RECEPTOR MEDIATED TARGETING OF LIPOSOMES

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CERTIFICATION OF SUPERVISOR

In terms of paragraph eight of "General regulations for the degree of Ph.D." I as supervisor of the candidate, M.H.Friede, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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SUMMARY

The targeting of liposomes to cells and the delivery of the liposomal contents into the cells have been investigated using either α-melanocyte stimulating hormone or Ricin-B-chain as ligands for promoting the binding of liposomes to cells.

α-melanocyte stimulating hormone has been conjugated to liposomes and to Ricin-A-chain via the Lys_{11} residue without significant loss of biological activity. The resulting conjugates were found to bind to B16 melanoma cells which express receptors for the hormone. Hormone targeted ricin was shown to be toxic to the cells, indicating receptor mediated internalisation of the conjugate. The hormone targeted liposomes however were unable to mediate the delivery of cytotoxic levels of methotrexate.

Ricin-B-chain, a lectin which mediates membrane translocation of the toxic ricin-A-chain, was examined for its applicability for targeting of liposomes to cells. This lectin was shown to promote the binding of liposomes to cells and to mediate the delivery of cytotoxic concentrations of methotrexate.

Further evidence of functional ricin-B-chain mediated intracellular delivery of the liposomal contents was shown by liposome mediated transformation of cells, and delivery of nuclease into the cell resultin in digestion of genomic DNA.

The study demonstrates that α-melanocyte stimulating hormone is unsuitable as a ligand by which to achieve delivery of large quantities of material into cells, although cell-specific targeting can be achieved. Ricin-B-chain is however ideally suited for this task, though is less cell-specific.

This finding may be of use in studies in which investigators wish to achieve intracellular delivery of compounds.

ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	3',5' cyclic adenosine monophosphate
CM-MSH	Carboxymethyl MSH
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DNase 1	Deoxyribonuclease 1
DPPC	Dipalmitoylphosphatidylcholine
DPPE	Dipalmitoylphosphatidylethanolamine
DPPS	Dipalmitoylphosphatidylserine
DSC	Differential scanning colorimetry
DTBP	Dithiobispropionimidate
DTNB	Dithiobisnitrobenzoic acid
DTT	Dithiothreitol
EDTA	Disodium ethylenediamine-tetraacetate
EYPC	Egg-yolk phophatidylcholine
FCS	Fetal calf serum
G418	Geneticin
HPLC	High pressure liquid chromatography
LIP-MSH	MSH conjugated to liposomes
LIP-RTB	RTB conjugated to liposomes
LUV	Large unilammelar vesicles
MPB-PE	N-[4-(p-maleimidophenyl)butyryl] phosphatidyl ethanolamine
MSH	α-melanocyte stimulating hormone
β-MSH	β-melanocyte stimulating hormone
MSH-SH	MSH thiolated at Lys_{11}
MTX	Methotrexate
PBS	Dulbeccos phophate buffered saline
PBS/Ca/Mg	PBS containing 1 mM Ca^{++} and 1mM Mg^{++}
PC	Phophatidylcholine
PDP-PE	N-[3-(2-pyridyldithio)propionyl]phosphatidyl ethanolamine
PE	Phosphatidylethanolamine
PHA	Phytohemagglutinin
PS	Phosphatidylserine
REV	Reverse phase evaporation vesicles
RNA	Ribonucleic acid
RCA	Ricinnus communis agglutinin
RTA	Ricin-A-chain
RTA-MSH	Ricin-A-chain - MSH conjugate
RTB	Ricin-B-chain
SPDP	N-succinimidyl-[3,2-pyridyldithio] propionate
SUV	Small unilammelar vesicles
TCA	Trichloracetic acid
TEA	Triethylamine
TFA	Trifluoracetic acid
TLC	Thin layer chromatography
WGA	Wheat germ agglutinin

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Chapter 1

RECEPTOR MEDIATED TARGETING OF LIPOSOMES

Introduction

In the last decade there has been a tremendous amount of interest in the use of liposomes as agents for the delivery of drugs or other biologically active materials into cells both in medicine and in molecular biology. Several comprehensive monographs on the subject have been published (Knight 1981, Gregoriadis 1984) so I shall not attempt to review the entire field, but rather to provide an overview of some aspects of it, and to provide a background to the arguments presented later in this thesis.

Ever since its first postulation by Paul Ehrlich, the concept of the 'magic bullet', a construction that delivers a drug directly to the required site without its uptake in the rest of the body has been the holy grail of pharmacologists and physicians. Much of the research has focused on the linkage of cytotoxic or radio-opaque drugs directly to antibodies which then carry the drug to the desired site, however such constructs have limited usefulness in that an antibody molecule can carry only a limited number of drug molecules, and the construct is exposed to degradative conditions in the body.
Liposomes were recognised as being potentially superior building blocks of such magic-bullets as they completely enclosed the entrapped drug or enzyme, protecting it from degradation while circulating, and minimising side-reactions such as toxicity and allergy. Furthermore a

liposomes is capable of carrying several thousand fold more drug than a single antibody molecule.

Most of the early work demonstrated that liposomes injected *in vivo* were rapidly taken up by the reticuloendothelial system (RES), which while useful for treatment of certain ailments such as lysosomal parasites eg. Leishmaniasis (Alving and Swartz 1974), severely restricted their use for delivery to the rest of the body. The possibility of redirecting the liposomes to the required cells was first investigated by modifying the liposomal surface with antibodies recognising determinants on the target cell surface (Gregoriadis and Neerunjun 1975). Since then many modifications to the liposomal surface have been investigated, including hormones, lectins, carbohydrates and a host of antibodies, however the use of such targeted liposomes *in vivo* has remained hampered by the rapid clearance of many preparations by the RES, and the inaccessibility of the target cells to the circulating liposomes (Poste 1984). Recently liposomes with modified size distributions and lipid compositions have been shown to evade the RES (Allen and Everest 1983, Allen et al 1988) and to have enhanced uptake by tumors (Gabizon and Papahadjopopoulos 1988). That such liposomes have immediate potential for diagnostic or therapeutic use is testified by the growing number of commercial liposome companies. This targeting however stems from the physical nature of the circulatory system, rather than a recognition by the liposomes of specific chemical determinants on the desired target.

While active targeting of liposomes by recognition of surface determinants is hampered in therapy by undesired uptake of the liposomes and inaccessibility of the target to the circulating liposomes, it holds great potential as a tool in cell biology where it is able to promote the uptake

of drugs or macromolecules into cells. Successful use of this application has allowed *inter alia* ; the rescue or elimination of a subpopulation of cells (Leserman), the delivery of cAMP dependant protein kinase inhibitors into cells and investigation of the role of cAMP in hormone release (Reisine et al. 1986), and transformation of eukaryotic cells by liposome entrapped DNA (Machy et al. 1988).

Unfortunately the successful use of liposomes to achieve functional cellular uptake of the entrapped material is dependant on a number of factors including; liposome size and composition, the ligand attached to the liposome surface, the number and nature of the receptors for the ligand on the cell, the type of cell, and the entrapped material.

Liposomes

The preparation and characterisation of liposomes have been reviewed (Szoka and Papahadjopopoulos 1981), the following is a brief outline of liposomal parameters and how they affect liposome targeting.

Composition

Liposomes can be prepared from a wide variety of lipids, however the interaction of the resulting liposomes with cells and serum is highly dependant upon the choice of lipids.

There exists within the literature two principle philosophies regarding the use of liposomes to deliver molecules into cells. The first relies on the virtually insignificant interaction of untargeted liposomes with cells and the use of ligands attached to the liposomes to increase

the interaction with a specific type of cell, and the second relies on a natural affinity of liposomes without any ligands for cells. The essential difference between the two approaches lies in the ionic charge of the lipid used.

Most liposome preparations contain at least 50% (mol%) phosphatidylcholine (PC). Normally egg yolk phosphatidylcholine (EYPC) is used, which has a transition temperature (Tc) of -7 to -15 °C (Ladbrook and Chapman 1969) resulting in liposomes which are in a liquid crystal state above -7 °C. It is occasionally advantageous to use a defined lipid with a defined transition temperature, such as in the preparation of liposomes sensitive to temperature changes (Magin and Weinstein 1984), in which case pure synthetic lipids such as dipalmitoyl phosphatidylcholine (DPPC) are employed. Liposomes composed primarily of phosphatidylcholine do not, in the absence of additional ligands, bind significantly to cell membranes.

Additional phospholipids or charged amphiphiles may be added to alter the surface charge of the liposomes and their interaction with cells :

Phosphatidylserine (PS), which can be used to replace the phosphatidylcholine completely, induces a net negative charge on the liposomes. Liposomes composed of only PS and cholesterol bind to, and are endocytosed by, certain cell types (CV-1 and L929) very readily yet with other cell lines (T-lymphoma) associate very weakly (Straubinger et al. 1983). Liposomes composed primarily of PS have also been shown to be superior to positively or neutrally charged vesicles for the delivery of macromolecules into cells (Fraley et al. 1981), ascribed to an ability of negatively charged fluid vesicles to fuse with cells, whereas neutral vesicles, or negatively charged solid vesicles being taken up primarily by endocytosis (Poste and Papahadjopopoulos

1976). Contrasting to this argument is the demonstration that endocytosis is also the primary route of uptake for phosphatidylserine vesicles (Straubinger et al. 1983). The discrepancy in the proposed routes of uptake is ascribed to the assays employed to determine endocytosis and fusion and on detailed analysis, it would appear that endocytosis is indeed the primary route of uptake of liposomes, independent of the charge. It is thought that the binding of negatively charged liposomes to cells may be mediated by the presence of receptors for multiply negatively charged molecules on some cells (Fraley et al. 1981).

The incorporation of stearylamine imparts a positive charge to the liposomes, and in contrast to the above, it has been shown that positively charged liposomes rather than neutral or negatively charged, interact preferentially with cells (Szoka et al. 1980, Potter et al. 1985). The apparent discrepancy between the findings by various authors on the role of lipid charge on liposome cell interaction may result partially from the different assays employed to determine the interaction, and also from the condition of the cells, as it has been reported that the binding of positively charged liposomes to cells is highly dependant on whether the cells are in suspension or growing as monolayers (Fraley et al. 1981).

Pure phosphatidylethanolamine does not readily form liposomes, however, if stabilised with acylated molecules such as antibodies modified with palmitic acid, vesicle formation is possible. The binding of the stabilising molecules to cell surfaces results in the destabilisation of the liposomes and subsequent release of their contents. Such target sensitive liposomes have been shown to deliver greater amounts of entrapped drugs than comparable insensitive liposomes (Ho et al. 1987) and under conditions

where delivery of the contents of bound liposomes to cells is limiting, may be the liposomes of choice..

If the incubations with cells are to be performed in the presence of serum, it is necessary to include cholesterol in the liposomes. Cholesterol up to 50 mol% affords significant protection of the liposomes to serum lipoproteins by tightening the packing of phospholipid molecules in the bilayer (Allen and Cleland, 1980)

Preparation

There exist a large variety of methods for preparing liposomes, each with its own advantages and disadvantages for a particular application , however two of the main types of liposomes in the literature on targeting are Small Unilamellar Vesicles (SUV) prepared by sonicating phospholipid dispersions (Papahadjopopoulos and Millar, 1967), and large unilamellar vesicles (LUV) prepared either by the reverse phase evaporation procedure (REV)(Szoka and Papahadjopopoulos 1978) or calcium chelation (Papahadjopopoulos et al. 1975).

The SUV have diameters between 20 and 50 nm. Liposomes this small are more readily endocytosed by some cells than are large liposomes (Machy and Leserman 1983, Matthay et al. 1984) leading occasionally to situations in which only targeted SUV are able to deliver their contents into the cells, whereas LUV, while binding to the cells, do not exert any effect (Watanabe and Osawa 1987).
SUV however have several disadvantages over LUV (which are typically within a size range of 200 to 500 nm). These disadvantages are ;

1) The encapsulation volume and efficiency are very low, typically in the range of 0.2-1 l per mole lipid, with an efficiency of 0.1-1% of the required material being entrapped. LUV by comparison entrap between 15 and 50% of the aqueous space, with a volume of 10 - 20 l per mol lipid. The small aqueous space of SUV minimises their usefulness in encapsulating macromolecules, and the low efficiency can present problems if encapsulating expensive material. Furthermore the low internal volume implies that many liposomes have to be internalised to result in the equivalent uptake of one large vesicle. Since volume is related to the cube of the radius a five fold difference in radius represents a 125 fold difference in volume. Even with this difference in volume it is possible that greater delivery may be achieved in certain circumstances with SUV rather than LUV because of the ease of uptake of SUV rather than LUV.

2) The preparation of SUV requires extensive sonication to which the material to be entrapped may be sensitive. While slight sonication is required for REV preparation it can be reduced to a level at which even DNA is not affected (Fraley et al. 1980).

3) SUV are thermodynamically unstable structures, able to convert with time to larger structures (Larrabee 1979), a process which can be promoted by proteins (Utsumi et al. 1989) and in the case of phosphatidylserine liposomes, by cations (Wilschut et al 1985). It is therefore conceivable that preparations of SUV change properties with time.

The disadvantage of the REV type of liposomes over SUV is that the material to be entrapped comes into contact with organic solvents, which may cause denaturation. Furthermore diethylether, which is the most commonly used solvent for this purpose is frequently contaminated with peroxides, which, if not removed prior to liposome preparation,

promotes both oxidation of the lipid and any entrapped protein.

By contrast, the calcium chelation procedure is extremely mild, avoiding exposure to both organic solvents and sonication. It is however limited by the type of lipids which can be used, normally phosphatidylserine, and as noted above, liposomes prepared from this lipid interact non-specifically with cell membranes, and hence in targeting experiments where binding to non-target cells is deleterious, this liposome type is not suitable.

Linkage procedures

Successful attachment of proteins to the outer surface of liposomes has been achieved by several methods. There are three principle techniques;

1) Direct chemical coupling by the use of cross-linking agents such as EDC. This has been employed to link streptavidin at high density to PE containing liposomes (Rosenburg et al. 1987). These authors point out the possibility of contaminants within the organic solvents used for the liposome preparation interfering with the EDC reaction. In contrast to these results the binding of IgG to liposomes by EDC is not significantly greater than by adsorbtion (Endoh et al 1981).

2) Acylation of the protein permits its binding to liposomes if present during liposome preparation (Huang et al. 1982, Weissig et al. 1986), or if added subsequent to liposome formation (Sinha and Karush 1979). This technique requires the exposure of the ligand to detergent. An alternative procedure based on the same philosophy is the coupling of ligands to empty liposomes via diazotised stearylamide, and

the subsequent dehydration and reconstitution of the liposomes in the presence of the material to be entrapped (Garcon et al. 1986).This avoids the use of detergent in the preparation of the acylated protein.
The advantage of these procedures is that they do not expose the liposome contents to cross-linking reagents, and are capable of yielding very high efficiencies of linkage.

3) The covalent attachment of the protein to the liposome surface via interaction between a sulphydryl moeity on the protein and a sulphydryl-reactive group present on the liposomes. This resulting bond can be either a cleavable disulphide if the reaction was with a thiopyridyl group (Martin et al. 1981), or a stable thioether crosslink if a maleimido group was present on the liposomes (Martin and Papahadjopopoulos 1982).

The advantages of this technique are that it is mild, not exposing the liposomes or the internal contents to oxidising conditions, does not permit homopolymer formation, and if the site of the sulphydryl on the protein is removed from its functional site, is likely to permit binding without affecting functional activity. However not all proteins possess free sulphydryl groups hence chemical methods such as reaction with S-acetyl thioacetate (SATA) (Derksen and Scherphof 1985) are required to introduce sulphydryl groups into such proteins.

