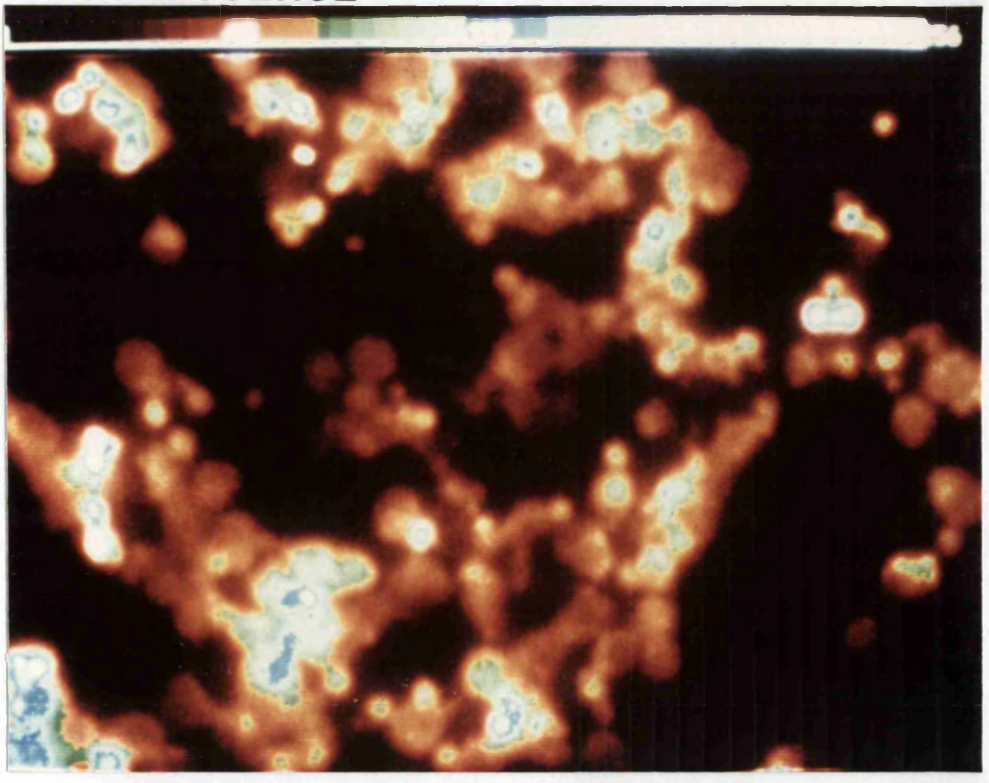


B 8A TARGETED

I) PHASE CONTRAST

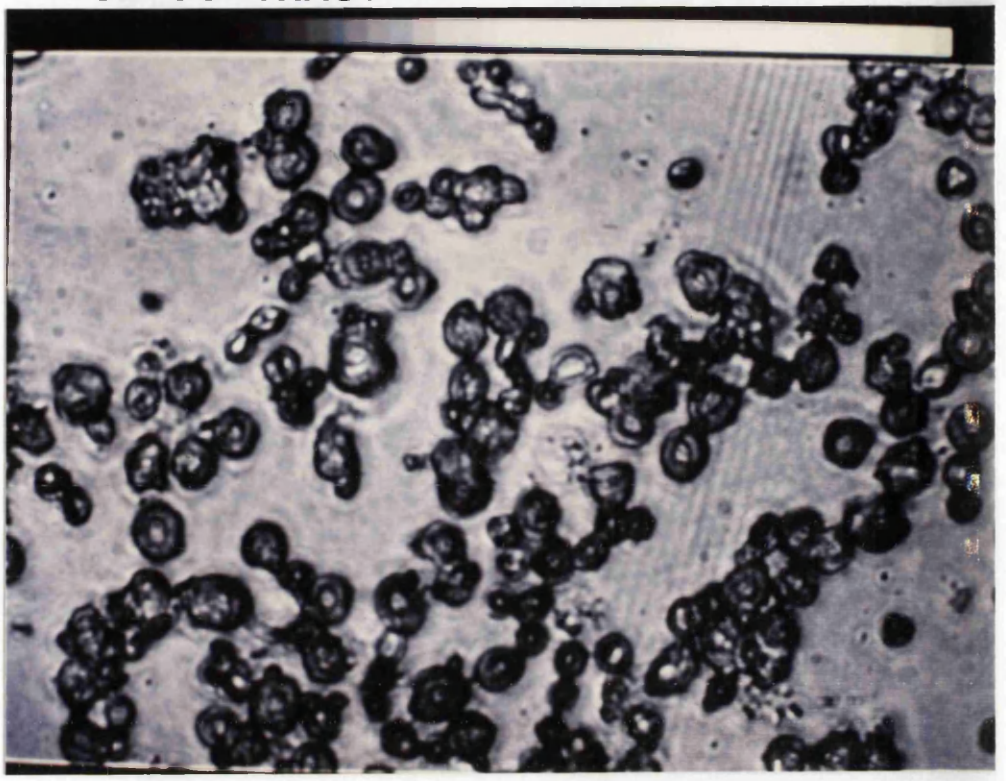


II) FLUORESCENCE

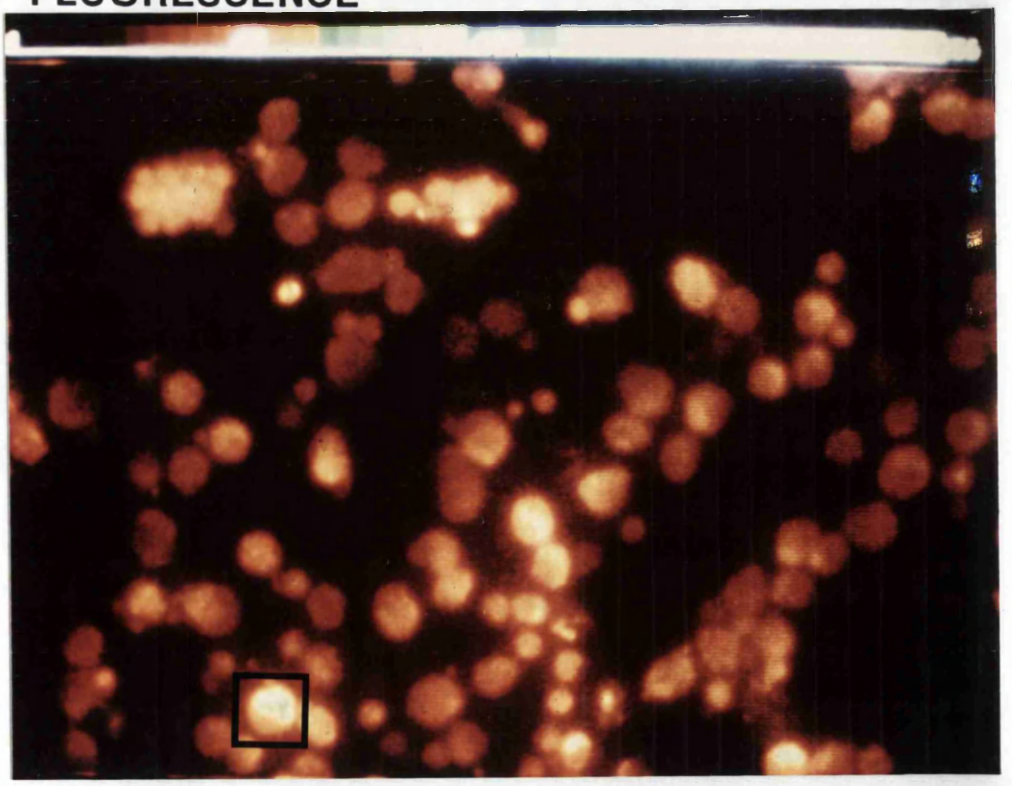


C UNTARGETED (LIPOSOMES ONLY)

I) PHASE CONTRAST



II) FLUORESCENCE



the fluorescence tended to be concentrated in clumps of cells. Bone marrow samples have a tendancy to clump, and no especial effort was made to obtain a single cell suspension. The fluorescent pattern could be due to the target cells themselves clustering naturally. Alternatively, liposomes may have become trapped in these clusters or bound non specifically to stromal material holding the cells together.