Several investigators employ an indirect method of targeting, in which free antibodies are bound to the target cells, and liposomes directed towards these antibodies either via protein A coupled to the liposomes (Leserman et al. 1980) or via a biotin-avidin bridge (Rosenberg et al. 1987, Trubeskya et al. 1988). The advantage of such methodology is that it obviates the requirement of linking each ligand of interest to the liposomes, hence several

different ligands can be investigated using the same preparation of liposomes.

Ligands

A variety of ligands have been attached to the surface of liposomes to promote their interaction with specific cell types. It is becoming apparent that the choice of the ligand is crucial in determining the final efficacy of targeted liposomes, some ligands promoting binding but not uptake of the liposomes.

Antibodies:

The majority of cell-specific liposome targeting has relied on immunological techniques using antibodies, and in particular, monoclonal antibodies. These have an advantage over hormones and lectins as ligands by which to target liposomes, in that any cell line can be specifically targeted towards provided it expresses a specific antigen. This has been reviewed (Toonen and Crommelin 1983) hence the following is not exhaustive, but a selection of more recent data, or conflicting results.

Much of the early work on antibody mediated targeting used antibodies directed against either the class 1 ($H2K^k$) (Huang et al. 1983, Heath et al. 1983, Machy et al. 1984, Leserman 1984) or class 2 (H-2 $I\text{-}A^k$ and $I\text{-}E^k$)(Machy et al. 1982) major histocompatability antigens. Apart from these more recent antibodies successfully used include antibodies against the carcinoembryonic antigen (Watanabe and Osawa 1987), the human endothelial cell surface (Trubetskya et al. 1988) and the neural cell adhesion molecule (Reisine et al. 1986).

The requirement for the antigen to be expressed for liposome binding and uptake to occur has been demonstrated by the increased binding and toxicity of liposomes bearing anti-H2K^{k} antibody towards mouse L-cells, compared to BALB cells which do not express the antigen (Heath et al. 1983). Similar results were obtained by Huang et al. (1983), however in testing the ability of the H2K antigen as a liposome target on different cell types (Machy et al. 1982) it has been found that identical antigens perform differently on different cell types, possibly as a function of the role of the antigen on that cell type . These authors found that binding of liposomes mediated via anti-H-2K antibodies was obtained on both T and B lymphocytes, and although both cell types were able to internalise liposomes targeted via other antibodies (H2 I-A), only T lymphocytes internalised these liposomes, indicating an internalising effect of the H-2K antigen on T lymphocytes absent in B lymphocytes. This indicates that the antigen plays an active functional role in the internalisation and delivery of liposomal contents.

An active role of the target is further demonstrated in which anti-trinitrophenyl antibody binds liposomes at a very high density to lymphocytes modified with trinitrophenyl. These liposomes do not deliver their contents into the cells (Weinstein et al 1978), a possible consequence of the antigen in this case being non-specifically chemically modified membrane proteins and not a specifically endocytosed marker.

In contrast to this however, it has been observed that anti-HRBC (horse red blood cell) antibody specifically binds liposomes to erythrocytes (Martin and Papahadjopopoulos 1981), and such binding is able to promote the delivery of

entrapped anti-malarial drug into the erythrocytes (Agrawal et al. 1987) significantly enhancing the efficacy of the drug. Since erythrocytes are unlikely to undergo endocytosis, this suggests that under certain conditions, internalisation of the liposomal contents can be achieved without the requirement for the antigen to be endocytosed. This leads to the question of the route of internalisation, discussed in the next section.

Hormones

Hormones offer several potential advantages over antibodies as ligands by which to promote the cell specific targeting of liposomes :

Hormones frequently express very strong affinity for their receptors, with dissociation constants in the range of 10^{-9} to 10^{-12} M. This is in comparison to typical K_D of 10^{-6} to 10^{-9} M for antibodies. This thousand fold difference in affinity may permit the specific binding of targeted liposomes to cells at a fraction of the concentration of antibody targeted liposomes.

Since most peptide hormones are thought to be endocytosed subsequent to their interaction with the receptor, it is likely that the carriers to which they are bound will become internalised. By contrast, many antigenic determinants do not become endocytosed.

The receptors for many hormones are restricted to specific cell types and recognition is often independent of species of origin of the cells.

In spite of these advantages, very little research has been done on the use of hormones for targeting liposomes. Hormones have been extensively and successfully used for the

direct targeting of toxins to cells (Oeltmann 1985, Cawley et al. 1980) however liposome targeting has been limited to interacting avidin labelled liposomes with cells exposed to biotinylated nerve growth factor (NGF) (Rosenberg et al 1987). These authors demonstrate hormone mediated binding of the liposomes to the cells, however the increase in binding above background is only four to five fold, and at a concentration of liposomes much higher than that expected from the affinity constant of avidin for biotin (approximately 10^{-18} M).

Insulin has been successfully used to target Sendai virus envelopes to cells deficient in Sendai virus receptors (Gitman et al. 1985), however although it has been linked to liposomes (Cantenys et al. 1983) it has not been used to target the liposomes to cells.

Lectins

A wide variety of lectins are available and their specificities for sugars make them an interesting alternative to either antibodies or hormones. All cells exhibit glycoproteins on their cell surface, yet different cells frequently exhibit different sugars on the glycoproteins, a difference that could be exploited by lectin targeting.

A variety of lectins have been used to target either drugs or liposomes to cells. Daunomycin has been directly linked to Concanavalin-A (Con-A) and targeted to tumor cells *in vivo*. Significant increase in survival time demonstrates successful targeting towards the tumor (Kitao and Hattori 1977). Wheat germ agglutinin (WGA), Phytohemagglutinin (PHA) and *Riccinus communis* agglutinin (RCA) have been used to promote the attachment of sialoglycoprotein bearing liposomes to erythrocytes (Juliano and Stamp 1976) by first

incubating the cells with the lectin, and then adding the glycoprotein-bearing liposomes. No investigation of uptake of the liposomes was performed, however the ability of the free hapten sugar to release bound liposomes suggests that WGA and PHA promote internalisation, whereas RCA does not.

Liposomes have been targeted to cells using concanavalin A (Con A) or Phaseolus vulgarus phytohaemagglutinin (PHA) (Salame and Patel 1986), however most of the lectin-bound liposomes could be removed by treatment with trypsin, indicating that internalisation of the liposomes had not been promoted by the lectin. In this experiment the liposomes used were MLV, and no selection for size is reported, hence the liposomes may have been too large for endocytosis.

WGA linked to SUV via a thioether linkage has been shown to promote the binding of liposomes to a glycophorin, to which the lectin binds, and the targeting efficiency dependant on the degree of coupling of the lectin to the liposomes (Hutchinson and Jones 1988).

Routes and assays of internalisation

The choice of the internal contents of the liposomes, and hence also of the assay for the delivery of liposomal contents into the cells, can have a very significant effect on the conclusions drawn from the experiment.

Possibly the simplest assaying procedure is the inclusion of a fluorescent dye within the liposomes, and the microscopic and spectrophotometric determination of fluorescence associated with the cells subsequent to incubation. The two most commonly used materials for this are calcein and carboxy-fluorescein (CF), however the two dyes behave differently upon internalisation (Straubinger et al. 1983) .

CF, although charged at neutral pH, losses its charge at intralysosomal pH and is able to diffuse into the cytoplasm, resulting in the appearance of diffuse fluorescence, an event which is inhibited by the addition of lysosomotropic amines such as ammonium chloride or chloroquin . The diffuse fluorescence, which may be incorrectly interpreted as being due to fusion of liposomes with the cells, is not observed when calcein, which is more highly charged and does not loss its charge in acidic environment, is used. These authors conclude that the punctate fluorescence observed with calcein, and with CF in the presence of NH_4Cl is due to endocytosis of the liposomes, and that liposome-plasma membrane fusion is a rare event.

The self quenching of carboxyfluorescein which results from its dimeric association at high concentrations (Chen and Knutson 1988), and which is relaxed when the dye becomes diluted, provides a sensetive assay for the release of the dye from lumen of the liposome. Total cell associated fluorescence can be determined by lysing the cells and the cell-associated liposomes in detergent, and comparison with the fluorescence prior to lysis provides an indication of what proportion of the cell associated fluorescence was not due to liposomally entrapped dye. Such an assay has been used to monitor the fusion of liposomes with cells (Weinstein et al. 1984), however, as stated above, the ability of the dye to escape from endocytotic vesicles into the cytoplasm hinders the interpretation of results from this assay.

A widely used assay of liposomal uptake is the inclusion within the liposomes of the cytotoxic drug methotrexate (MTX), the effect of which is easily determined by monitoring the uptake of radiolabelled nucleotides. Using this assay, several investigators (Heath et al. 1983, Huang et al. 1983, Machy et al. 1982) reach the conclusion that

endocytosis is the primary route of uptake of liposomes by cells since the addition of lysosomotropic agents such as NH_4Cl or chloroquin afford the cell some degree of protection against the liposomally entrapped drug. Such agents have no effect on the toxicity of the free drug. A problem with the use of drugs such as methotrexate, is that they are toxic to the cell in the absence of liposomes and hence toxicity due to leakage from the liposomes prior to uptake by the cells is difficult to distinguish. For this reason it is advantageous to use drugs modified so that they are unable to enter the cells unaided. Heath et al. (1983) have adopted this protocol by using a modified MTX which is unable to be taken up by the cells. Interestingly these authors are able to obtain almost 100% protection of toxicity with NH_4Cl, whereas other authors using underivatised MTX obtain between 20 and 50% protection. This discrepancy might be due to liposomal leakage at the site of attachment to the cell, and subsequent entry of the native drug, which is at a locally high concentration, through the plasma membrane.

Since erythrocytes are not actively endocytotic, they are unlikely to take up liposomes by endocytosis, and hence the observation that anti-malarial drugs in liposomes targeted towards erythrocytes have an enhanced efficacy (Agrawal et al. 1987) suggests that either fusion of the liposomes with the cells, or leakage from the bound liposomes into the cell, is taking place.

A similar approach has been taken by Watanabe and Osawa (1987) who encapsulate either the toxic lectin ricin, or its enzymatic component ricin-A-chain in liposomes. The A-chain component is highly toxic once inside the cell, but is unable to enter the cells unaided, making it an excellent monitor of liposomal effect. The lack of toxicity of targeted liposomes containing A-chain compared to that of

liposomes containing ricin indicates that leakage of bound liposomes does occur.

The cellular expression of liposomally entrapped DNA or RNA provides an unequivocal demonstration of functional delivery of the liposomal contents into the cells (reviewed later on in the thesis) as it is unlikely that genetic material can enter the cell unaided. The assay involves either the encapsulation of viral DNA or RNA such as SV40 (Fraley et al. 1980), or TMV-RNA (Nagata 1984), and monitoring the infectivity , or the encapsulation of a plasmid carrying the gene for an enzyme which confers resistance against a nutritional or chemical restraint eg. Thymidine kinase (Schaefer-Ridder et al. 1982) or phosphotransferase (Nakatsuji et al. 1987).

Summary

It is evident that although the simple targeting of liposomes to cells presents no problem *in vitro*, the subsequent functional delivery of the liposomal contents to the cells is highly dependant on the nature of the liposome, the choice of the target to which the liposome is directed, and on the material entrapped within the liposomes.

CHAPTER 2

MELANOCYTE STIMULATING HORMONE TARGETING OF LIPOSOMES

This project was initiated to study the feasibility of using small peptide hormones as a means of targeting liposomes to cells which carry receptors for the hormones. The advantages which hormones should have over antibodies as ligands for targeting have been discussed in chapter 1. At the time this project started there were no reports in the literature of hormone mediated targeting of liposomes. Insulin had been bound to liposomes (Cantenys et al. 1983), but no demonstration of biological activity of the resulting conjugate was demonstrated, and no attempts had been made to bind the liposomes to cells.

α-Melanocyte stimulating hormone (α-MSH) was chosen as a model peptide to study this approach because it is a small peptide whose physical and chemical properties have been fairly well characterized, and there exists a wide variety of melanoma cell lines which bear receptors to the hormone. The published figures for the binding constant of β-MSH (K_D = 3 X 10^{-9} M Varga et al. 1974) suggested that the affinity of the receptor for the peptide was several orders of magnitude greater than most antibodies express towards antigens and hence targeting should be achievable at lower concentrations of liposomes than for antibody mediated targeting. Furthermore, since melanoma is a pathological condition, and melanoma cells exhibit receptors for α-MSH, it was hoped that investigations into α-MSH mediated targeting of liposomes *in vitro*, might shed light on the possibility of utilising a similar approach *in vivo*.

Melanocyte stimulating hormone.

α-MSH is a linear tridecapeptide with the sequence - Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. (Haris and Lerner 1957). It is synthesised in, and secreted from the pars intermedia of the vertebrate pituitary, and is responsible for a variety of physiological effects, the most well characterized of which is control of skin pigmentation (for a review see: Sawyer et al. 1983). It also plays a role on tissue lipolysis (Ramachandran et al. 1976) and pain and behavior (Walker et al. 1980).

Biological activity assays.

The melanotropic effect of α-MSH is exerted by either melanosome dispersion, in the case of dermal melanocytes, or melanin synthesis in epidermal melanocytes and melanoma cells. The net effect of this is darkening of the cells, which can be measured by light reflectance either *in vitro* using frog (*Rana pipiens*) skin (Shitzume et al 1954) or lizard (*Anolis carolinensis*) skin (Goldman and Hadley 1969), or *in vivo* (Hogben and Slome 1931, Castrucci et al. 1984). In tissue culture, using melanoma cells the biological effect can be assayed by :

1) Spectrophotometric assay for the melanin content of the cells (Lee et al. 1972). This assay requires the cells to be grown for up to 48 hours in the presence of the hormone before the increase in melanin content becomes significant, and is subject to a number of unknown factors including serum components (Jerdan et al 1985) and melanogenic potential (Niles and Makarski 1978).

2) Tyrosinase activity assay (Pomerantz 1966). The process of melanin synthesis within the cell requires an increase in

the activity of the enzyme tyrosine hydroxylase, which converts L-Tyrosine to L-dopa, the precursor of melanin. This activity is expressed in cells within 30 minutes of exposing the cells to α-MSH and reaches a plateau after 40 hours (Legros et al. 1981). The assay for this activity relies on the conversion of 3,5-^{3}H-tyrosine to dopa, resulting in the formation of tritiated water. The tritiated water is separated from the tritiated tyrosine still in the reaction mixture by passage through a charcoal column. A second purification step of passage through a Dowex-50 column (Korner and Pawalek 1977) improves the accuracy of the measurement. This assay can be performed on living cells in tissue culture medium, however it is then affected by the rate of tyrosine transport into the cells (Pawalek 1979), and should be compared to assays of the cell homogenate.

3) Adenylate cyclase assay (Kreiner et al. 1973). When melanoma cells are exposed to α-MSH there is a rapid (within 10 minutes) increase in the levels of cAMP. In the case of Cloudman S-91 cells this increase is only found in those cells in the G2 phase of the cell cycle (Varga et al. 1974) indicating that the receptor to the hormone is only expressed in those cells during that phase. The accumulation of cAMP in the cells is directly linked to melanogenesis, as cAMP alone is able to induce an increase in the tyrosinase activity, and this increase is independent of the cell cycle (Varga et al. 1974), and as such has been implicated as the intracellular mediator for the response of melanocytes to α-MSH (for review see: Sawyer et al. 1983). Contrasting this hypothesis is the observation that α-MSH-tobacco mosaic virus conjugates are capable of tyrosinase activation independently of adenylate cyclase activation (Wunderlin 1984). It is possible that cAMP accumulation in melanoma cells in response to α-MSH is similar to its accumulation in PC12 cells exposed to NGF (Cho et al. 1989), mediating some but not all of the hormonal effects, possibly acting as one

of a number of required second messengers . Intracellular cAMP levels are also linked to the metastatic potential of the cells (Sheppard et al. 1984) although the physiological relevance of this remains unclear.

The increase in the intracellular concentration of cAMP is readily monitored using one of several commercially available kits (Amersham, NEN). The assay is simple and rapid, and can be performed on a large number of samples in one day. That the cells need only be exposed to the hormone for several minutes obviates the need for strict sterility which is required for the other assays.

While there is a wide variety of biological assays available, not all the assays yield the same results when comparing the potency of α-MSH and its derivatives, possibly due to slightly differing structures of the receptors between frog skin and lizard skin (Wilkes et al 1983),and inaccessibility to the receptor in skin for large conjugates (DiPasquale et al. 1978).

Receptor characterisation

The characterisation of the α-MSH receptor has been achieved primarily by interaction of Cloudman S91 cells with iodinated β-MSH (Varga et al. 1974). These authors observed that binding to the receptor occurs only in the G2 phase of the cell cycle, and consequently, in asynchronous culture, only a fraction of the cells bind the hormone. Binding of labelled hormone to the receptor has been shown to occur in discreet areas on the cell membrane, thought to be associated with an area that is related to the Golgi complex, and internalisation of the hormone results in the localisation of the label within the Golgi complex (Varga et al. 1976). The receptor has also been shown to be sensitive

to proteolysis, and treatment of the cells with trypsin or chymotrypsin resulted in loss of hormone binding capacity. It is likely that results obtained from studies with β-MSH hold true for α-MSH as the effects of the two hormones are non-additive (van Calker et al. 1983), suggesting the presence of a single receptor type.

Quantitation of the receptor yields approximately 10^4 receptors per cell, with an affinity constant of 3×10^8 l/mol. Similar figures have been obtained using indirect labelling of the Cloudman S91 MSH receptor with fluorescent conjugates of α-MSH and ferritin (DiPasquale et al. 1977).

Apart from Cloudman S91 cells, many other cell lines have been found that exhibit a response to α-MSH, including B16 mouse melanoma cells (Lee et al. 1972), human melanoma cells (Legross et al 1981, Fuller and Meyskens 1981), and brain cells (van Calker et al. 1983).

Attempts to use iodinated α-MSH as a probe for the receptor have been hampered by the tremendous loss of biological activity that occurs when α-MSH is iodinated. The inactivation is due both to the use of oxidising conditions during the iodination, resulting in the oxidation of Met_4, and the presence of a bulky group on the Tyr_2 (Sawyer et al. 1977). The excellent purification properties of HPLC have however permitted the preparation of monoiodo- α-MSH with 35% of its biological activity still retained (Eberle and Hubscher 1979) and monoiodo β-MSH with full biological activity (Lambert et al. 1982).

Synthetic Derivatives of α-MSH

If α-MSH is to be used as a ligand to target liposomes, it will have to be linked to the liposomes. The position on the peptide at which to achieve this without destroying the affinity for the receptor is best chosen by examining which parts of the peptide can be modified without concomitant loss of biological activity.

α-MSH, partial sequences of α-MSH, and analogues in which some of the residues have been replaced with alternative amino acids have been synthetically prepared (Eberle et al. 1975) and tested for biological activity in a number of systems. This has resulted in the elucidation of the presence of two message sequences within α-MSH that are able to trigger the hormonal response on amphibian skin, the first being the central region of the peptide -His-Phe-Arg-Trp- and the second being the C-terminal Gly-Lys-Pro-Val-NH_2. Replacement of Met_4 with Nle and replacement of the L-Phe_7 with D-Phe results in a melanotropin with 60 times the potency of native α-MSH in the frog skin assay, and ten times the potency in the lizard skin assay (Wilkes et al. 1983). A cyclic derivative in which the Met_4 and Gly_{10} were replaced by cysteine was found to have a 10000 fold greater potency than α-MSH in darkening frog skin, and substantially prolonged activity. In contrast it was only 30 fold more active than the native peptide in the lizard skin assay, and only three fold more potent than α-MSH in stimulating adenylate cyclase in Cloudman S91 melanoma cells. Cyclic lactam analogues of the peptide have also been synthesised and their structure and biological potency elucidated (Sugg et al. 1988).
These results suggest that across species, receptors to melanotropins differ in their conformational requirements for recognition of the melanotropin, but that a generally preferred conformation exists (Sawyer et al. 1983). Proton

NMR analysis of the aqueous solution conformation of melanotropin analogues (Sugg et al. 1986) has shown this conformation to comprise a left handed turn of the backbone going from His_6 to Trp_9, and a close spatial relationship between the side chains of His_6 and Phe_7.
Any modification or alteration to the peptide which destabilises this preferred conformation is thus likely to have the effect of reducing its biological activity. Thus loss of a positive charge by substituting either Ser or Nle at Lys_{11} , which is often used for linking the peptide to carriers, results in the activity being significantly reduced to approximately 5% of the original, however replacement of the Lys_{11} with ornithine, which does not result in loss of positive charge, causes only a slight (35%) decrease in activity (Eberle 1981).

Therefore if biological activity is desired, the preparation of any conjugate of α-MSH should be done in a manner to not cause significant alterations to the preferred conformation. This is best achieved by linking the peptide to the carrier via its amino terminus which is not involved in receptor recognition, however the absence of a primary amino group at the amino terminus of the native hormone necessitates that for this protocol to be followed the peptide must be prepared synthetically.

MSH conjugates

MSH has been conjugated to a variety of carriers, and the biological activities of the resulting conjugates assayed.

α-MSH has been covalently linked to ferritin by carbodiimide activation of a ferritin-FITC and conjugation to α-MSH via

the Lys_{11} (DiPasquale et al. 1978). The resulting conjugate contained 5 α-MSH molecules per ferritin molecule. It exhibited tyrosinase activity stimulation of 0.3 , and frog skin darkening potency of 0.1 compared to that of the native peptide. Furthermore it bound specifically to Cloudman S91 cells, and the binding could be inhibited by free α-MSH. The authors take great care to ensure that the observed biological activity is not due to release of hormone from the conjugate. By this conjugation procedure the charge on Lys_{11} is lost, yet a fairly high biological activity remains.

In contrast to these results conjugation of α-MSH to human serum albumin to yield a similar ratio of 5 hormone molecules per carrier molecule has been achieved by linkage through the Lys_{11} position and the N-terminal position (Eberle 1981). In this case when the conjugate was linked via the Lys_{11} position the biological activity (both tyrosinase and frog skin melanophore dispersion) was 0.01 that of free native α-MSH. When the linkage was through the N-terminus (achieved using a synthetically prepared peptide containing a maleimido group at the N-terminus and conjugating to thiolated albumin) the conjugate had activity equal to that of the native hormone. The linkage via the lysine was achieved by condensing N-maleimido-Lys_{11}-α-MSH with thiolated albumin, resulting in a thioester linkage in which the charge on the lysine is lost and a maleimide group introduced.

If the α-MSH was linked via its N-terminus to tobacco mosaic virus resulting in a conjugate with 300-500 hormone molecules bound per carrier (Kriwaczek et al. 1978, Eberle 1981) the biological activity was significantly increased. This increase in activity is best explained by a cooperative affinity phenomenon (Kriwaczek 1978) in which one carrier molecule is able to interact with several receptors

simultaneously, and the receptors experience a high local concentration of the hormone because several hormone molecules are held in place.

Several conjugates have also been made using β-MSH which being a larger peptide (Asp-Glu-Gly--Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Lys-Asp) is more amenable to modification without affecting biological activity. it is likely that the receptors for α-MSH and β-MSH are the same.

β-MSH has been covalently coupled to Sepharose beads (Varga et al. 1974) and the resulting beads stimulated both tyrosinase activity and melanisation at a concentration of hormone similar to that of the free peptide. Since the major surface area on a sepharose bead is internal (our estimate in this laboratory is in the order of 98%- results not shown), most bound hormone would be inaccessible to the receptor, hence the high biological activity must represent release (possibly enzymatic) of a biologically active portion of the β-MSH from the conjugate and its subsequent interaction with the cells. Since the incubation was performed for 48 hours under conditions in which proteolytic cleavage is possible, this warns that interpretation of the results of biological activity assays with hormone-carrier complexes should bear in mind the possibility of fragments of the peptide being cleaved off or being released due to solvolysis (Tesser et al. 1974). β-MSH being a longer molecule, is possibly more susceptible to proteolytic cleavage resulting in a biologically active fragment.

Conjugates of β-MSH with daunomycin (Varga et al. 1977, Varga 1985) have been prepared and found to be specifically toxic to melanoma cells, and the toxicity can be inhibited by the presence of free hormone. This suggests that the receptor for MSH does permit the specific internalisation of the conjugate. That the cells obtain protection from the

conjugate by the addition of lysosomotropic amines suggests that the conjugate is taken up by an endocytotic process, and passes through the lysosomes, where its processing is required for it to be toxic. The concentration of conjugate required to kill 50% of the cells was two orders of magnitude greater than the dissociation constant of the native peptide. Since 50% of the receptors should be occupied at a concentration equal to the dissociation constant, this indicates that there has been a decrease in the affinity of the hormone for the receptor.

Conclusion

α-MSH is a peptide hormone with a very high affinity (compared to antibodies) for a receptor which is expressed on several cell types. Chemical modification of the peptide is possible without significant loss of biological activity, and evidence from β-MSH suggests that the receptor promotes binding and internalisation of hormone conjugates, making α-MSH an ideal candidate as a peptide ligand by which to target liposomes.

CHAPTER 3

MSH MEDIATED TARGETING OF LIPOSOMES

Introduction

Lacking the facilities with which to synthesise derivatives of MSH carrying chemically active moieties at the amino terminus (eg maleimido groups), I decided to investigate the possibility of linking the peptide to the carrier via the Lys_{11} but maintaining the positive charge. This was based on the observation that substitution of Ornithine for Lysine at position 11 did not drastically reduce the biological activity (Eberle 1981). To achieve this, MSH was derivatised with dithiobispropionimidate (DTBP), a cleavable cross linker which interacts with amino groups via its imidate moiety leaving a positive charge, although displaced, and can be cleaved with DTT to expose a thiol group. This thiol group could then be used to conjugate the peptide to a carrier.

Peptide thiolation

MSH was modified with DTBP and reduced as described in methods and purified on HPLC. The resulting peaks from HPLC (fig 3.1) were analysed for peptide content by amino acid analysis and for sulphydryl content by colorimetric determination with DTNB (dithiobisnitrobenzoic acid).

A requirement of the linkage procedure is that the thiolated peptide is not contaminated with free thiol groups (eg DTT). As shown in fig 3.1 HPLC gives excellent separation of the products and reactants. Only a single peak

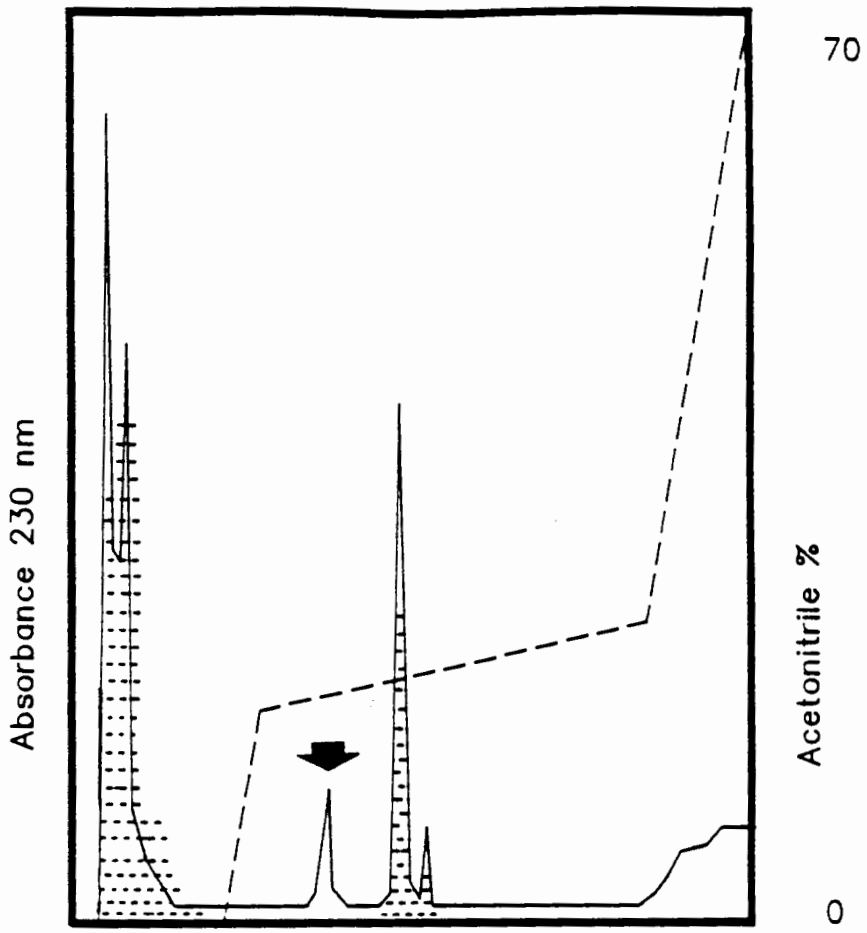

Fig 3.1 : HPLC of MSH modified with dithiobis propionimidate. The peptide and its derivatives were eluted off C_{18} μ-Bondapak column with a 0-70% acetonitrile gradient in 0.05% trifluoracetic acid. Absorbance (lower trace) monitored at 230 nm. Sulphydryl content (dotted area) monitored by reaction of aliquots with DTNB. Arrow shows elution position of MSH.

contained both peptide and thiol, and this peak which was assumed to represent the modified peptide was clearly separated from any unmodified peptide and reducing agent. Since the elution buffer contained 0.05% TFA the oxidation of the exposed thiol is extremely unlikely, however the thiolated peptide was prepared fresh whenever needed.

Biological activity

To determine whether modifying MSH with DTBP had altered its ability to be recognised by the receptor, the thiolated peptide (MSH-SH) was assayed for biological activity by monitoring peptide induced stimulation of both adenylate cyclase activity and tyrosine hydroxylase activity.

Since the time response of MSH mediated adenylate cyclase activation varies between cell lines it was necessary to establish the time response of the cell line used. The cell line used was the B16-F_1 subclone of the B16 mouse melanoma line.

Fig 3.2 shows the effect of 10^{-7} M MSH or MSH-SH on the accumulation of cAMP in B16-F_1 cells with time. Maximal stimulation is achieved by both the native and modified peptide after 30 minutes of incubation, a significantly lower time response than that published for cultured brain cells (van Calker et al 1983), however similar to that for Cloudman S91 cells (Fuller and Viskochil 1979). For subsequent experiments an incubation time of 30 minutes was employed.