In the absence of antibody, some fluorescence was observed on a few cells in the samples. These were probably monocytic cells, which have a natural propensity for engulfing particulate matter such as liposomes. It was unlikely that liposomes were taken up by progenitor cells since there was no phototoxicity when similar samples were also treated with red light and grown on agar (Section 4.5.2.).

For more accurate identification of the cells which take up AlSPc liposomes non specifically, double staining methods could be used. Double staining could be done either on cytospins, or by flow cytometry, using antibodies against other antigens on the surface of the different cell types. Using single cell suspensions by careful disaggregation of the bone marrow would also help to resolve the ambiguity of non specific uptake of liposomes in cell clumps.

CHAPTER 6

GENERAL DISCUSSION

6.1. INTRODUCTION

In this study AlSPc was used as the drug for delivery to cells by antibody targeted liposomes, therefore, as well as the problems and benefits encountered using liposome and antibody, there will also be those arising from the use of AlSPc. Targeting and light mediated toxicity of cells by AlSPc-containing liposomes as used in this investigation will be discussed in relation to the present state of knowledge of liposomes as drug carriers in vivo and in vitro, and of photosensitisers as tumour selective agents. Similarities and differences in the effects of free photosensitiser compared to liposome encapsulated and antibody targeted AlSPc will be emphasised with reference to tumour specificity, sites of phototoxic action, and the mechanism of cell death. The importance of other factors such as quenching agents and the degree of sulphonation of phthalocyanine in the free and liposomal forms on cell uptake, photoactivity and specificity will also be referred to.

Finally, after a brief overview of the present state of photodynamic therapy, some of the situations in which antibody targeted photosensitive liposomes might be therapeutically useful will be described.

6.2. USE OF LIPOSOMES IN VITRO AND IN VIVO

Using liposomes as a drug carrier is a potentially useful method:

a) for protecting normal tissues and cells from toxic effects or non specific uptake of the encapsulated drug. For example, the

distribution and pharmacokinetics of liposome encapsulated drug is altered from that of the free drug, being reduced in the gut, kidney and cardiac and skeletal muscle (Juliano and Stamp, 1978), thus reducing any toxic effects in these organs.

b) as an amplification system since large numbers of drug molecules can be encapsulated in liposomes, either in the aqueous compartment or the lipid bilayer or both.

c) as a targeting agent when combined with an antibody against the target tissue, or by passive targeting to cells such as macrophages.

Consequently, much greater specificity is theoretically possible. Lower amounts of drug can be used to give the same effect when encapsulated in antibody targeted liposomes compared with the free form, and side effects that might occur with the latter are diminished.

However, in practice and particularly in vivo, use of liposomes as carriers, whether or not targeted by antibody is not so simple. In vitro, liposomes with antibody attached can target drugs specifically to cells and under the right conditions deliver the contents to the cell interior (Yemul et al, 1987; Matthay et al, 1986; Gray et al, 1988). In vivo, however, when injected into the bloodstream, or intraperitoneally, liposomes and their contents tend to accumulate in tissues rich in reticulo- endothelial cells.

There is also some tendency for liposomes to accumulate spontaneously in tumours. This is due to several factors, and the amount of such accumulation can be improved in many ways. To deliver liposomes more specifically to tumours, several investigators have modified their characteristics. Richardson et al, 1977 found negatively charged small liposomes targeted better, and using an anti-tumour antibody attached to the liposome surface also improved tumour localising properties (Neerjun et al, 1977). Others found that small negatively charged fluid membrane, or neutral small liposomes (Richardson et al, 1978), associated best with tumours and also gave lowest uptake in liver and spleen (Proffitt et al, 1983). Serum stable liposomes have a longer half life in the circulation and therefore more opportunity to eventually become trapped in tumour tissue (Ogihara-Umeda and Kojima, 1988).

Encapsulating photosensitisers in liposomes might limit to some extent their use in vivo. There are, however, some situations in which liposomes might be a suitable choice for drug or photosensitiser administration, and these will be discussed later (Section 6.11.).

More recent work has resulted in development of large unilamellar liposomes containing monosialylganglioside, which have prolonged circulation lifetimes, and decreased uptake into the spleen and liver. These have been termed Stealth liposomes (Allen et al, 1987). Gabizon and Papahadjopoulus (1988), made similar observations with liposomes containing saturated phosphatidylinositol, and also noticed enhanced uptake into

implanted tumours in mice. With monoclonal antibodies raised against antigens on the surface of cancer cells attached to the 'stealth' liposomes, even higher tumour specificity was achieved (Papahadjopoulus and Gabizon, 1987: Straubinger et al, 1988).

With such liposomal preparations available, some of the previous disadvantages of drug delivery by liposomes can be overcome. However, there will still be limited accessibility of liposomes targeted to cells enclosed in specialised membranes which restrict the passage of particles the size of liposomes.

Bugelski et al, 1981, described certain characteristics of tumours such as the presence of tumour macrophages, poor lymphatic drainage and leaky vasculature due to new and rapid growth which would partly account for liposome accumulation. Because of their size, liposomes cannot easily pass the basement membrane of blood vessels unless there are large spaces between the cells of the basement membrane. The above combination of factors in tumours both aids liposomal access and decreases their elimination.

6.3. SELECTIVITY OF PHOTOSENSITISERS IN VIVO

Some photosensitisers have been delivered by liposomes to tumours in vivo. Porphyrins incorporated in liposomes were delivered preferentially to tumours over normal tissues (Jori et al, 1983). Similarly, zinc phthalocyanine in liposomes accumulated in tumours and caused damage to both neoplastic cells and the vascular system (Milanesi et al, 1987). The proposed delivery system for these photosensitisers is via low density

lipoproteins (LDL) in the blood with which the liposomes react. Some tumour tissues have large numbers of LDL receptors, which could be a reflection of their requirement for lipid for membrane synthesis, due to rapid growth. Some examples are leukaemic cells (Vitols et al, 1984), and melanoma in which the receptor numbers are elevated at the level of both tumour and endothelial cells (Netland et al, 1985), Moreover, LDL are natural carriers for protoporphyrin and uroporphyrin (Reyftman et al, 1984) and can act as carriers for these and other photosensitisers to cells with LDL receptors, which deliver them to the lysosomal compartment of the cells (Santus et al, 1981).

A large part of photodynamically induced tumour necrosis in vivo appears to be a consequence of vascular damage resulting in a cut off of the blood, and consequently the oxygen supply to the tissues, rather than a direct photodynamic effect on the neoplastic cells themselves (Selman et al, 1986: Berenbaum et al, 1986; Henderson et al, 1984; Henderson and Fingar, 1989). These latter two papers showed that tumour cells removed immediately post light treatment grew well in vitro whereas delaying removal of cells inhibited their subsequent growth.

The factors which influence tumour uptake of liposomes are probably also responsible in part for tumour selectivity of photosensitisers, whether administered in liposomes or not. Thus, tissue constraints of the tumour favour photosensitiser retention and injected free photosensitisers are likely to bind to serum components, including LDL and be delivered by some of the same pathways as liposomes. The fate of the photosensitiser

depends partly on its lipophilicity. More hydrophobic compounds such as tetrasulphonatophenylporphine (TPPS) may bind to serum proteins or the outer layers of LDL (Milanesi et al, 1987), whereas lipophilic compounds such as zinc phthalocyanine bind to the more hydrophobic core (Valduga et al, 1987). Binding of porphyrins (Oenbrink et al, 1988) and phthalocyanines (Ben-Hur and Rosenthal, 1985a; Chan et al, 1986; personal observations) to serum proteins is a well observed phenomenon, and may affect their ultimate destination both in vivo and in vitro. However, other factors such as charge distribution on the free photosensitiser molecule can also affect distribution among plasma proteins (Kongshaug et al, 1989).

Uptake of chloro aluminium phthalocyanine by monocytes has been observed in vitro (Singer et al, 1987) and also in vivo when tumour macrophages took up large amounts of the dye (Chan et al, 1988). These findings suggest that localisation in tumour tissue is not entirely due to a preferential affinity for tumour cells themselves, but are, in part, due to a number of other reasons.

Because these localisation effects are all rather non specific, attaching an antibody to the liposomal surface offers the potential for much more specificity.

6.4. ANTIBODY TARGETED PHOTOSENSITIVE LIPOSOMES

In this study liposomes containing photosensitiser were directed to cells by antibody in a simple in vitro model. Conditions were further simplified by the discovery that the liposomal contents need not be internalised for effective action

(Section 3.2.3.), unlike models in which the photosensitisers incorporated into liposomes were released into the cell (pyrene, Yemul et al, 1986; porphyrins, Milanesi et al, 1989; zinc phthalocyanine, Firey et al, 1988). For effective cytotoxicity, previous classes of targeted drugs have needed to gain access to the cell interior, whether presented in liposomes (eg. methotrexate, Leserman et al, 1981: Gray et al, 1988), or attached to the antibody as an immunotoxin (eg. ricin, Thorpe and Ross, 1982). The Ag/Ab/drug complex must be endocytosed for effective action. However not all antigens or receptors are internalised (Machy et al, 1982), the rate of internalisation varies widely (Machy et al 1982a; Truneh et al, 1983) and some antigens are shed from the surface, and are thus unsuitable for drug delivery. Use of an externally effective agent thus gives a far greater choice of targeting moiety.

AlSPc and other antibody targeted photosensitisers, for example chlorin e_6 (Oseroff et al, 1986) and pyrene (Yemul et al, 1987) do not necessarily need internalisation to be effective cytotoxic agents.

The target antigen on 791T cells can internalise slowly by pinocytosis (Huehns, 1986) but conditions were used in my experiments, (incubation at $4^{o}C$), that would not favour the kinetics of endocytosis or antigen shedding. AlSPc would not therefore gain access to the interior of the cell but would remain bound to the cell surface, and produce its cytotoxic action from a distance.

6.5. TARGET SITES FOR EXTRACELLULARLY GENERATED SINGLET OXYGEN

The effect of targeting the AlSPc in liposomes seems to be to concentrate the photosensitiser in sufficient proximity to the cell for it to have a cytotoxic action after light activation. Singlet oxygen is readily able to diffuse from its site of generation within the liposome to the target cells (Rodgers and Bates, 1982). It is possible that the liposome membrane is destroyed in the process, though no evidence to support this was obtained. Saturated phospholipids, which are much less susceptible to oxidation than unsaturated phospholipids were used in the preparation of the liposomes, and no evidence of peroxides was observed by MDA assay after illumination of AlSPc liposomes (Section 4.6.). However, cholesterol, which can be attacked by singlet oxygen, resulting in its oxidised product 3 -hydroxy-5 cholest-6-ene 5-hydroperoxide (Langlois et al, 1986), was also included in the liposomes. This oxidised product is not detectable in the MDA assay but could contribute to destabilising the liposome bilayer.

Since singlet oxygen is produced outside the cell, cytotoxic effects are likely to be similar to those of other photosensitisers (in the free form) that accumulate primarily at the cell membrane. The main target sites are thus the cell membrane, and nearby cytoplasmic components (Kessel, 1977), which are attacked by singlet oxygen after its generation and diffusion from the liposome (Eisenberg and Taylor, 1989). The cell membrane components which are susceptible are more likely to be membrane proteins than lipids, at least during the first 24 hours post

illumination (when the majority of cell death occurs, Section 3), since no lipid peroxidation was detected during this time after PDT of cells targeted with AlSPc liposomes (Section 4.6). However, cholesterol is a large component of cell membrane and the cholesterol photoperoxide content was not analysed. Photochemically generated cholesterol hydroperoxides in erythrocyte ghosts have been shown to be a poor substrate for glutathione peroxidase detoxification compared to other lipid peroxides (Thomas and Girotti, 1989a) and are therefore available for peroxidative damage and may contribute to cell death.

Some authors have suggested that singlet oxygen generated externally to cells is not of great importance in the phototoxic reaction of photosensitisers, and it is bound photosensitiser that is effective. Suwa et al, 1977, used a liposome as a model for haematoporphyrin generated oxidative damage of membrane, and found that haematoporphyrin was much more effective at photo-oxidising cholesterol when incorporated in the membrane than when adsorbed outside. No effect was observed when cholesterol was dispersed in an aqueous solution of haematoporphyrin. This was explained by the lifetime of singlet oxygen in the lipid environment being long enough to interact with cholesterol, whereas in the aqueous medium singlet oxygen was quenched by water before it could reach its target. Sonoda et al, 1987 used haemolysis of red blood cells by photosensitised phthalocyanine sulphonates to demonstrate membrane damage. They similarly concluded that photoactivated haemolysis was only effective with bound phthalocyanine. Furthermore, in studies using several

porphyrins (Kessel, 1977); Moan and Christenson, 1981), it was discovered that only sensitiser bound to or taken up into cells was able to affect cell viability.

In the present study, experiments with the CCD camera (Section 5.3), indicated the presence of small amounts of AlSPc bound to the cell non specifically; this is probably attached to the cell membrane, though the degree of penetration of the cells is still uncertain. The amounts bound were not enough to cause significant toxicity unless the concentration of AlSPc encapsulated in the liposomes was high, and there were no quenching agents present in the medium (Section 4.3.). This agrees with Suwa's results with adsorbed free haematoporphyrin. However, in all the papers quoted above, the external sensitiser was relatively dilute while in this thesis although the total amount of photosensitiser present was comparable to that used by the other investigators, it was presented to the cells in relatively concentrated 'pockets'. When similar amounts and even ten times as much AlSPc than was contained in the antibody targeted liposomes was presented in a free form to cells, there was no light mediated toxicity. These results are in agreement with those of other authors (Suwa et al, 1977; Sonoda et al, 1987; Kessel, 1977; Moan and Christenson, 1981). Obviously the use of closely associated concentrations of AlSPc which can produce a 'shower' or flux of singlet oxygen is of importance for an externally generated phototoxic response.

Most work on singlet oxygen generated from liposomes has been done on photosensitisers incorporated into the lipid bilayer

or adsorbed to its outside. Singlet oxygen has been produced from both these sites and shown to have an oxidative effect on cholesterol, which was lower for externally adsorbed photosensitiser (Suwa et al, 1977). Little work has been done on the generation and effects of singlet oxygen from water soluble photosensitiser encapsulated in the aqueous compartment of liposomes, so at the moment it is not possible to know the effects of singlet oxygen on the cholesterol contained in the liposomal membranes used in the present experiments. For cholesterol oxidation by singlet oxygen to be efficient, it appears that the photosensitiser needs to be bound closely to the lipid, which is not the case for water soluble photosensitisers (Bachowski et al, 1988). However, it seems likely that at least some of the cholesterol will be oxidised as the singlet oxygen diffuses through the liposomal layer, thus making the liposomes more susceptible to peroxidation and destabilisation.