Interestingly, although the magnitude of stimulation was approximately the same for both the native and modified peptide, the decay of cAMP in the cell was significantly slower for the thiolated peptide than for MSH. This suggests

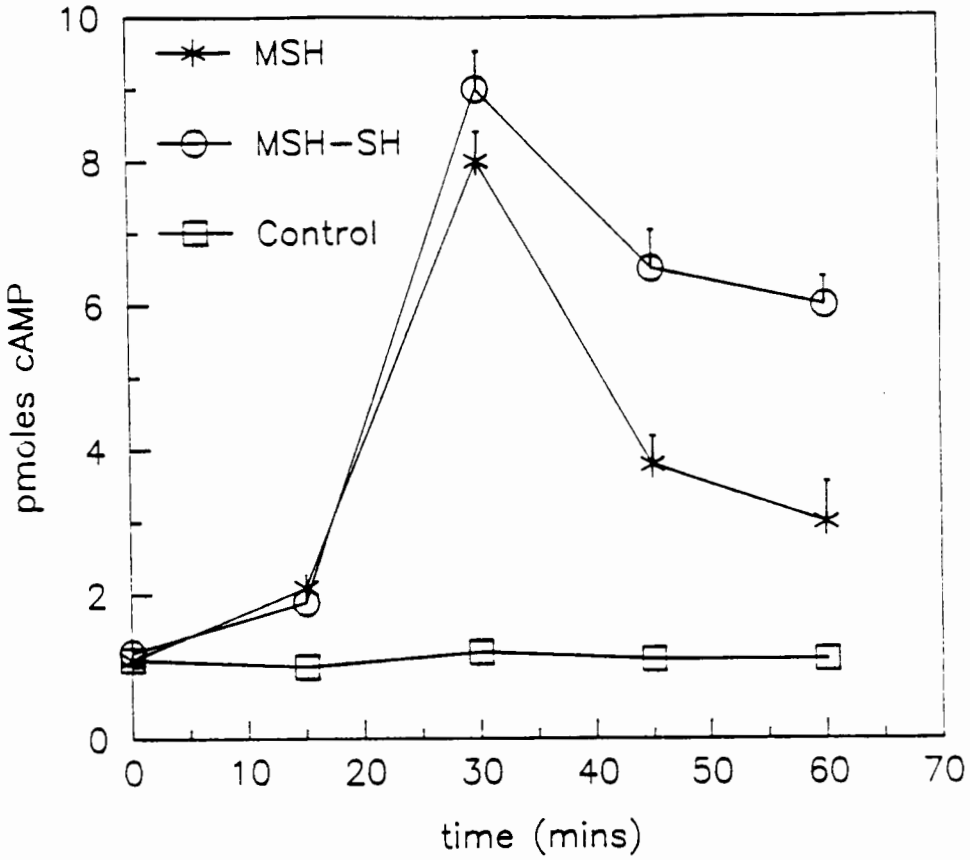

Fig 3.2: Time course of the increase in the level of cAMP evoked in B16-F_1 mouse melanoma cells by 10^{-7} M MSH, MSH-SH or a control of no peptide. At 15 minute intervals after adding peptide to the cells the medium was decanted and cAMP extracted with 0.2M HCl in ethanol. The quantitation of the cAMP was performed using a commercial cAMP assay kit (Amersham) and expressed as pmoles cAMP per well. Each point in the control was assayed once. Each point in the experiment is the average of duplicates.

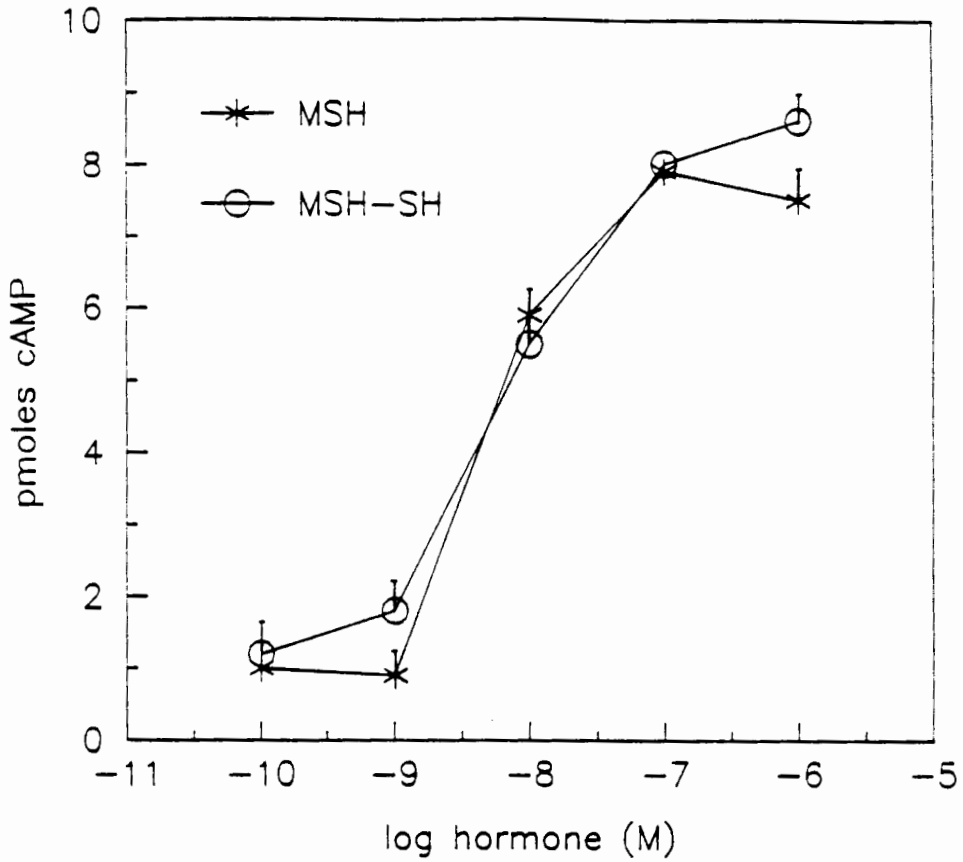

Fig 3.3: Dose-response curves of the accumulation of cAMP evoked by MSH or MSH-SH in B16-F_1 cells. The response is expressed as pmoles cAMP per well. Each well contained 10^5 cells. Results shown are the average of duplicates performed on the same sample of cells. Variability within each assay was low, however between assays performed on different days the absolute values varied. This is explained in the text.

that there may be a slightly altered interaction of the thiolated derivative with the receptor compared to that of the native peptide; possibly a slower dissociation of receptor and peptide resulting in a longer stimulation of adenylate cyclase and hence a slower decay of intracellular cAMP.

The dose-response of adenylate cyclase to both MSH and thiolated MSH is shown in fig 3.3, demonstrating that the modification of the peptide has not caused a decrease in its ability to stimulate adenylate cyclase via the MSH receptor. Since the adenylate cyclase stimulation is an immediate response to the hormone, it is indicative of hormone-receptor interaction, and hence it appears that the modified hormone is able to interact with the MSH receptor as well as the native peptide. The peptides express an EC_{50} of 9×10^{-9} M, within the range of most published figures.

That the modified peptide is able to interact with the receptor is not sufficient proof that it is able to exert full biological effect, and hence the ability of both peptides to stimulate tyrosinase activity were investigated. The results are shown in fig 3.4, demonstrating that the modified peptide is slightly less able to promote tyrosinase stimulation than MSH is. The native peptide exhibits an EC_{50} of 8 nM, compared to the thiolated derivative with an EC_{50} of 20 nM. Since both peptides are able to stimulate adenylate cyclase to the same extent, yet differ in their abilities to stimulate tyrosinase, it is possible that their interaction with the receptor is identical, but interact differently with a post-receptor processing element. β-MSH-Daunomycin conjugates have also been shown to be recognised by the receptor and yet to exhibit no tyrosinase stimulation, a phenomena ascribed to decreased susceptibility of the modified peptide to proteolysis within the cell, and hence the absence of active peptide fragments

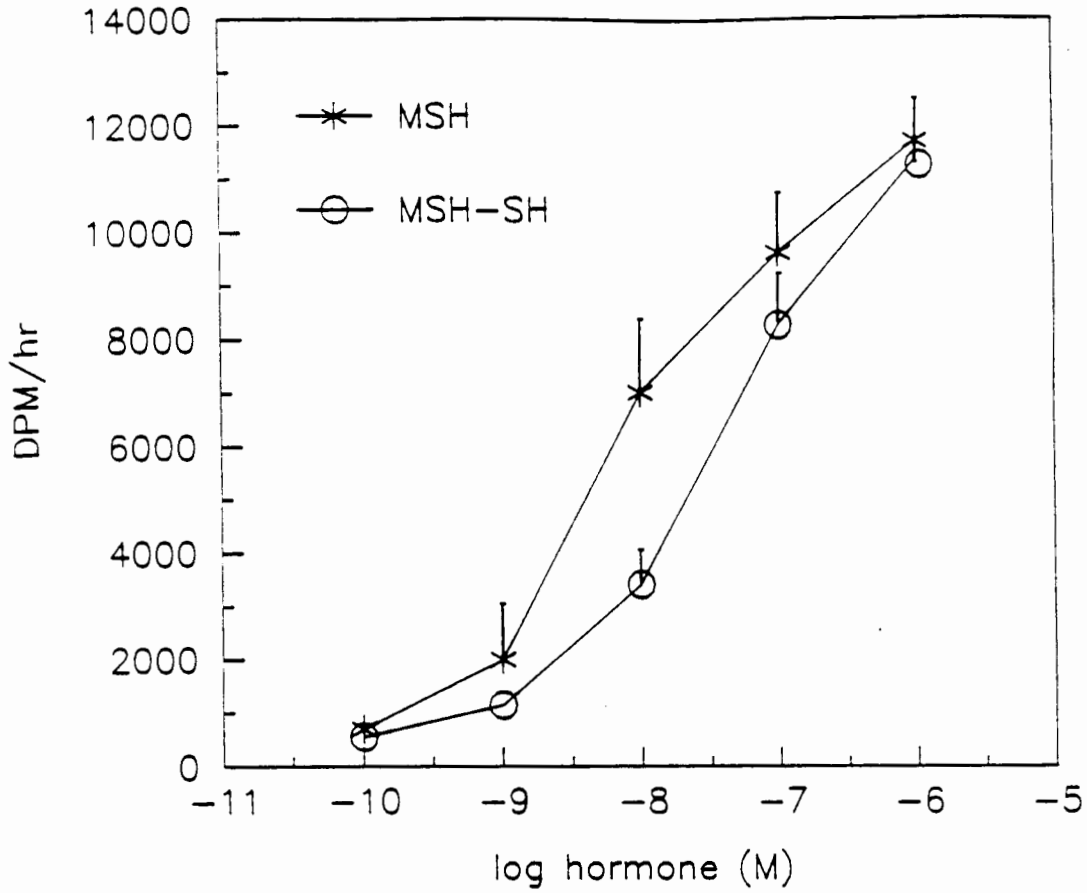

Fig 3.4 : Dose-response curves of tyrosinase activity in B16-F_1 cells. Cells were incubated in McCoys 5A medium suplemented with 10% fetal calf serum with increasing concentrations of hormone. After 24 hrs 0.5 µCi of L-[3,5-^{3}H]tyrosine was added and incubation continued for a further 24 hrs at which time the amount of ^3H_2O in the medium was assayed. The results are expressed as dpm ^3H_2O per hr per well averaged on duplicates.

which may be required for full biological activity to be exerted (Varga 1985).

These results show that the modified MSH is able to interact with the MSH receptor as strongly as the native peptide, and hence appears that it may be able to function as a ligand by which to target liposomes to cells.

That there has not been a decrease in the biological activity is surprising. Although the positive charge has been maintained one would expect the introduction of a larger group at the lysine to cause some decrease in activity by hindering the formation of the turn in the Phe-Arg-Trp region. Two possible explanations exist for the maintenance of full adenylate cyclase activation potential.

1) The displacement of the positive charge from the epsilon-amino of Lys_{11} three atoms further away onto the imidate of the modifying reagent DTBP, may allow closer, and hence stronger, electrostatic interaction of the positive charge on Lys_{11} with the negative charge on Glu_5. This may function to stabilise the turn in the peptide, and hence maintain its affinity for the receptor.

2) The exposed thiol on the modified peptide may be able to interact with a similar moiety within the receptor, increasing the strength of interaction of the peptide with the receptor, and thus canceling the decrease in affinity due to the introduction of a large side-group. A reaction with the receptor may also account for the slower decay of adenylate cyclase activity observed with the thiolated peptide.

It was noted during these studies that occasionally preparations of the modified peptide exhibited biological activity of five to ten fold that of native MSH, and were

able to induce the adenylate cyclase to a much higher level than MSH alone. Quantitation of the sulphydryl content of these samples showed it to exceed the peptide content, indicating the presence of a contaminating thiol compound. This compound was identified to be a contaminant introduced with the mercaptoethanol which co-eluted with the MSH-SH on the HPLC. To avoid this all further preparations were prepared using DTT as a reducing agent. This observation, and the published reports of thiol compounds in stimulating melanogenisis (Horowitz 1957), and the non-identical biological behavior of thio-ether and disulphide conjugates of MSH-TMV (Wunderlin et al. 1985) supported this argument.

Since the modified peptide contained a free sulphydryl, it is possible that this moeity was responsible for the observed high biological activity, possibly through an interaction with a sulphydryl within the receptor. If this were the case then blocking the sulphydryl on the peptide should result in a lower biological activity.

Carboxymethyl-MSH (CM-MSH).

To ascertain whether the high biological activity was due to the presence of a sulphydryl, the sulphydryl moeity of MSH-SH was blocked on an analytical scale using radiolabelled iodoacetic acid as described in methods.

Purification of the reaction mixture on HPLC (Fig 3.5 A), and monitoring of the radioactive content of the fractions, demonstrated that MSH-SH exposes a sulphydryl which can be carboxymethylated to nearly 100% efficiency resulting in a radiolabelled, blocked, modified peptide. To confirm that the modification was due to the sulphydryl, MSH was subject to identicle reaction conditions and purification (Fig 3.5 B). No significant labelling of the peptide peak occurred

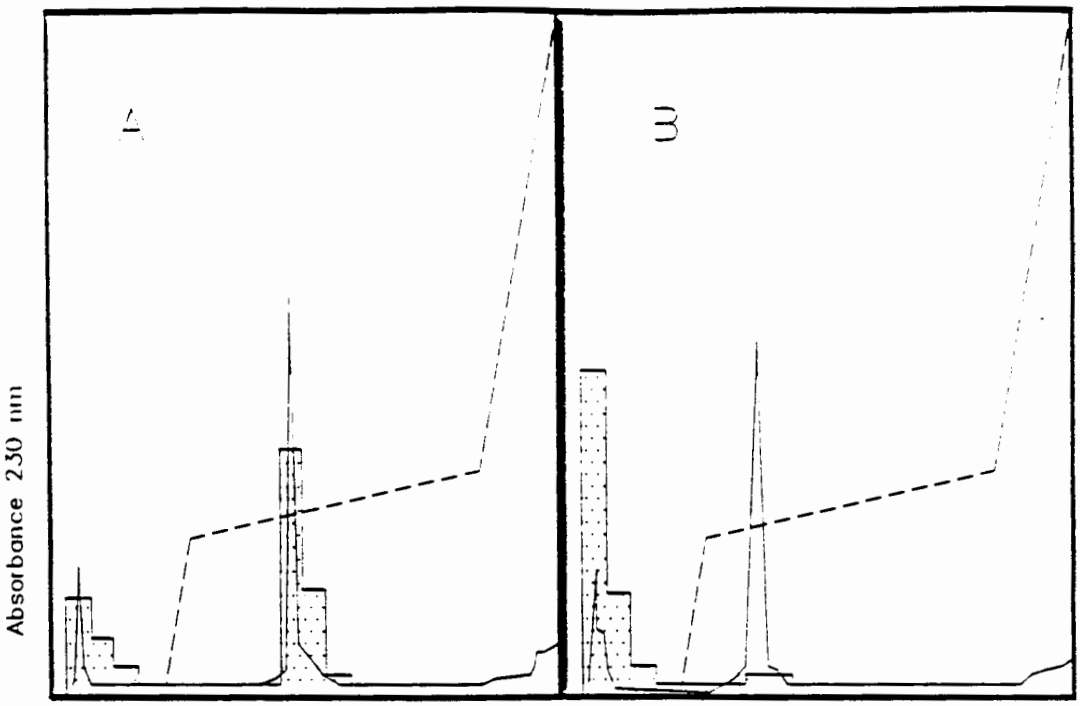

Fig 3.5 : HPLC purification of products after interaction of MSH-SH (A) or MSH (B) with ^{3}H-iodoacetic acid. Lower trace is absorbance monitored at 230 nm, hatched area is radioactivity, monitored by counting aliquots in scintillation fluid. Dashed line indicates the gradient of acetonitrile in 0.05% TFA.

indicating that there was an exposed thiol group on MSH-SH, and that on interaction with iodoacetic acid, this group became carboxymethylated.