6.6. MECHANISM OF CELL DEATH

The mechanisms leading to cell death are uncertain, though most evidence suggests some form of lytic action which compromises the integrity of the cell membrane is involved. Photosensitisers, unlike many cytotoxic drugs, are therefore able to destroy resting or slowly growing cancer cells as well as actively dividing cells, which suggests that the potential of a potent anti-cancer agent.

Early experiments (Table 7), showed the presence of non viable cells as early as one hour after light treatment of

targeted cells. This death was not due to lipid peroxidation subsequent to singlet oxidation of unsaturated membrane phospholipids since there was no evidence of peroxides as thiobarbituric acid reactive material even 24 hours post illumination. However, increased cell permeability was demonstrated by the uptake of trypan blue. Others have also demonstrated increased permeability using ethidium bromide, consequent to free radical damage, (without indications of lipid peroxidation, Dean, 1987), and to photodynamic treatment (Oseroff et al, 1986).

Further evidence of cell membrane damage caused by photosensitised reactions was a reduction in membrane transport of α-amino isobutyrate and increased release of chromate by cells induced by haematoporphyrin derivative sensitisation (Moan et al, 1983). The former being due to damage of the active transport system and the latter to increased permeability of cells.

The effects of phototoxicity at a cellular level were studied by Moan et al, 1979. A few minutes after haematoporphyrin and light treatment they observed cellular swelling and membrane damage. The swelling was probably due to inactivation of pumping and carrier proteins, thus affecting ionic homeostasis (Dean, 1987).

However, the actual cytological effects vary with the photosensitiser location in the cell (Kessel et al, 1989), which further depends on its time of incubation with cells (in vitro) and its physical properties. The events observed above are more often observed with shorter rather than longer incubation times

and hydrophilic rather than hydrophobic compounds, before the photosensitiser has been able to penetrate the cell more fully.

For liposome targeted cells, phototoxic effects are likely to be similar to those mentioned above since the liposomes are bound to the cell membranes, and damage will occur in those molecules which are accessible to diffused singlet oxygen. Photogenerated singlet oxygen is able to diffuse freely through different micellar phases (Jori and Spikes, 1981), unilammelar vesicles (Rodgers and Bates, 1982), and water-lipid phases in liposomes (Valduga et al, 1987) with an average range of 60-160 nm in aqueous and hydrocarbon media (Jori and Spikes, 1981). Singlet oxygen should therefore be able to penetrate well into the typical cell membrane which is 5-8 nm deep.

6.7. IMPORTANCE OF SULPHONATION OF AlSPc IN ANTIBODY TARGETED LIPOSOME MEDIATED PHOTOTOXICITY

In the search for the most efficient photosensitisers for PDT, it appears that the most toxic ones administered in the free form are those that are best taken up by cells. These are generally lipophilic compounds which, because of their poor solubility in water are not suitable for use in cellular systems unless dispersed in a suitable carrier medium such as liposomes or detergents, or dissolved first in a non-polar solvent and then diluted in buffer solution. However, there are some photosensitisers which are water soluble including the dye AlSPc. AlSPc can contain from one to four sulphonate groups with a concomitant increase in solubility in water. The toxicity of free

AlSPc decreases with the number of sulphonate groups (with the exception of the mono-sulphonated form) which parallels its uptake into cells in vitro (Berg et al, 1989). The active phototactive species is monomeric AlSPc, which is why mono-sulphonated AlSPc does not fit the general pattern since it tends to aggregate more than the more highly sulphonated species. Another factor influencing cell uptake and hence phototoxicity is molecular structure. Di-sulphonated AlSPc can occur in two basic forms (with several isomers) in which the sulphonate groups are:

a) on on adjacent benzene rings, and

b) on opposite sides of the molecule.

The structure of the two forms affects the lipophilicity of the molecules, the former being more and the latter being less lipophilic. The adjacent form is taken up by cells more easily. HPLC purified AlSPc enriched for these two forms of the di-sulphonate gave good uptake of the adjacent form (Paquette et al, 1988), and the highly purified Gallium di-sulphonate in the opposite form was non-toxic (Brasseur et al, 1987). The differences in toxicity were attributated to the amphiphilic nature of the adjacent form, favouring cellular uptake, a property not shared with the opposite form or with the tri- and tetra-sulphonates.

However, when AlSPc is targeted to cells by liposomes and antibody, no uptake into the cell is required for toxicity, so the lipophilic properties of the molecules are not important and indeed may be detrimental to the specificity of targeting. The desirable characteristics of AlSPc for use as a liposome

encapsulated and antibody targeted agent have now become those which affect:

a) the specificity of the liposomes, and

b) the efficiency of production of singlet oxygen in aqueous solution within the liposome.

It was shown in Section 4.4. that liposomes containing HPLC purified di-sulphonated AlSPc became incorporated in the lipid bilayer and bound to cells non specifically. The di-sulphonate consisted of a mixture of all its isomers but the non specific binding was probably due to the lipophilic forms. Non specific binding was far less for the more highly sulphonated and hydrophilic tri- and tetra- species. Possibly use of the di-sulphonate isomers with opposing sulphonate substituents would also result in low non specific binding, but these experiments await more highly purified compounds.

Apart from the hydrophilic effect, tetra- and tri-sulphonated AlSPc are less quenched in solution and are therefore more efficient at producing singlet oxygen for a given AlSPc concentration. There is no published data as yet for quenching of the individual components of the di-sulphonates, but if quenching reflects hydrophilicity as it seems to at least in part, then it is likely that the trans-orientated di-sulphonate will also be relatively unquenched in aqueous medium.

In contrast to most of the published literature on differentially sulphonated AlSPc which indicates that the best photosensitiser in the free form is the di-sulphonate, the most

suitable sulphonates for use in antibody targeted phototoxicity by AlSPc liposomes are the tri- and tetra-sulphonated molecules.

6.8. IMPORTANCE OF QUENCHING AGENTS

Another important factor for effective phototoxicity by externally targeted AlSPc containing liposomes is the absence of quenching agents such as FCS and phenol red in the medium. This was demonstrated by the use of a cell line (C170) which expressed low levels of target antigen. When liposomes were bound directly to C170 cells by monoclonal antibody in the presence of phenol red a higher liposome concentration was required for maximum phototoxicity compared with 791T cells, which express more antigen (Section 4.1). However, in the absence of the quenchers, equal phototoxicity of C170 and 791T cells was observed (Section 4.3.), unless very low concentrations (0.05 mM) of AlSPC were contained in the liposomes, when there was a slight difference ($p < 0.025$).

This implies that targeted AlSPc liposomes are as toxic for cells expressing low antigen numbers as those with high numbers of target antigens in the range used in these experiments (0.5 to 2.2 million target sites per cell), provided the singlet oxygen produced on illumination is able to reach the target molecules before being quenched. This is an important factor to be considered when designing a treatment protocol.