MSH-SH was then carboxymethylated on a preparative scale to prepare CM-MSH for biological activity assays. Blocking procedure was as above but using non-radioactive iodoacetic acid, and 30 ug of peptide. The peak isolated from HPLC was quantitated by amino acid analysis and assayed for biological activity.

Biological activity of CM-MSH

CM-MSH was compared against MSH-SH for its ability to activate adenylate cyclase.

The results are shown in fig 3.6, demonstrating that the carboxymethylated derivative does express a lower biological activity. EC50 for MSH-SH is 8 X 10^{-9} M whereas for CM-MSH it is 1.5 X 10^{-8} M, or 0.63 times as active.

This decrease in activity could be due to blocking of the sulphydryl moeity, preventing its interaction with the receptor if such an interaction does occur, or due to the introduction of a further bulky group onto the hormone, altering the conformation and interfering with the hormone-receptor interaction.
This line of investigation was not pursued further as it was felt that the CM-MSH closely resembled the likely structural configuration of MSH-SH linked to a carrier via a thioether linkage, and that a biological activity of 0.63 proved that it was recognised by, and interacted with the receptor.

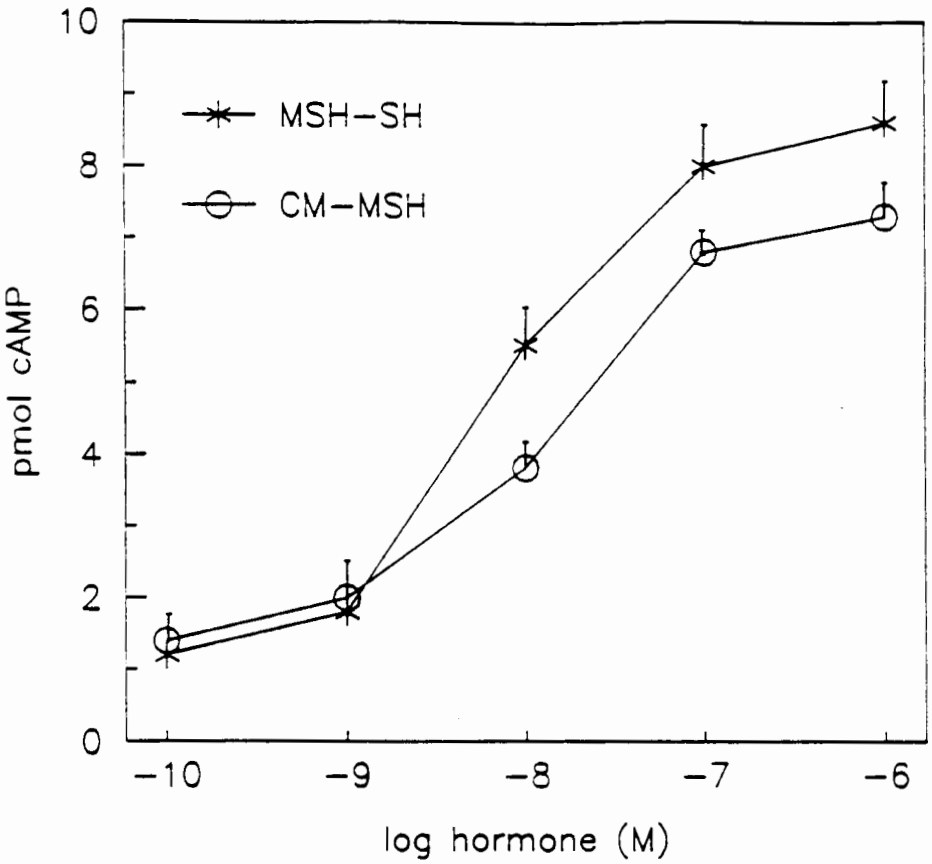

Fig 3.6: Dose-response curve for adenylate cyclase activation of B16 cells by MSH-SH or its carboxymethylated derivative CM-MSH. Data points for MSH-SH are averages of duplicates, for CM-MSH averages of triplicates.

Comparison of biological activity assays.

It was found that the adenylate cyclase assay gave far more reproducible results, could be performed on a larger number of samples, and was technically easier to perform than the tyrosinase activity assay, and hence was used for all subsequent assays of biological activity. Furthermore, as discussed previously, the adenylate cyclase activity assay provides an indication of interaction with the receptor, whereas the tyrosinase or melanisation assays appear to require additional post-receptor processing and hence may not be indicative of receptor interaction.

The increase in the level of adenylate cyclase activity was found to be highly dependant on the recent history of the cells. Cells in mid-log phase which had been regularly subcultured expressed a ten fold stimulation of basal cellular cAMP, however if they had been allowed to grow to confluence prior to subculturing into the flask from which they were assayed, the response was only a two fold stimulation (results not shown). Likewise if the cells were left in the multi-well dishes for several days and assayed during the stationary phase of their growth, the response was significantly diminished.

The variable response of the B16 cell line to MSH has been investigated by Sheppard et al.(1984) who found that clones of the B16 cell line that were highly metastatic also displayed a large (50 to 70 fold) increase in basal cAMP levels in response to MSH or forskolin exposure. Clones with low metastatic potential displayed only a 2 to 3 fold increase in basal level. Their conclusion is that cAMP metabolism is linked with biochemical pathways responsible for formation of metastasis. Their finding contrasts that of Niles and Makarski (1978), who, in monitoring tyrosine

hydroxylase increase in response to MSH found that cells of high metastatic potential were not significantly responsive to MSH, whereas those of low metastatic potential were. In the paper by Sheppard et al.(1984) this disparity is explained as being due to the use by the earlier authors, of heterogenous polyclonal B16 lines in which changes to the balance of cellular subpopulations may have occurred. The cell line used in these experiments was the B16-F_1 subclone, supposedly of low metastatic potential and hence of low MSH responsiveness. The results presented here do however show that the cell line was responsive to MSH, although with a lower magnitude of response than that published for highly metastatic subclones. This, and the variable responsiveness to MSH with cell history was therefore assumed to be due to the presence of both responsive and non-responsive subpopulations within the cell line.

For this reason the passaging and assaying procedure was standardised to maximise the observed response. If the control (MSH) response in any experiment resulted in less than six-fold stimulation of basal cAMP, the results were discarded and the experiment repeated using cells from a lower passage number.

Chapter 4

CONJUGATION OF α-MSH TO CARRIERS

MSH containing a small amount of tracer ^{125}I MSH (prepared by the lactoperoxidase method and purified as described in methods) was derivatised with DTBP and purified as described. The radiolabelled fraction of the sample eluted from the HPLC slightly later and hence broader fractions had to be collected to ensure the presence of the tracer with the hormone (Fig 4.1). The MSH-SH thus obtained had a specific activity of 30 mCi/mmol. After drying and redisolving in PBS it was used for conjugation to liposomes.

Liposome preparation:

Liposomes prepared by the reverse phase evaporation procedure (REV) (Szoka and Papahadjopopoulos 1978) were chosen because they fulfilled the criteria required for the experiments : They are large unilammelar liposomes with a high entrapment efficiency, capable of encapsulating macromolecules.

The liposomes were prepared to contain 50 mol% cholesterol to minimise leakage induced by serum proteins, 45 mol % Phosphatidylcholine (PC) to minimise nonspecific binding, and 2.5 mol % N-[3-(2-pyridyldithio)propionyl] phosphatidylethanolamine (PDP-PE) for coupling of thiolated ligands to liposomes through a disulphide linkage (Martin et al. 1981). Dicetyl phosphate (2.5 mol%) was also included to minimise aggregation of liposomes (Rosenberg et al. 1987). It was found that the inclusion of the dicetyl phosphate

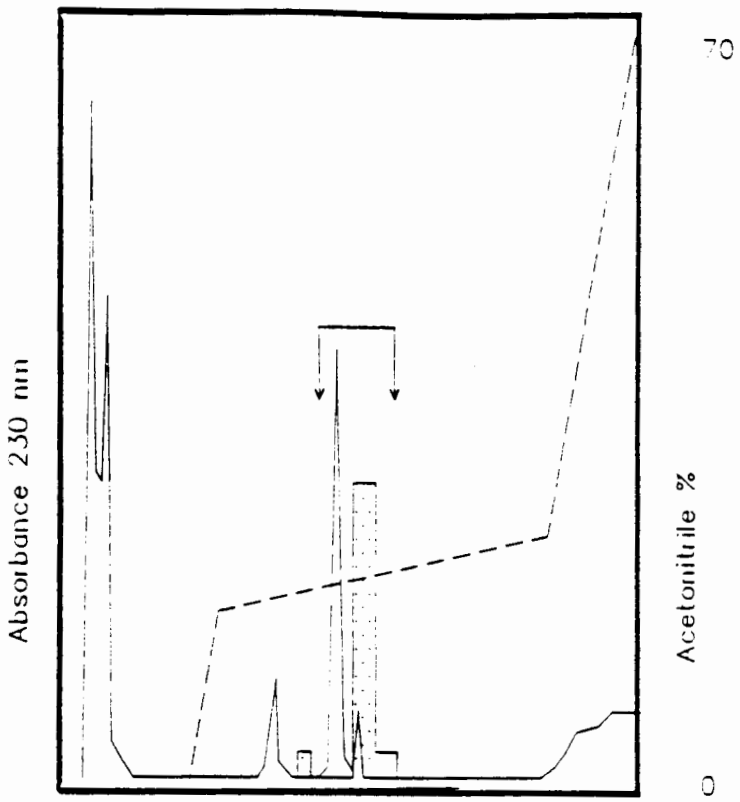

Fig 4.1: HPLC trace of preparation of radioiodinated MSH-SH. MSH containing a trace of ^{125}I-MSH was derivatised with DTBP and reduced as described in methods. Lower trace is absorbance at 230 nm, dotted area is dpm of ^{125}I determined by counting 0.2 ml aliquots. The fraction collected and used as preparative ^{125}I-MSH-SH is shown bracketed.

which imparts a slight negative charge to the liposomes significantly facilitated liposome preparation, and purification. Although it is possible that the negative charge may increase the non-specific binding of liposomes to cells, the aggregation of liposomes which occasionally occurred when liposomes were prepared without this component was avoided.

The liposomes were extruded through sterile 0.2 μm polycarbonate membranes immediately after preparation. This served to decrease the heterogeneity of the liposome size distribution, and also to sterilise the liposomes and hence minimise any degradation that may occur due to bacterial contamination. Prior to conjugation the liposomes were separated from unentrapped material by flotation on Ficoll gradients (Fraley et al. 1981).

Characterisation of the liposomes was achieved by freeze fracture electron microscopy, which showed them to be unilamellar and of an average size of 200 nm (Fig 4.2). Confirmation of the size distribution performed by scanning electron microscopy of liposomes on 0.2 μm polycarbonate membranes and on cells is shown later on in the thesis.

The inclusion of a fluorescent marker (Calcein) within the liposomes during their preparation allowed the assessment of the encapsulation efficiency (see methods) which was shown to be between 15 and 20 % with an entrapment of approximately 5 μl/μmol lipid and also permitted a rapid analysis of the stability of the liposomes. The theoretical encapsulation efficiency of 200 nm diameter liposomes based on the calculations of Guiot and Baudhuin (1984) is 14%, hence the efficiency of encapsulation confirms the electron microscopy findings.

Fig 4.2: Freeze fracture electron micrograph of reverse phase evaporation vesicle (REV) demonstrating unilamelar nature of vesicle. Conditions for microscopy are described in methods. (Bar = 200 nm).

Conjugation to liposomes

100 ug of MSH-SH with tracer ^{125}I-MSH-SH was added to liposomes composed of PC / Chol / DCP / PDP-PE (45:50:2.5:2.5 mol%, 10 µmoles total phospholipid) as described in methods, incubated overnight and dialysed to remove unbound peptide. Under optimal conditions 60 % of the peptide could be bound to the vesicles, representing approximately 2000 molecules of MSH per liposome. The ratio of PDP-PE to peptide is 10. Increasing this figure did not increase the amount of peptide which bound to the liposomes (results not shown).

Dansyl derivatives of MSH and ACTH have been shown to bind to lipid vesicles from aqueous solution (Schwyzer 1979) and $ACTH_{1-24}$ has been shown to spontaneously insert into lipid membranes (Gremlich et al. 1983), hence it is possible that the binding of the MSH to the liposomes was not due to covalent coupling to the PE-DTP, but due to a hydrophobic association with the bilayer. Incubation of the conjugated liposomes in 10 mM DTT for 4 hours followed by dialysis to remove the released peptide resulted in 97% of the radioactivity appearing in the dialysis buffer, demonstrating that the peptide could be quantitatively released from the liposomes under reducing conditions, and the association is therefore not due to membrane insertion.

To assay whether the conjugation of MSH to the liposomes perturbed the bilayer, liposomes were prepared containing 100 mM Calcein and were conjugated to MSH. The rate of leakage of calcein from the liposomes was assayed by monitoring the increase in fluorescence due to the unquenching of the fluorescent dye. The results are shown in Table 4.1, indicating that the binding of MSH to the surface

of the liposomes does not promote leakage, and in fact appears to provide a small degree of protection from serum induced leakage, possibly by acting as a protective 'shell' on the liposome surface decreasing the ease of access of serum lipoproteins to the bilayer.

	PBS	PBS+FCS
Lip	4.5%	19.0%
Lip-MSH	4.3%	16.5%

Table 4.1 : % efflux of calcein over a 24 hour period from liposomes with or without MSH bound to the surface in PBS with or without 10% fetal calf serum. 10 nmoles lipid was incubated in 1.0 ml buffer or buffer with FCS. Fluorescence after 24 hours was compared to fluorescence after adding 10 µl of 10% Triton X-100 which lysed the liposomes and resulted in 100% leakage.

Biological activity of liposome-MSH conjugate

Having bound the hormone to the liposomes it was necessary to test whether the bound hormone was able to be recognised by the hormone receptor, and to elicit any biological response. This was achieved by assaying the ability of the liposomes to elicit adenylate cyclase activity in B16 cells by the same protocol as for MSH activity determination. Fig 4.3 shows the comparison of the adenylate cyclase stimulation of both MSH and of MSH bound to liposomes. For the MSH bound to liposomes the hormone concentration is based on the total number of MSH molecules bound to the liposomes.