6.9. AMPLIFICATION

One of the reasons for encapsulating AlSPc in liposomes was so that amplification could be achieved by targeting large numbers of molecules per antibody to cells. Indeed, up to 60 molecules (and more are possible) of AlSPc have been enclosed in a liposome and targeted to a single cell surface antigen. Uptake of free AlSPc by cells in vitro can be considerably greater than that bound in liposomes by antibody (personal observations) but since maximum phototoxicity can be achieved by using low AlSPc concentrations in liposomes, great amplification is not always necessary. The importance of amplification is probably in the degree of concentration found at each target site on the cell in the liposomes rather than in the average concentration throughout a cell. In tissues, if photosensitiser concentrations are too high, they may absorb photoactivating light, and reduce the depth of its penetration into a tumour mass, thus leaving untreated areas (Wilson et al, 1986). This is particularly true of phthalocyanines which have high absorption at the chosen treatment wavelength (Bown et al, 1986). However, this may be less of a problem with liposomes because their internal concentration can be controlled and they bind to cells in a single layer.

By using different concentrations of AlSPc in liposomes it was shown (Section 4.3.) that maximum toxicity was obtained with lower levels of encapsulated AlSPc than expected. This is partly because a photosensitiser molecule has an inherent amplification system, being reactivated after it decays from an excited state,

provided light of a suitable wavelength is available. This applies to both free and liposome encapsulated AlSPc.

In these experiments the illumination time was long, so amplification by increasing the number of encapsulated molecules was of less importance than reactivation of molecules recycled to the ground state. Further experiments need to be done to determine the importance of maximum amplification. Also, at the higher AlSPc concentrations more aggregation occurs, even of the relatively polar tri- and tetra-sulphonated AlSPc. This decreases the efficiency of production of singlet oxygen even if the total singlet oxygen production is greater.

In most of the experiments done for this thesis, the light source was fluorescent tubes which produce low intensities over a fairly wide range of the spectrum. Use of a laser could probably produce much more efficient activation with shorter exposure times. In this situation high concentrations of AlSPc in the liposomes might be more important.

The most reasonable balance to produce effective phototoxicity is likely to be an intermediate concentration of AlSPc in liposomes, combined with short intense illumination times. This should give a good amplification without too much aggregation, but would not affect selectivity by non specific binding. The balance would obviously vary among cell types and those with lower antigen expression would require a longer light exposure for a similar phototoxic effect to those cells with high antigen expression, since fewer liposomes would be bound. For low expression cells too, a higher AlSPc concentration would be more

suitable because photobleaching would release monomeric species from dimeric aggregates during illumination (Berg, 1989), whereas if low AlSPc concentrations were used, photobleaching would decrease the AlSPc available for excitation, possibly below the threshold for cell death.

6.10. CLINICAL USE OF PHOTODYNAMIC THERAPY

Most photodynamic research and clinical treatment have used porphyrins as photosensitisers (reviewed by Spikes and Jori, 1986). Its main use has been for detection and treatment of neoplasms, but its use is also developing in other fields such as the treatment of viral (Sieber et al, 1987) and microbial infections, psoriasis, and atheroma.

The main porphyrins used clinically, HpD and its purified form PF II are in some respects unsatisfactory. They have low absorption at red wavelengths, are inhomogeneous, have poor localising properties, and cause skin photosensitivity. Consequently, more suitable compounds are being looked for and developed, of which AlSPc, among others, shows promise. Different ways of delivering the photosensitiser are also being investigated in an attempt to improve tumour selectivity. These include use of antibody-photosensitiser conjugates and liposome-incorporated photosensitiser. The present investigation has used the combination of liposome and antibody with the aim of increasing specificity further.

Tumour to normal tissue ratios of sensitiser are generally

low so there is bound to be some damage to the latter. Barr et al, 1987 studied healing of normal rat colon after free AlSPc and laser treatment and found that despite full thickness necrosis, the lesions regenerated with no loss of strength. In contrast, bowel healed after thermal laser treatment was weakened. The recovery was attributed to lack of reactivity with collagen, in the photodynamic treatment whereas it is affected detrimentally by heat. Similarly loss of the full normal bladder epithelial layer in response to AlSPc mediated photo-treatment resulted in full regeneration of normality (H. Lottman, personal communication). It appears that normal tissues are less sensitive than tumour tissues to the destructive effects of photosensitised radiation.

For cancer treatment, photodynamic therapy is most effective for early and superficial tumours such as bladder and lung cancer, both of which are easily accessible to a laser probe. For more advanced tumours it is mainly palliative at this stage of research, but has the advantage of few side effects apart from skin photosensitivity (Ash and Brown, 1989). With advanced cancer, metastases would not be destroyed unless they were also exposed to light. However, combination therapy, with photodynamic treatment plus chemotherapy could have far better effects than use of the individual modalities. Straight et al, 1989, showed that adriamycin and PF II when used alone, inhibited, but did not cure a neuroblastoma in mice but when they were used together 100% cure was obtained.

All these results were obtained using free photosensitisers.

No clinical and very little animal work has been done with liposome encapsulated photosensitisers. However, since liposomes tend to accumulate in tumours, and with the addition of anti-tumour antibody attached to their surfaces, they might be expected to bind more selectively to tumour cells than free sensitiser which tend to remain in the tumour blood vessels. The degree of accessibility of tumour cells by liposomes is uncertain, and will depend on factors such as the tumour structure, the size of the liposomes and the avidity of the targeting antibody for the tumour antigen. Tumour antigens are often expressed on the stroma as well as the tumour cells and this may inhibit liposomal access.

Some other side effects possible with photodynamic therapy are post treatment mutagenesis and various immune reactions.

Although many sensitisers do not reach or enter the nucleus, some cause DNA strand breaks, possibly during mitosis. Sensitisation of Chinese Hamster fibroblasts with AlPc resulted in increased mutations over background after light treatment but this was not statistically significant. Therefore the mutagenic potential of photodynamic therapy is small (Ben-Hur et al, 1987).

An effect of photodynamic therapy which has recently emerged is its influence on the immune system. Reversible systemic immune suppression was observed after peritoneal photodynamic therapy with PF II (Elmets and Bowen, 1986; Jolles et al, 1988). The immunosupression could be adoptively transferred and was mediated by cells of the macrophage lineage (Lynch et al, 1989).

Also, splenic natural killer (NK) cells from mice treated

with AlSPc and laser, showed inhibited cytotoxic activity in vitro. Macrophages from similarly treated mice showed variable impairment of antitumour activity (Marshall et al, 1989).

The mode of action of these effects is uncertain. One possibility for NK suppression could be related to induced release of suppressive factors, or inhibition of release of stimulatory cytokines by macrophages. Monocytes are accessory cells for many immune mediated reactions and it seems likely that immune suppression was due to indirect effects of photodynamic therapy on this population. For example, sub-lethal HpD and DHE photosensitisation selectively inhibits binding of IgG 2a antibodies to FC R1 (high affinity receptors for the Fc portion of IgG molecules on human peripheral monocytes) by altering the structure of the receptor. Photosensitised monocytes were then unable to function as accessory cells for CD3 induced proliferation of T lymphocytes (Krutmann et al, 1989). Any interleukin 2 (IL2) induced responses that might be induced as a result of T lymphocyte proliferation such as stimulation of NK cytotoxic activity, would then be inhibited.

An interesting use of HpD was in the non photoirradiated rescue of leukopaenic mice after lethal irradiation and chemotherapy. This was due to the induction of hyperplasia in in spleen and bone marrow, by an unknown mechanism (Canti et al, 1984).

The immune effects were observed with free sensitisers and were relatively small, particularly when compared with the profound immunosuppression observed with many anti-cancer

therapies. However some of the cells affected have their own role against tumour activity which may be compromised, so an awareness of these factors is important. Phototherapy involving liposomes is likely to have a similar immunosuppressive action, particularly since it is mediated by macrophages which engulf liposomes in vivo.

6.11. CLINICAL SITUATIONS IN WHICH ANTIBODY TARGETED LIPOSOMES COULD BE POTENTIALLY USEFUL.

Most drug-antibody conjugates or liposome-antibody targeted drugs have had reasonable success as cytotoxic agents in vitro, but not in vivo, due to rapid uptake by the reticulo endothelial system, which prevents the drug reaching the target (Weinstein, 1984). Some success has been achieved by exploiting the uptake of phthalocyanine containing liposomes by low density lipoproteins (LDL), and their subsequent delivery to tumour tissues with high LDL receptor expression (Reddi et al, 1987). However, liposomes can still have an important role to playin some situations, such as: the treatment of malignancies affecting the body cavities, the ex-vivo manipulations ('purging') of cells in autologous bone marrow transplantation,or where the affected organ is accessible to instillation, such as the bladder. This is particularly so in conditions where current treatment is inadequate, such as advanced ovarian carcinoma involving the serous cavities, or in multiple myeloma for purging in autologous bone marrow transplantation (ABMT).

6.11.1. INTRAPERITONEAL PHOTODYNAMIC THERAPY

Intraperitoneal treatment of ovarian carcinoma has been attempted with limited success by administration of chemotherapeutic agents at much higher concentrations than would be given systemically (Dedrick et al, 1978; Richardson et al, 1985). An advantage of such treatment is lower systemic toxicity, but there are local side effects due to free cytotoxic drug. Several studies have been performed with liposome encapsulated drugs, antibody directed drug conjugates or liposomes, and photodynamic therapies with a view to improving the therapeutic indices of the particular treatment agent used.

Intraperitoneal injection of antibody conjugated ^{131}I has been used for radiotherapy of ovarian and colon cancer in early clinical studies (Riva et al, 1988; Stewart et al, 1988; Ward et al, 1988). Some good responses were observed for tumours involving ascites, but poor results were obtained for solid tumours. Ascites resolution was antibody dependent, with no response in a patient with antibody negative ascitic cells (Ward et al, 1988). Some problems encountered were firstly, bone marrow suppression, due to 25% of the radioactive dose reaching the circulation, and secondly, the development of anti-mouse antibodies,

Preliminary studies in nude mice (Straubinger et al, 1988), showed localisation in ascitic cells of liposome encapsulated drugs directed by antibody to human ovarian carcinoma. The liposomes were toxic to the carcinoma cells in vitro.

The photosensitiser, haematoporphyrin derivative (HpD), was

effectively used intraperitonealy in a murine ascitic tumour with laser light delivered by a quartz fibre (Tochner et al, 1986). Of 20 mice treated, 17 were alive 11 months after treatment. Untreated controls died within 25 days. The treated mice that died developed subcutaneous tumour along the line of the entry of the laser probe. The photodynamic results were superior to the intraperitoneal treatment of the same tumour model with adriamycin. Normal tissue in the peritoneal cavity took up substantial levels of HpD, but levels were relatively much higher in the tumour cells soon after injection, which was when light was administered. The light wavelength used was 514 nm, at which HpD absorption is high, but this wavelength has poor tissue penetration, which is probably why there were no side effects on normal tissue.

If the liposome system developed in this thesis were used for intraperitoneal photodynamic therapy, tumour cells could be targeted by monoclonal antibodies and the normal peritoneum protected from non specific uptake by the insulating effects of the encapsulating lipid. There would be no problems with systemic toxicity or bone marrow suppression as with chemotherapeutic drugs and radionuclides, because AlSPc is not toxic when unactivated, and smaller amounts could be administered than in the free form. Furthermore, there is evidence that liposomes administered intraperitoneally are retained for longer than free drugs (McDougall et al, 1975; Parker et al, 1982). This may be assisted by compromised lymphatic drainage, which often occurs in ovarian carcinoma (Feldman et al, 1972 and 1974).

Photodynamic therapy of ovarian cancer would be feasible by intraperitoneal injection of antibody targeted AlSPc liposomes followed by laser light delivered by quartz fibre for photoactivation. Its usefulness would probably be restricted to palliation of ascites, since there is evidence of poor penetration of solid tumour by particulate matter such as liposomes, or even antibody conjugates. However, the low toxicity of AlSPc has much to recommend it and also suggests the possibility of its use in combination with more toxic regimes. Patients who do not respond to or relapse after initial chemotherapy often have disease which is refractory to conventional treatments. These patients might benefit from novel agents with different activity to conventional drug types (Cody and Slevin, 1989). AlSPc is one such agent which has the potential of offering some relief to patients who would otherwise have little hope of further amelioration.

Similar treatment could equally be applied to other serous cavities affected by carcinoma, for example pleural effusions resulting from breast cancer.

6.11.2. PURGING IN AUTOLOGOUS BONE MARROW TRANSPLANTATION

This technique may also be widely applicable for the ex vivo purging of residual disease from the bone marrow of patients undergoing ABMT after high dose chemotherapy or total body irradiation to eliminate host disease. Pharmacological agents have been used to purge marrow from patients with lymphoma and leukaemia, but they lack selectivity and are equally toxic to

normal haemopoietic cells (Singer and Linch, 1987). Photosensitisers have been shown to have differential toxicity on progenitor and tumour cells, (merocyanine, Sieber et al, 1987; AlSPc, Singer et al, 1987 and 1988). With both dyes, CFU-GM recovery was low, less than 50%, which compares unfavourably with the 100% results achieved with the antibody targeted AlSPc liposomes (Section 4.5). Combination therapy with merocyanine and adriamycin was even more toxic in preliminary experiments, and only 8% of CFU-GM survived (Gulliva, 1988).

Purging is not thought to be necessary for ABMT where bulk disease can be satisfactorily eliminated and there is no detectable disease in the marrow. However, where there is marked infiltration of tumour cells in the bone marrow, as in multiple myeloma, then purging with antibody targeted AlSPc liposomes might offer a better chance of recovery without relapse. With the right combination of monoclonal antibodies it should be possible to target and destroy tumour cells in bone marrow while sparing the normal haemopoietic precursor cells necessary for re-engraftment.

In vitro purging has been performed by Gobi et al, using an immunotoxin with the same anti-B-cell antibody, 8A, used in Section 4.5. The toxin, momordin, a ribosome inactivating protein was used as the cytotoxic moiety with good killing of tumour cell lines and 90% recovery of CFU-GM (Gobbi et al, 1988). This has since been followed by treatment of three patients with multiple myeloma by AMBT with purging using the immunoconjugate (Gobbi et al, 1989). The results were reported post ABMT for one patient

who was doing well with no evidence of multiple myeloma, and at 21 and 7 days for the others. It is still early to comment on the efficacy of the purging treatment or to speculate whether or not an AlSPc liposome purging would be more effective. One possible advantage is that toxins need to enter the cell cytoplasm to be effective, whereas the AlSPc liposomes are effective when bound to the outside of the cell.

6.11.3. PHOTODYNAMIC THERAPY OF CARCINOMA OF THE BLADDER

One disease that offers itself to photodynamic therapy probably more than any other is carcinoma of the bladder, because it is easily accessible to a laser probe. The optimum situation would be carcinoma in situ, because it does not present the problems of more solid tumours (see below).