Within the time period of the assay the liposomes were able to maximally stimulate adenylate cyclase to an activity of

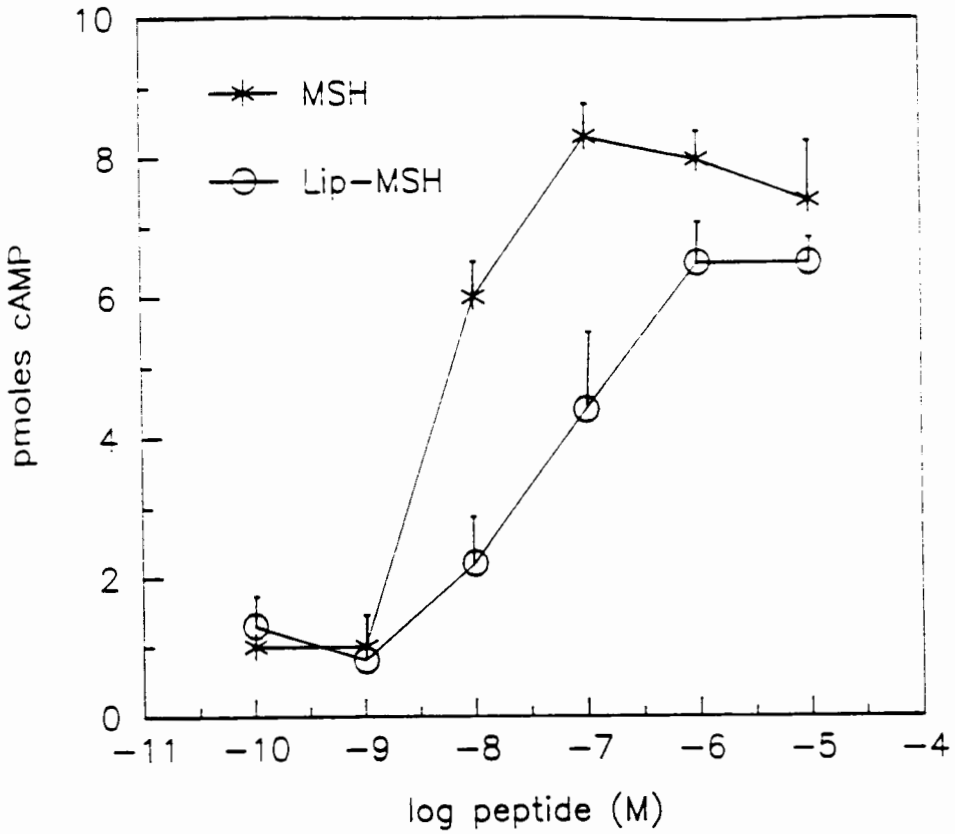

Fig 4.3: Adenylate cyclase activation of B16 cells by MSH and by MSH bound to liposomes (LIP-MSH). The incubation was performed in PBS/Ca^{++}/Mg^{++} for 30 minutes and cAMP determined as described in methods. For LIP-MSH the MSH concentration is expressed as the total amount of MSH present. Data points for MSH are from duplicates, for LIP-MSH from quadruplicates.

only 73 % of that achieved by free MSH, and required 20 fold as much MSH to do so.

That there is adenylate cyclase activation by the liposomally bound MSH shows that the immobilised hormone is recognised by the receptor, however a quantitative comparison of the biological activity of immobilised hormone with that of the free hormone is not valid because of the reasons outlined below.

1) On a liposome the bound peptide should be evenly distributed, covering the entire sphere, and hence only a small proportion will be available at the receptor site. Most of the hormone is therefore not available for interaction and does not contribute towards the biological activity. Since lateral mobility is possible within the liposomal membrane there may be accumulation of hormone at the receptor, it is however impossible to ascertain what proportion of the peptide is available to interact at the plasma membrane surface. If several ligands on the same liposome each react with a receptor (which is possible), this will increase the effective local concentration of the ligands at the receptor and hence increase the apparent association constant in a manner similar to the super-hormone activity of TMV-MSH (Kriwaczek et al. 1978). This is discussed later.

2) The large size of the liposomes compared with receptor may prevent many of the receptors from interacting with liposomes, possibly explaining the lower maximal response as being due to only a proportion of the receptors being occupied. The magnitude of this effect will be increased if the receptors are locally concentrated, and not spread out over the entire cell surface.

3) Sterically constrained MSH may interact with the receptors in a manner not conducive to activation of the adenylate cyclase. Thus, If receptor aggregation is a prerequisite for adenylate cyclase activation then liposome-bound MSH, because of the relatively large size of the liposomes may inhibit this. Since the liposomal membrane is in a liquid crystal state at the temperatures of the incubation, there is a certain degree of mobility of the peptide on the surface of the liposome which might permit aggregation of hormone-occupied receptor, but at a slower rate than for free hormone. This would slow down the activation. It is interesting to compare the lower adenylate cyclase activation observed here with the absence of adenylate cyclase activation observed in the case of TMV-bound MSH (Wunderlin et al. 1985) for which, due to the ridgidity of the complex, aggregation may be impossible, preventing activation. Alternatively, if receptor internalisation is a prerequisite for adenylate cyclase activation, the immobilisation of the hormone on liposomes could hinder this activation, accounting for the lower level of stimulation.

4) The observed activity could be due to hormone released from the liposomes. To determine whether here had been any release of hormone from the surface of the liposomes the supernatant from the cell-liposome incubation above was passed over a sepharose This was checked by passing the incubation medium over a Sepharose 4B column. 1.2 X 10^6 DPM (4 ug peptide in 2 ml) were applied to the column, and 900 DPM eluted in the outer volume, indicating a 0.075 % release of peptide, equivalent to 0.003 ug. This could not account for the 5% observed activity.

Hence a biological activity of 5% with respect to ligand present is meaningful only as far as confirming that there is interaction of the liposomes with the hormone receptor,

suggesting the possibility of hormone mediating binding of the liposomes to the receptors.

Binding to cells

MSH-SH was coupled to liposomes that contained the fluorescent marker Calcein. These liposomes, and a control of liposomes to which no MSH had been linked were added at increasing concentrations to B16 cells in PBS/Ca/Mg and incubated for 30 minutes at 37 degrees,and then washed four times with ice-cold PBS/Ca/Mg. The cells and bound liposomes were then lysed with 1% Triton-X-100 and analysed for fluorescence.

Fig 4.4 shows the results of this experiment expressed as the number of liposomes bound per cell. The figure of 1.2 X 10^{13} liposomes / 10 µmol phospholipid is calculated according to Guiot and Baudhuin (1984) assuming an average diameter of 200 nm.

The results indicate that at low concentration of liposomes there is a definite specific binding of the targeted liposomes to the cells, however at higher concentrations of lipid, the non-specific binding becomes increasingly more significant. Subtracting the binding of un-targeted liposomes from that of targeted liposomes gives the figure for specific (hormone mediated) binding which reaches a maximum at a lipid concentration of 200 µM, corresponding to a hormone concentration of 7.2 X 10^{-7} M.

The affinity of the binding calculated from the specific binding curve gives a K_D of 2.7 X 10^{-7} M with respect to the hormone, two orders of magnitude lower than that of MSH. A direct comparison of this figure with that of the K_D of free MSH is meaningless because of the multimeric nature of the construct. Since the hormone is distributed over the entire

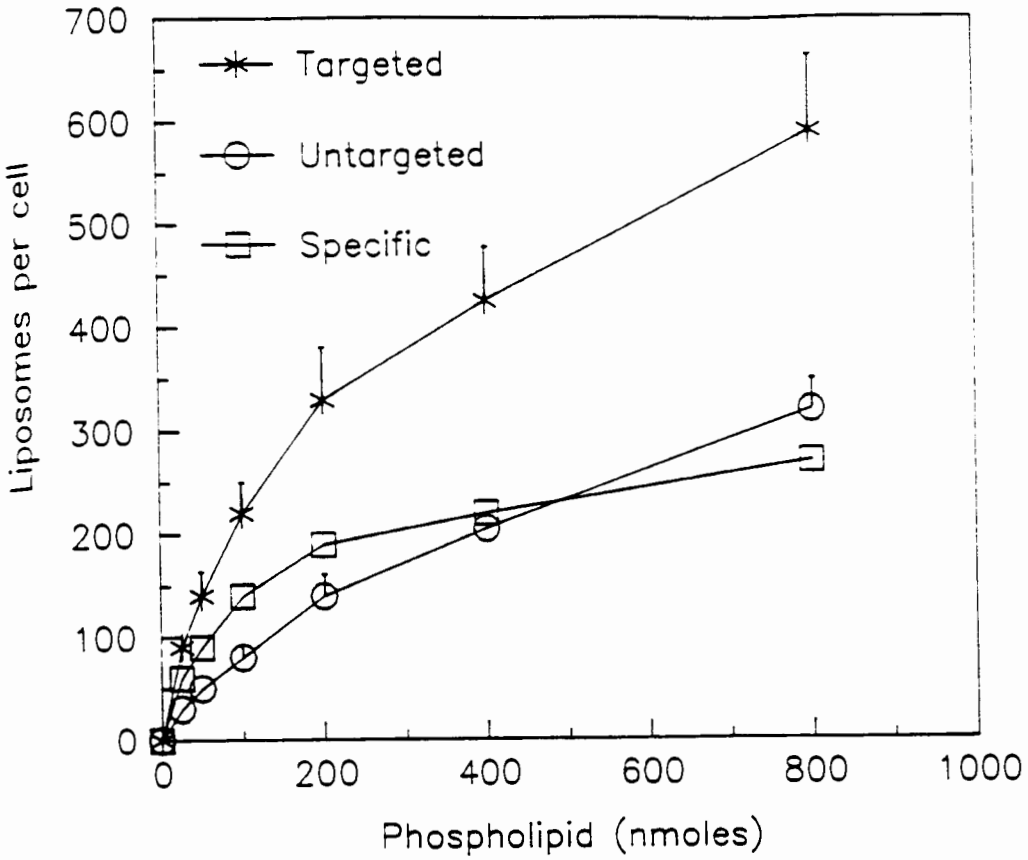

Fig 4.4: Binding of liposomes to B16 cells. 10^5 cells in monolayer were incubated in 1.0 ml PBS/Ca/Mg with increasing concentrations of either targeted (LIP-MSH) or untargeted (LIP) liposomes containing 100 mM calcein for 30 minutes at 37°C. The number of liposomes bound per cell was calculated from the fluorescence associated with the cell and assuming the number of vesicles in the preparation to be 1.2×10^{13}. Subtraction of the figures for untargeted binding from targeted binding gives specific binding. Results shown are the average of triplicates performed on the same sample.

surface of the liposome, only a small proportion can be available at any moment for interaction with the receptor. This quantitiy is unknown and hence the calculation of the K_D, which assumes all the hormone present to be available for binding, is severely underestimated. A more valid assesment of the affinity of the conjugate for the receptor is obtained from inhibition studies presented later.

The interpretation of the increase in binding due to the targeting, and also of the number of liposomes bound per cell requires cognizance of the cell cycle dependant expression, and hence liposome binding, of the cells. As published (Varga et al. 1974), and as shown by fluorescence micrographs later in this thesis, only 20 to 30% of the cells in an asynchronous culture bind MSH. The non-specific binding of liposomes is however independent of the cell cycle, and hence the figure for specific binding obtained by subtracting targeted from untargeted binding is correct for the entire cell population, but is only 20 to 30% of the correct figure for those cells participating in the targeted binding. Thus, while the entire sample exhibits a maximum increase in binding due to targeting of only three fold, and a saturation of specific binding of 230 liposomes per cell, when calculated for the proportion of cells which actually participate in MSH mediated targeting, the figures become; a 12 fold increase in binding due to targeting, and a maximum specific binding of approximately 1000 liposomes per cell.

Since the cell surface should be able to accommodate at least 10000 liposomes by area, the plateau reached at 1000 liposomes per cell is indicative of either ;
1) The number of receptors being very much lower that the published figure of 10^4 per cell for Cloudman S91 cells (Varga et al. 1974) or;

2) Only a proportion of the receptors are in a conformation in which they are able to bind liposomes, or;
3) Only a small proportion of the receptors are available to bind the liposomes because of a patchy distribution and steric hindrance by the liposomes.

Inhibition of binding :

To further prove that the binding of the liposomes to the cells was due to a recognition of the bound MSH by the MSH receptor, inhibition studies with free MSH were performed by incubating targeted liposomes with cells in the presence of increasing concentrations of free MSH. The results are shown in fig 4.5, which shows that the binding can be inhibited by the addition of an excess of free MSH, indicating that the binding is due to hormone-receptor interactions, however the amount of MSH required to inhibit the binding is large. At a concentration of 3×10^{-5} M (30 nmoles free MSH, three orders of magnitude greater than the K_D), which represents a 40 fold excess of free MSH, only 60 % of the specific binding has been inhibited, and a stoichometric quantity of free MSH does not decrease the binding at all.

The requirement of non-stoichometric quantities of free ligand to inhibit the binding of targeted liposomes has previously been shown in the experiments of Heath et al. (1983) where a 20 fold excess of free antibody is required to inhibit antibody mediated binding of liposomes by 75%, leading these authors to speculate that liposomally bound antibody has a higher affinity for the antigen than the free antibody.

If we apply such reasoning to this experiment, then it implies that the K_D of the MSH bound to the liposomes is at least 40 times stronger than that of the free hormone,

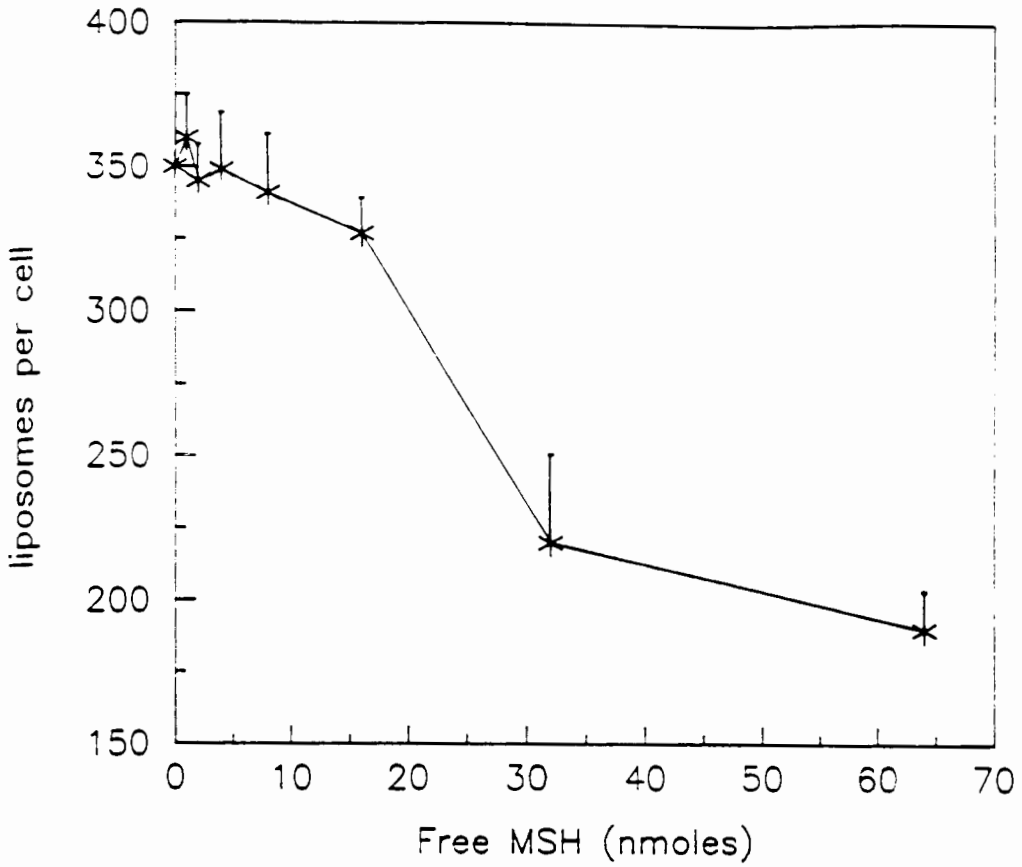

Fig 4.5: Inhibition of binding of targeted liposomes by MSH. Targeted liposomes (200 nmoles phospholipid, 0.72 nmoles bound MSH per ml) were incubated with cells in 1 ml PBS/Ca/Mg containing increasing quantities of free MSH and binding determined as for the previous experiment. At this concentration of liposomes a non-specific binding of 140-150 liposomes / cell occurs (see fig 4.4). Data points are average of duplicates.

giving it a K_D of approximately 1 X 10^{-10} M. From the direct binding studies, the liposome complex as a whole exhibits a K_D of 2.7 X 10^{-7} M with respect to all of the MSH on the liposome, of which only a small proportion actually interacts with the receptor. Since there are on average 2000 MSH molecules per liposome, dividing the K_D of the LIP-MSH complex by the number of ligands yields an individual K_D of 1.3 X 10^{-10} M, very close to the figure obtained from the inhibition studies, suggesting that each liposome is able to interact with only one receptor at any one moment.