Several photosensitisers have been used with varying degrees of success for photodynamic therapy of transplantable bladder tumours grown subcutaneously (purpurin derivatives, Morgan et al, 1987; metallopurpurin derivatives, Morgan et al, 1988; AlSPc, Selman et al, 1986) or bladder carcinoma cell lines (HpD, Miyoshi et al, 1984; Hisazumi et al 1984; rhodamine, Shea et al,1989). HpD has produced good clinical results for treatment of carcinoma in situ of the bladder (Hisazumi et al, 1984a). However, because HpD was given intra-venously, there were problems associated with skin photosensitivity. There was also uptake into the normal underlying layers of the bladder with damage to these tissues resulting in poor function which only resolved slowly. Presumably the use of photosensitisers which cause less skin sensitivity, or

direct bladder instillation of the sensitiser might prove more suitable. Damage to normal bladder tissue with severe lack of compliance was also observed in rats treated with intravenous AlSPc (Lottman et al, 1988). AlSPc has the advantage of causing less skin photosensitivity than haematoporphyrin (Tralau et al, 1989), so this was not a problem.

Bladder instillation of HpD showed lack of tumour specificity (Lin et al, 1984). Fluorescence distribution due to HpD did not correspond to tumour, being most intense in areas of oedema, necrosis and cell degeneration. Similar distributions were observed in multicellular spheroids of tumour cells, the photosensitiser partitioning into the central necrotic areas (Christenson et al, 1984), with decreased phototoxicity compared with monolayers of the same cells. Instillation of AlSPc into normal rat bladder resulted in patchy uptake by the urothelial lining, but was not taken up by the underlying muscular layers (A.Pope, personal communication).

It has been shown that monoclonal antibodies (produced against antigens of human bladder transitional cell carcinoma) bind preferentially to tumour rather than normal mucosa after intravesical injection, (Chopin and DeKernion, 1986). Targeting AlSPc-liposomes with such antibodies could provide an alternative route for treatment of bladder tumours, considering the problems that have been encountered with free photosensitisers with both intrabladder and intravenous administration using free photosensitisers.

A further problem with laser treatment of the bladder is the

difficulty of illuminating relatively inaccessible areas such as the neck, where tumour could escape irradiation because of spatial effects. This can beovercome by instilling a non toxic light scattering medium such as fat emulsion which results in homogeneous irradiation of the whole bladder (Jocham et al, 1984).

6.12. CONCLUSIONS AND PROSPECTIVE WORK

AlSPc liposomes have been shown to be potent sensitisers for antibody targeted photokilling. They appear to be effective at low AlSPc concentrations, and on cells with low antigen expression. They are effective when bound to the outside of cells, and presumably also effective when internalised, though the later has not been studied here.

A comparison of some of the advantages and disadvantages of free and liposome encapsulated AlSPc is summarised in Table 13.

They are most specific using the polar tri- or tetra-sulphonated aluminium phthalocyanines, the latter being the simplest to synthesise in a homogeneous form. They are easy to synthesise from non-toxic components, can be simply sterilised by filtration, and have a long shelf life.

They have considerable potential in their present form for the treatment of the 'contained' diseases described in Section 6.11. With the recent advances in liposome composition producing the 'stealth' liposome, and the availability of diverse stable linking agents for attachment of antibody, they might eventually be used for many of the diseases presently treated with free

TABLE 13

COMPARISON OF FREE AND LIPOSOME ENCAPSULATED ANTIBODY TARGETED AlSPc

FREE AlSPc	TARGETED AlSPc LIPOSOMES
1) Not very selective	Highly selective
2) High non specific binding Binds avidly to proteins	Low non specific binding Protected from binding to proteins By the insulating lipid bilayer
3) Large amounts needed for phototoxicity	Small amounts needed for phototoxicity
4) No amplification	High amplication
5) No especial preparation	Requires synthesis Simple but time consuming
6) Internalisation necessary for cell phototoxicity	No internalisation necessary for cell phototoxicity
7) Site of action throughout cell	Site of action mainly at cell surface
8) Less polar fractions most suitable	More polar fractions most suitable

photosensitisers with greater specificity.

Their specificity is dependent on both the presence of determinants on the surface of cells and the antibodies raised against them. A drawback to the use of antibodies with AlSPc liposomes, as with any other immune mediated therapy is the induction of immune responses which prevents long term treatment. Again, advances in recombinant technology mean that less immunogenic antibodies can be produced in large quantities.

Much work still needs to be done on the optimisation of doses of AlSPc, light, and antibody, with particular reference to the effects of bleaching, quenching, and light intensity. It would be interesting to find out how low antigen expression can be before photodynamic toxicity is not feasible.

The actual targets of singlet oxygen produced outside the cell are not known, and may differ from those damaged by activated photosensitiser in the cell membrane or in the interior of the cell. There is a possibility that in vivo the damage produced by externally bound liposomes may be of less consequence and more easily repaired than in vitro.

In vivo events can only be determined by using the AlSPc liposomes in an animal model. There are good ovarian carcinoma and myeloma models in nude rats and mice which would be suitable for testing the AlSPc liposome. Presently most progress is occurring in the direction of bladder carcinoma. In collaboration with Drs. H Lottman and D Chopin at the Henri Mondor Hospital in Paris, monoclonal antibodies are being screened for their suitability for targeting to bladder carcinomas. They are being

tested against human bladder carcinoma cell lines in a phototoxicity assay for the best single or combination of antibodies. Instillation of the chosen antibodies and liposome preparations into bladders will then determine tumour specificity in vivo and finally photodynamic therapy can commence.

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APPENDICES

APPENDIX 1

ESTIMATION OF IMMUNOGLOBULIN CONCENTRATION SPECTROPHOTOMETRICALLY

The molar concentration of a substance can be measured spectrophotometrically at a particular wavelength by the following equation:

$$A = c\varepsilon l$$

when A = absorbance

c = molar concentration

ε = molar extinction coefficient, which is a constant for any particular compound at a fixed wavelength

l = pathlength of the cuvette in cm.

For a 1 cm pathlength, and rearranging the equation:

$$A/\varepsilon = c \text{ (molar)}$$

The greatest sensitivity is obtained when the substance is measured at a wavelength where it has a high absorbance.

For immunoglobulin there is a good absorbance peak at 280 nm. The main contributions to the absorbance come from tryptophan and tyrosine residues in the primary protein structure, the former predominating. For IgG these are reasonably constant and:

$$\varepsilon_{280} = 9.3 \times 10^{-6} \text{ M}^{-1}\text{cm}^{-1}$$

Some proteins do not contain sufficient tryptophan residues and cannot therefor be measured in this way.

Sometimes the protein concentration is required in mg/ml. In this case a simple constant can be introduced which gives the result

required directly.

For example: if a sample of IgG solution gave an absorbance of 1 at 280 nm:

$$c = 1/9.3 \times 10^{-6}$$
$$= 1.07526 \times 10^{5} \text{ molar.}$$

To convert to mg/ml the molar concentration must be divided by the molecular weight of IgG, which is approximately 150,000 (1.5×10^5)

Thus:

$$1.07526 \times 10^5 / 1.5 \times 10^5 = 0.7168 \text{ mg/ml}$$

To reach this result directly from the absorbance value a constant (k) must be substituted into the equation:

$$A = kC$$

Since A = 1

and C = 0.7168 (IgG concentration in mg/ml)

$$1 = k \times 0.7168$$

thus $k = 1/ 0.7168$

$$= \mathbf{1.395}$$

or as an approximation $k = \mathbf{1.4}$

The direct formula is therefore:

$$\mathbf{A/1.4 = C \text{ mg/ml}}$$

APPENDIX 2

ESTIMATION OF THE NUMBER OF 2-PYRIDYL DISULPHIDE RESIDUES SUBSTITUTED PER PROTEIN MOLECULE

Each protein molecule that has been reacted with SPDP will contain 2-pyridyl disulphide groups (2PS), attached by thiol bonds. The number of 2PS groups present can be calculated by measuring the concentration of pyridine-2-thione (P2T) released from the protein at 343 nm by reduction of the thiol bonds on addition of dithiothreitol (DTT) (to a final concentration of 50 mM) according to the method of Carlsson (1978).

This is done using the following simple equation:

$$A = \varepsilon c \qquad \text{for a 1 cm pathlenghth}$$

When A = absorbance, ε = molar extinction coefficient at 343 nm for P2T ($8.08 \times 10^{3}\ M^{-1}\ cm^{-1}$)

For this situation the difference in A at 343 nm on addition of DTT gives the absorbance of P2T and the equation becomes:

$$(2PS) \quad c = \frac{A_2 - A_1}{\varepsilon} \text{ Molar}$$

Protein concentration can be measured:

a) by absorbance at 280 nm (for immunoglobulin). See Appendix 1.

or

b) by radioactive trace (for Protein A or immunoglobulin).

For the absorbance method, a correction must be made for the concentration of P2T at 280 nm:

$$A_{280} \text{ due to protein} = A_{280} - (B \times 5.1 \times 10^3)$$

where B is the molar concentration of P2T as calculated above and the molar extimction coefficient of P2T at 280 nm is 5.1×10^3.

The concentration of protein in mg/ml may then be calculated by dividing the corrected absorbance by 1.4 and converted to the molar concentration by the molecular weight of the protein (150,000 for immunoglobulin).

Finally the number of 2PS residues per protein molecule is calculated by dividing the molar concentration of 2PS by the molar concentration of protein.

For the protein derivations under the conditions used in this thesis the average number of 2PS residues per protein molecule was 3-9 for protein A and 9-13 for antibodies.

APPENDIX 3

STATISTICS

CORRELATION COEFFICIENTS

A correlation is the average relationship between 2 or more sets of observations, and the correlation coefficient, r, denotes the measurement of the correlation on a scale of -1 to 1. No correlation between the observations is 0, positive correlation (one variable increasing as the other increases) is between 0 and 1 and negative correlation (one variable decreasing as the other increases) is between -1 and 0. The closer r is to 1, the better the positive correlation.

The correlation coefficient is calculated by the following equation:

$$r = \frac{\Sigma(x-\bar{x})\ (y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\Sigma(y-\bar{y})^2}}$$

x represents the independent variable (in Section 2.2.12 this was the cell number).

y represents the dependent variable (in Section 2.2.12 this was the number of radioactive counts per minute).

STUDENTS T TEST

Paired and unpaired student's t tests were calculated using the Apple Mackintosh computer with the statistics software programme Statview.

APPENDIX 4

INHIBITION OF PHA STIMULATED MITOGENESIS BY ANTI-CD3 ANTIBODY

When peripheral blood lymphocytes (PBL) were stimulated to proliferate with the plant lectin PHA in the phototoxicity assays (Chapter 3), it was observed that when liposomes were targeted to the T lymphocytes with a CD3 antibody, there was some inhibition of mitogenesis. A series of simple proliferation assays were done separately to discover if this was significant. Both PHA M (a crude preparation) and PHA P (a more purified version) were used as mitogens to see if this had any effect.

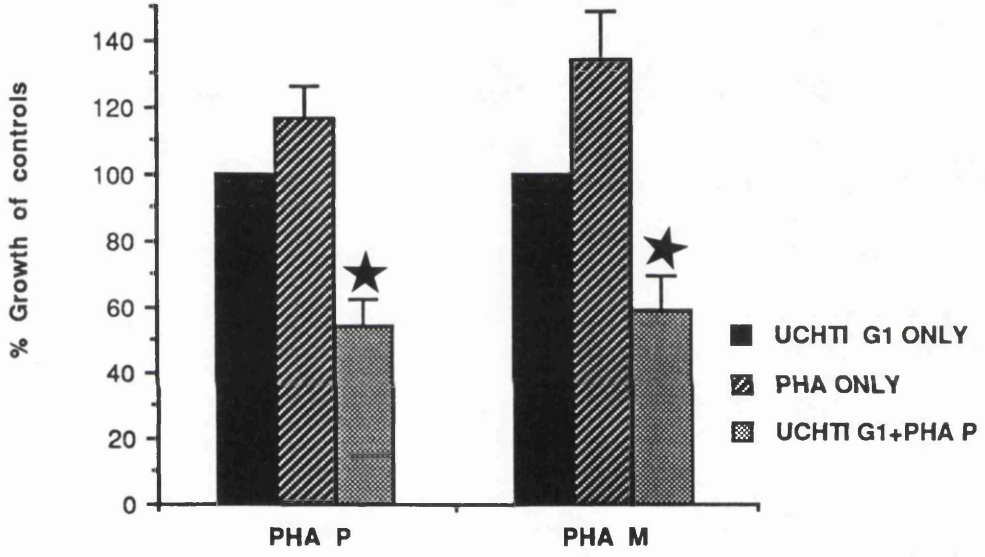
To samples of PBL in triplicate (2×10^5 cells per round-bottomed well), were added RPMI plus 10% FCS containing either PHA alone, UCHT1 G1 alone, or both together. The PHA (both types) was at a concentration which produced the maximum proliferation index of PBL as measured by uptake of ^{3}Hthymidine in the last 6 hours of a 72 hours incubation at 37^{o}C. The UCHT1 was at 2 μg/ml. Background samples contained neither PHA or UCHT1 and the radioactivity was subtracted from the test smples.

Six experiments were done for the PHA P combinations and five for the PHA M combinations. The UCHT1 stimulated cells were designated the controls and denoted as 100% growth. Growth of the PHA and combination samples was then calculated as a percentage of these.

The results in Figure 41 show a clear inhibition when the combination is used ($p < 0.005$ for both PHA P and PHA M). Six experiments were done for PHA P and five for PHA M, with triplicates at each point.

FIGURE 41

INHIBITION OF PHA P AND PHA M STIMULATED MITOGENESIS OF T LYMPHOCYTES BY THE ANTI-CD3 ANTIBODY UCHT1



LEGEND

Cells were treated as described in the text.

Results are Means +/- S.E.M. of 6 experiments with triplicates at each point.

★ $p \leq 0.005$

PAPERS PUBLISHED THAT CONTAIN SOME OF THE WORK DONE DURING THE EXECUTION OF THIS THESIS

GRAY AG, MORGAN J, LINCH DC and HUEHNS ER (1988). Uptake of antibody directed cytotoxic liposomes by CD3 on human T cells. Clin. Exp. Immunol. **72** 168-173.

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ABBREVIATIONS

AlSPc	-Sulphonated aluminium phthalocyanine
CF	-Carboxyfluorescein.
CFU-GM	-Colony forming units- granulocytes and macrophages
DPPC	-Dipalmitoyl phosphatidyl choline
DPPE	-Dipalmitoyl phosphatidyl ethanolamine
DTP	-Dithiopyridine
DTT	-Dithiothreitol
FCS	-Foetal calf serum
HpD	-Haematoporphyrin derivative
HPLC	-High pressure liquid chromatography
ISC	-Intersystem crossing
LDL	-Low density lipoprotein
MDA	-Malondialdehyde
PA	-Protein A
PBM	-Peripheral blood mononuclear cells
PDT	-Photodynamic therapy
PF II	-Photofrin II
SαMIg	-Sheep anti-mouse Immunoglobulin
S.E.M.	-Standard error of the mean
SPDP	-N-hydroxysuccinimidyl3-(2-Pyridyldithio)propionate
SUV	-Small unilamellar liposomes
TBA	-Thiobarbituric acid
TCA	-Tricarboxylic acid
Tc	-Transition temperature
TLC	-Thin layer chromatography
TMP	-Tetramethoxypropane