Fluorescence microscopy of MSH-targeted liposomes

To obtain some idea of the morphological distribution of the liposomes on the cells, fluorescence microscopy of calcein loaded liposomes interacting with the cells was performed. Liposomes at 100 μM lipid which gives maximal difference between specific and non-specific binding were incubated with cells growing on glass cover slips for 1 hour at 37°C. The time-period and temperature were chosen so that any internalisation of the liposomes by an endocytotic pathway could take place and hopefully be detected. Fig 4.6 is a typicall field of view, demonstrating that the binding of the liposomes to the cells occurs primarily on the cell body, although some binding to the dendritic extrusions is also evident. The appearance of very bright fluorescence in the middle of the cell body suggest that the receptors are not evenly distributed, and that the bright fluorescence is due to the binding of a large number of liposomes to an area of high receptor density. Such binding is simmilar to that observed by Varga et al. (1976), who found binding of MSH to melanoma cells occurred in a region of the cell surface overlying the golgi complex. It is possible that the bright fluorescence observed here is representative of a similar area.

That the fluorescence is primarily punctate suggests that the liposomes are still bound to the surface of the cell, and any calcein that may have been internalised is trapped within vesicles in the cell. Calcein is a highly acidic molecule and as such unlikely to lose its charge and be able to leak out of acidic vesicles. Hence, unless there has been fusion of the vesicles with the cell, internalised calcein will appear punctate. It was not readily discernable whether the non-peripheral fluorescence was on the cell or within it.

Not all of the cells aquired fluorescence, and in any field of view, only 20 to 30 % of the cells present could be detected by fluorescence. This is particularly evident from the fluorescent micrographs of FITC-labelled MSH-ricin-A-chain conjugates (chapter 5) in which both the fluorescent and non-fluorescent cells appear in the same micrograph. That the hormone mediated binding of liposomes occurred on only 20 to 30 % of the cells suggest that the B16 cell line, similar to the Cloudman S91 line, exhibits a cell-cycle dependant expression of the MSH receptor (Varga et al. 1974), and hence in an asynchronous culture, approximately only 30% of the cells express the receptor at any time.

Anomalous binding

It was observed that occasionally (the event was noticed five times out of an estimated 1 X 10^7 cells observed) a cell appeared within the B16 cell line that had a significantly higher affinity for the targeted liposomes and were much larger than the normal B16 cell. These cells were noticed because they bound very significantly more liposomes than their neighboring cells. Once located by fluorescence microscopy (fig 4.6 A), phase contrast microscopy (fig 4.6 B) showed these cells to be up to five times the diameter of

Fig 4.6: Fluorescence microscopy of binding of calcein loaded MSH-targeted liposomes to B16 melanoma cells. Liposomes were incubated with cells growing on glass coverslips in PBS/Ca/Mg for 60 minutes at 37°C. Bar is 5 μm.

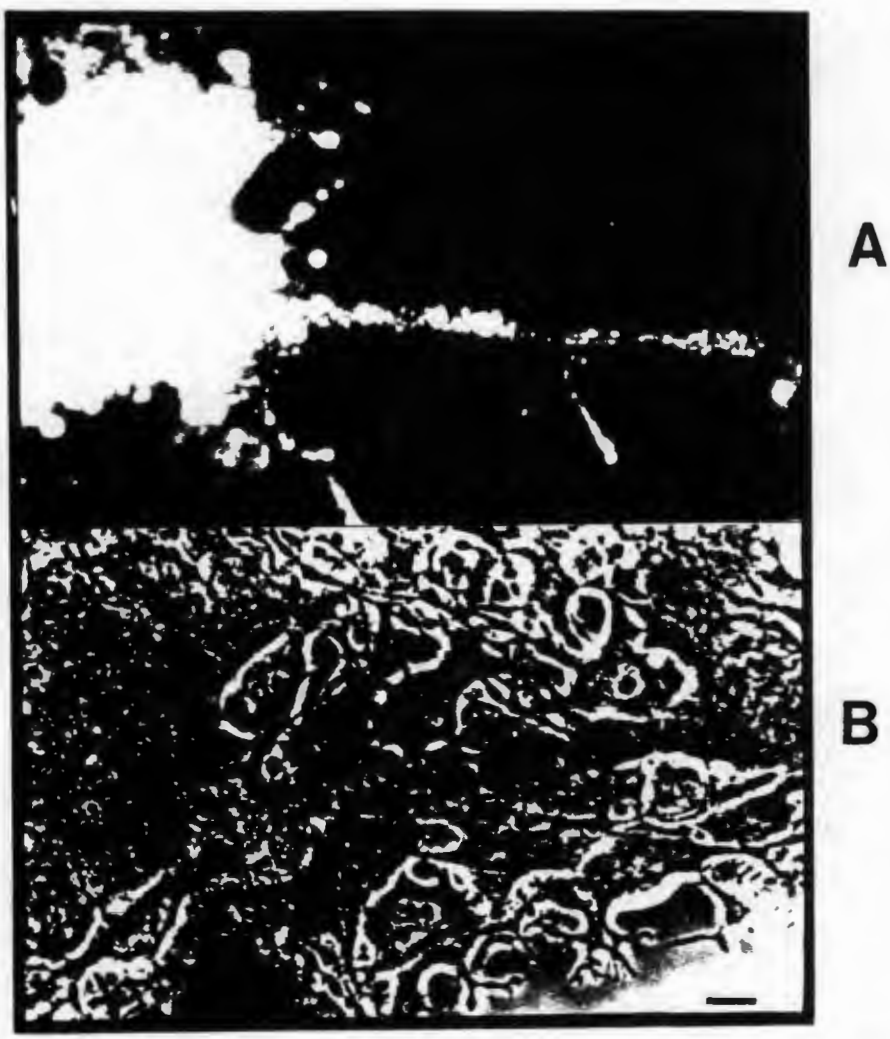

Fig 4.7: Microscopy of anomolous binding of MSH-targeted liposomes to a contaminant or mutant within the B16 melanoma cell line. Incubation was performed as described previously. (A) Fluorescence microscopy , (B) phase-contrast of same field. Bar is 5 μm.

the normal cells, and with a very large nucleus. The binding of fluorescent targeted liposomes to these cells is so much greater than the binding to the surrounding cells that features such as long axons growing over other cells are easily distinguished. The binding is primarily on the cell body, however distinct binding to the axons has occurred and some perinuclear diffuse fluorescence suggests that internalisation has occurred, however there is so much fluorescence that it is difficult to distinguish internalised from surface bound.

The very low frequency of observation of these cells (1 in 2 X 10^6) and the observation that there were never two together, suggests that these represent a spontaneous mutation of the cell, and are not a subpopulation within the cell line. The low frequency of observation also precludes the conclusion that because they were only observed with targeted liposomes, the increased binding is definitely due to an altered MSH receptor parameter. It is possible though unlikely, that they could be binding the liposomes via a different determinant, and were not observed in the control experiments because more time was spent observing the binding of targeted liposomes.
That such cells could occur within a population warns that spectroscopic data on liposome binding should always be confirmed with microscopy. These cells were not investigated further.

Targeted delivery of methotrexate

Having shown that the binding of MSH to the liposomes resulted in their specific binding to melanoma cells, it remained to be shown whether such binding promoted the delivery of the liposomal contents into the cells. The inhibition of ^{3}H-thymidine uptake into cellular DNA in

response to cytoplasmic accumulation of methotrexate, an inhibitor of the folate metabolism pathway, was chosen as the assay of liposomal delivery.

Liposomes were prepared containing 1 mM MTX, which, at 15% encapsulation resulted in an encapsulated drug : lipid ratio of 4 mmol/mol. The liposomes, both targeted and untargeted, and free MTX were tested for their ability to inhibit cellular proliferation of B16 cells. This was achieved by incubating the cells with increasing quantities of the drug for 24 hours, and then adding ^{3}H-thymidine and continuing incubation for a further 24 hours at which time the incorporation of the radio-labelled nucleotide was assayed.

The results are shown in fig 4.8, which demonstrates that the free drug is able to inhibit the incorporation of ^{3}H-Thy into cellular DNA with an ID_{50} of 500 nM. In comparison to this the ID_{50} for both targeted and untargeted liposomally entrapped MTX is 2100 nM. This indicates that there has been no significant increase in the delivery of the drug into the cells by targeting with MSH. Since the cells were shown to bind targeted liposomes specifically, this result initially suggests that the specifically bound liposomes are unable to deliver their contents into the cell, a possible consequence of the MSH receptors not mediating endocytosis, or of the liposomes being too large for MSH-mediated endocytosis.

A more detailed examination of the data, and of the experimental protocol provide some insight into the absence of target specific toxicity.

The very high ID_{50} of free MTX (500 nM) compared with that published for other cells which have an average ID_{50} of 10 nM (Piper et al. 1982) and that found for hepatoma cells (ID_{50} = 60 nM, see chapter 6), suggests that the B16 cells express some form of resistance to the drug. Cell lines are

known to become resistant to methotrexate by one of two routes ;
1) Increased intracellular levels of dihyrofolate reductase, the enzyme inhibited by MTX, or; 2) the loss of the active transport system by which MTX enters the cell.

The untargeted liposomes have an ID_{50} of 2.1 μM, this toxicity due to probably to leakage of the drug from the liposomes into the medium, and perhaps also to non-specific binding of the liposomes to the cells and delivery of the contents via either constitutive (non receptor-mediated) endocytosis or a fusion event. Assuming the rate of leakage of the MTX to be similar to that of calcein (a reasonable assumption since they are both charged molecules of approximately the same size), the leakage after 48 hours would yield a free drug concentration in the region of of 600 nM, however this concentration would not have been present during the entire time period as leakage is a time dependant process. Therefore, some of the toxicity is due to intracellular delivery of non-specifically bound liposomes, and since it is unlikely that this represents more than a few percent of the total liposomes present, this toxicity results from the delivery of only a small concentration of MTX.

These results are in qualitative agreement with those of Heath et al. (1983) who used a derivative of MTX still able to inhibit dihydrofolic acid reductase, but much less able to enter the cell than MTX. A similar ID_{50} of 1 to 2 μM is obtained for untargeted liposomal toxicity of this drug. To follow an argument these authors present, since the K_i of MTX on the enzyme dihyrofolic acid reductase is in the order of 5 nM (Piper et al. 1982), then an intracellular concentration of MTX of 5-10 nM should inhibit cellular growth, and such an internal concentration can be achieved by the cytosolic delivery of only a few liposomes. In this

experiment, the volume of the B16 cells (15 μm diameter) is 2.3 X10^6 times greater than that of a liposome (200 nm diameter), and since the liposomes contain 1 mM MTX, only 23 liposomes are required to deliver their contents into the cytoplasm to achieve a cellular concentration of 10 nM. There is however an assumption made here that the microenvironment of the DHFR enzyme is identicle to the gross cytoplasmic environment, which is not necessarily so, and any compartmentalisation of the enzyme could raise this figure by several orders of magnitude.

At the concentration at which the liposomally entrapped MTX exhibits toxicity, the lipid concentration is 500 μM. From the binding studies, at this concentration untargeted liposomes bind 230 liposomes per cell. The same number of liposomes can be bound in the case of targeted liposomes at a concentration of approximately 100 μM, hence it is surprising that the targeted liposomes do not exhibit at least a five fold increase in toxicity. Two postulates can be proposed to account for this.

The first is based on the experimental protocol. While several authors (eg. Huang et al. 1983) perform the above type of experiment by incubating the liposomes with the cells for a short time period followed by washing the cells and then continuing incubation, the cell cycle dependant expression of the receptor required that the liposomes were present in the medium for at least one doubling-time (approx 28 hrs). During this time period there may have been no further binding of targeted liposomes to the receptor, it being occupied, the non-specific binding however may have increased, being subject to no such constraints, resulting in insignificant differences in binding (and hence toxicity) between the two types of liposomes. The possible release of hormone from the liposomes in medium should not interfere

with the binding as it was shown previously that a vast excess of free hormone is required to inhibit binding.

The second postulate proposes that the MSH receptor is unable to promote the internalisation of the specifically bound liposomes, similar to the observation of binding without internalisation by Machy et al. (1982), whereas there is a chance of a non-specifically bound liposome being endocytosed by a constitutive process. It is also possible that some of the liposomes were specifically endocytosed, however because of the low number of specifically bound liposomes, this did not show above the background. Leserman et al (1983) have found that cells expressing a low copy number of the receptor, although still binding targeted liposomes, no longer exhibit sensitivity to the liposomes.

Conclusion

MSH can be modified and bound to a liposome without significantly altering its biological activity, and the resulting liposome expresses an affinity for the receptor greater than that of the free hormone. The liposomes can be specifically bound to cells expressing a receptor for the hormone, however the number of liposomes thus bound is small. Liposomes targeted to the cells do not appear to be able to promote the intracellular delivery of their contents to a level greater than that of non-specifically bound liposomes, however the reasons for this are not clear.

At this stage it was decided to investigate whether the MSH receptor on B16 cells was able to promote the internalisation of non-liposomal structures, to shed light on whether the failure to achieve specific toxicity was a function of the receptors endocytotic nature.

Chapter 5

MSH-Ricin Conjugates

Since MSH targeting of liposomes resulted in specific binding of the liposomes, albeit at a low level, yet no specific toxicity, it remained to be ascertained whether the MSH receptor on B16 cells was able to function as a receptor for internalising other targeted carriers, and hence whether the failure of the MSH targeted liposomes to deliver their contents to the cytoplasm was a function of the liposome characteristics rather than of the receptor. To investigate this, a hybrid toxin was constructed by conjugating the highly toxic A-chain of ricin (RTA) to the thiolated MSH, and testing the conjugate for cell-specific toxicity.

Introduction

Selective toxicity by specifically targeting drugs into cells is hampered by the high intracellular concentration of the drugs required to elicit an effect. In an attempt to overcome this problem many researchers have been investigating the targeting of toxic enzymes into the cell, where by nature of their enzymatic activity, only one or two molecules of the toxin should be sufficient to mediate a cytotoxic effect. A major advantage of using toxins over drugs is that unlike small drugs such as MTX, toxic enzymes are unable to enter the cells by themselves, and as such, have a very low toxicity unless associated with an agent which promotes their uptake. Any effective targeting is hence easily observed above the background toxicity.
The two toxins most widely investigated for this purpose have been ricin, a highly potent toxin isolated from the castor bean *Ricinus communis*, and Diptheria toxin, a toxin secreted by the pathogen *Corynebacterium diptheriae*.

Both of these toxins are comprised of two subunits ; An enzymatically active component, the A-chain, which by itself exhibits very little toxicity towards whole cells, being unable to gain entry into the cell, yet is highly toxic in cell-free toxicity assays, inactivating the protein biosynthetic machinery. In the case of ricin, by cleaving the N-glycosidic bond of A^{4324} of the 28S rRNA in a hydrolytic fashion (Endo and Tsurig 1987), and in the case of diptheria toxin by ADP-ribosylation of elongation factor 2 (Pappenheimer 1977).

A binding component, with specificity for cell surface receptors, is thought to be responsible for mediating the internalisation of the A-chain. In the case of ricin the B-chain is a lectin, exhibiting specificity for terminal non-reducing sugars, and hence delivery of the A-chain can be inhibited by the presence of lactose or galactose. The B-chain exhibits no enzymatic activity and is not toxic.

The concept behind the chimaeric toxin constructs is that replacing the B-chain with a ligand specific for a cell surface determinant will target the otherwise ineffective A-chain to the cell, and, hopefully promote its internalisation and hence mediate a specific toxicity. A large variety of such chimaeras have been investigated and it has been found that simply targeting the toxin to a cell does not necessarily result in toxicity.

A variety of monoclonal antibodies, with specificity for different antigens, have been compared for their efficacy in mediating RTA-antibody conjugate toxicity (Preijers et al. 1988). Large differences in cytotoxicity were found, correlating significantly with the degree of internalisation of the the antibody. This was found to be a function of the nature of the antigen, and not the magnitude of its surface expression. Hence some immunotoxins which bound to a large

extent were less toxic than other immunotoxins which had fewer conjugates bound per cell, but to an antigen that promoted the internalisation of the conjugate.

A comparison of two antibodies for the same antigen, but with differing affinities (Youle and Neville 1982) demonstrates that antibody affinity as well as number of available sites affects the rate of entry of the A-chain into the cell. By analysis of the kinetics of toxicity of RTA immunotoxins in the presence and absence of ricin B-chain, these authors further demonstrate that the ricin B-chain significantly enhances the cytosolic delivery of the A-chain, even if it was not responsible for the cell-surface binding of the A-chain. That this potentiation of the internalisation does not require the galactose-binding site of the B-chain nor its non-covalent association with the A-chain has been demonstrated by denaturation of the B-chain (Vitetta 1986, Wawrzynczak et al. 1988), however the mechanism by which it achieves this remains unclear.

Conjugation to hormones has also been achieved. The diptheria toxin A-chain (DTA) linked to human chorionic gonadotropin has been shown to be specifically toxic towards cells bearing receptors for this hormone, yet not toxic to cells lacking the receptor (Oeltmann 1985), demonstrating that the hCG receptor internalises the chimaera via a pathway that allows access of the toxin to the cytosol. DTA linked to EGF is however non-toxic to cells expressing EGF receptors even though binding of the conjugate to the cells occurs(Cawley et al. 1980). In contrast to this RTA-EGF conjugates were found to be highly toxic to the cells, demonstrating that internalisation and subsequent toxicity is also dependant on the toxin. The reason for the difference between these two conjugates is not clear, but could be due to the ability of RTA to associate with membranes by itself, or due to an ability to escape from the

endocytotic pathway into the cytosol. That Chloroquine is able to potentiate the toxicity of the RTA-EGF conjugate suggests that the toxin is internalised in endocytotic vesicles.

Simply binding the toxic component to the cell surface via the non-specific interaction of avidin with cell membranes has been shown to allow significantly increased binding, but no increase in the toxicity of biotinylated Diptheria toxin towards cells, indicating the requirement of specific receptors for translocation of toxins into the cytosol (Stenmark et al. 1988).

All of these conjugates discussed have been prepared synthetically, making use of the single exposed sulphydryl moeity on the toxin fragments to form a disulphide linkage to the ligands. The genetic construction and expression in *E.coli* of a fusion protein comprising diptheria toxin and MSH has been described (Murphy et al. 1986). The fusion gene directs the expression of a diptheria toxin related MSH hybrid protein in which the toxin receptor binding domain has been replaced with MSH sequences. The resulting protein was shown to be toxic towards melanoma cells yet not toxic towards cells lacking the MSH receptor. Toxicity can be calculated from the figures they quote as being in the order of 0.03-0.06 nM. This data suggests that in this cell line (NEL-M1) the MSH receptor promotes internalisation of a MSH targeted carrier. That NH_4Cl abolishes the cytotoxic activity shows that the internalised protein passes through an acidic vesicle, suggesting that the MSH receptor promotes internalisation through an endocytotic pathway. (Unlike ricin which becomes more toxic in the presence of lysosomotropic agents, diptheria toxin toxicity is inhibited by such compounds (Sandvig and Olsnes 1982)).

It was felt that the construction of a RTA-MSH conjugate using the sulphydryl of the MSH-SH to couple to the sulphydryl of the RTA, and the analysis of the constructs toxicity on B16 melanoma cells, would provide some insight into the use of the MSH receptor for targeting to these cells.

Conjugation

The protocol for conjugating thiolated MSH to RTA was adopted from the method of Youle and Neville (1982). The thiol on the MSH-SH (with tracer ^{125}I-MSH-SH)was reacted with 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB), and following separation on HPLC, conjugated to freshly reduced RTA.

The peptide was incubated with the toxin at a 1.5 fold molar excess. Hence after dialysis if 100% conjugation had occurred one would expect 66% of the added radioactivity to remain associated with the toxin. Of the 4 X 10^6 DPM added, only 2.1 X 10^6 remained bound to the toxin, indicating that considerable (75 %) conjugation had taken place, although the conjugation was not quantitative.

To confirm that the associated radioactivity represented covalently bound peptide an aliquot of the conjugate was treated with 50 mM DTT for 2 hours and then dialysed against PBS. Virtually quantitative release of the radioactivity into the dialysate was achieved indicating the conjugation to be covalent with no detectable non-covalent association.

Biological evaluation

Three quality control checks are required on newly synthesised toxin conjugates (Cumber et al. 1985).

1) Inhibition of protein biosynthesis in a cell free system to ensure that the A-chain retains enzymatic activity.

2) Antigen binding capacity, or in this case, recognition of the hormone by assaying biological activity of the conjugate, and the binding specificity of the labeled conjugate to cells bearing the receptor.

3) Cytotoxicity, in this case the toxicity of the conjugate to melanoma cells compared with the toxicity of the free A-chain.

Inhibition of protein biosynthesis

After incubation with 50 mM DTT for 1 hr the released A-chain was assayed for its ability to inhibit protein biosynthesis in the rabbit reticulocyte lysate assay as described in methods. Pure A-chain was assayed as a comparison. To ensure that they were being tested at the same concentration, the concentrations of the conjugate and A-chain were checked by monitoring absorbance at 280 nm. The results of the protein biosynthesis inhibition are shown in fig 5.1.

The A-chain released from the conjugate had an IC_{50} of approximately 15 to 20 ng/ml, compared with the IC_{50} of 10 ng/ml for the pure A-chain. This represents a slight decrease in the toxicity of the A-chain, possibly due to some enzymatic degradation during dialysis, however in light

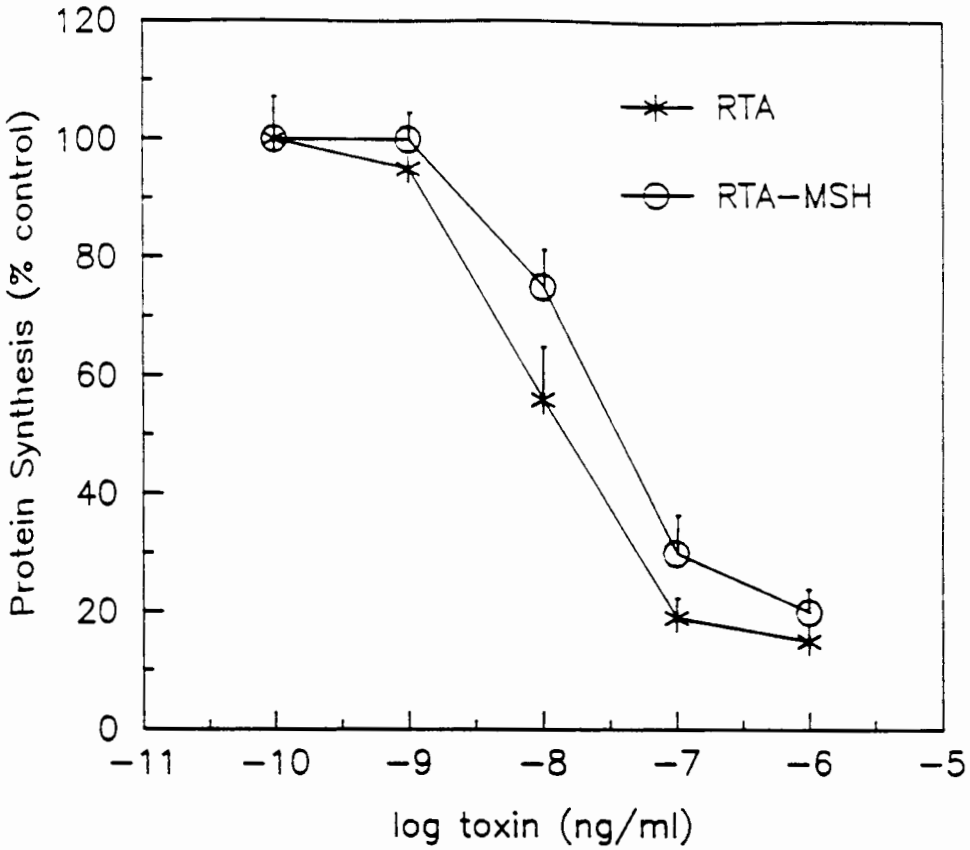

Fig 5.1 : Inhibition of protein biosynthesis in a cell free system by RTA and RTA-MSH. The toxins were reduced for 1 hr in 50 mM DTT before being incubated at increasing concentrations with a rabbit reticulocyte lysate assay. Trichloracetic acid precipitable material was determined for each incubation after 10, 30 and 60 minutes, and velocity (DPM/min) determined from the linear region of DPM vs. Time. The results are expressed as a percentage of the velocity of the control (no toxin). Two aliquots were counted for each time point, and the experiment was performed twice and the relative (% control) results averaged.

of the dilutions used for the assay, and the potency of the toxin, it was felt that this decrease in toxicity was not serious.

Biological activity

In order for the conjugate to bind to the cell specifically it must be recognised by the receptor. To determine whether the RTA-MSH conjugate was recognised by the MSH receptor, the ability of the conjugate to stimulate adenylate cyclase was assayed, and compared to that of native MSH. The results (fig 5.2) indicate that the conjugate has only 15 to 20 % of the adenylate cyclase stimulating activity that the free hormone has. This activity is lower than expected considering that the carboxymethyl derivative exhibited at least 50% activity, and the structure in the vicinity of the hormone is the same for both constructs. Several possible reasons exist which may account for the lower biological activity;

1) Association of the MSH with hydrophobic domains of the RTA, decreasing its accesability to the receptor.

2) Association of the RTA with the cell membrane once brought into the vicinity of the membrane by the MSH, decreasing the accesability of the MSH to the receptor.

As pointed out previously it is possible for hormone conjugates to interact fully with the receptor and yet exhibit no, or diminished hormonal biological effect because of an interaction of the molecule attached to the hormone with the pathway required for the biological effect to be elicited. Hence it is also possible that in this case there has been an interaction of the RTA with a component of the adenylate cyclase activation machinery, inhibiting the assay.

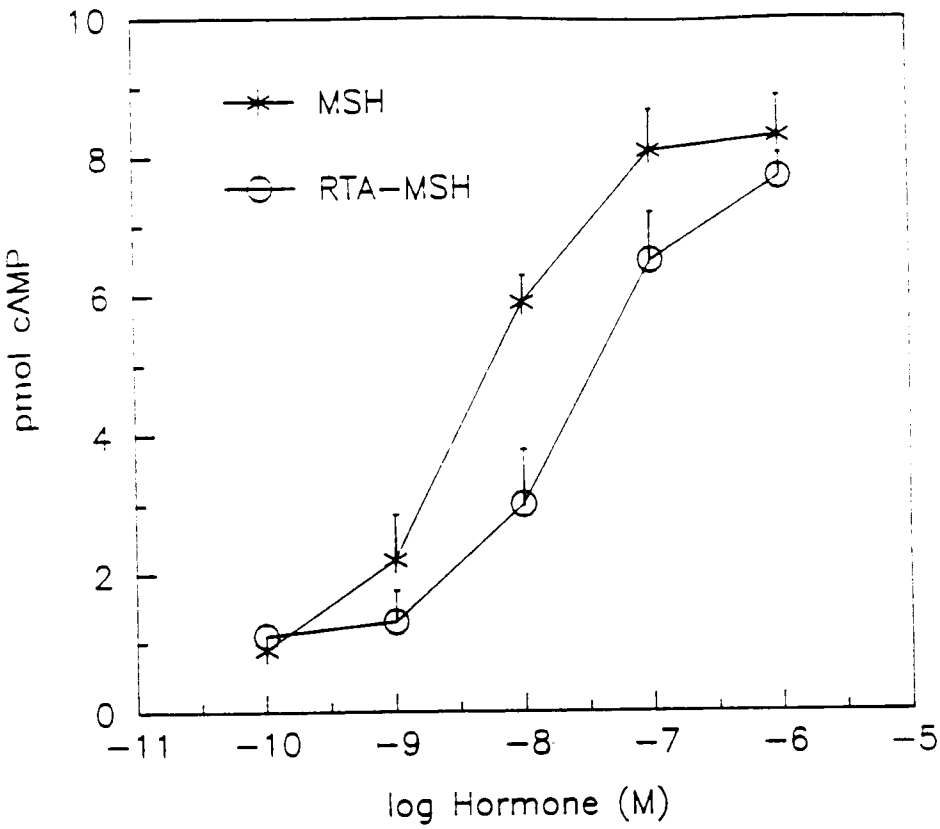

Fig 5.2: Adenylate cyclase activation by MSH and by MSH-RTA conjugate. B16 cells were exposed to increasing concentrations of the hormone or hormone conjugate for 30 minutes and the intracellular cAMP analysed as described in methods. Data points are average of triplicates.

Irrespective of which cause is responsible for the lower biological activity, that the conjugate exhibits biological activity suggests that it interacts with the receptor, and hence should target the toxin to the cell. This is best tested by monitoring the binding.

Binding

An aliquot of RTA-MSH was made fluorescent by treatment with FITC as described in methods. Incubation of the FITC-RTA-MSH conjugate with cells at 37 degrees for 3 hours resulted in approximately 30% of the cells aquiring fluorescence (see fig 5.3). That only a fraction of the cells aquire fluorescence is due probably to the cell-cycle-phase dependant expression of the MSH receptor, and hence binding of the fluorescent conjugate. The fluorescence appears to be fairly diffuse, suggesting that there may have been internalisation of the complex, although at the edges of the cells discreet punctate fluorescence is evident, indicative of compartmentalisation of the fluorescence, possibly in endocytotic vesicles. The fluorescence does not cover the cells evenly, being associated mainly with the cell body, and very little on the dendritic protrusions, a possible consequence of the distribution of the receptor.
To confirm that the uptake of fluorescence was due to the MSH an aliquot of the conjugate was treated with 50 mM DTT for 2 hrs prior to incubation with the cells. No significant fluorescence was seen to be associated with the cells (results not shown), although it was observed that after 3 hrs incubation there was a very low level of fluorescence associated with all of the cells, indicative of non-specific binding.

This result shows that MSH-mediated binding of the complex to the cells does occur, and within the time period of the

Fig 5.3: Fluorescence microscopy of MSH-RTA-FITC conjugates. B16 cells in mid-log-phase growing on glass cover slips were incubated with 10^{-8} M MSH-RTA-FITC for 3 hours at 37°C in PBS/Ca/Mg and then washed in PBS. Photography was performed by illuminating the cells with UV light only for 60 seconds and then illuminating with visible light for 10 seconds permitting non-fluorescent cells to be detected. Arrow A points to a fluorecent cell, arrow B to a non-fluorescent cell. Bar is 35 μm.

incubation appears to be much greater than the non-specific binding. Hence it is possible that the complex will exhibit specific receptor mediated toxicity.

Cytotoxicity

Ricin, ricin A-chain, and RTA-MSH were added to melanoma cells in complete medium with serum, and incubated for 48 hours at which time the cells were analysed for protein biosynthesis as described in methods.

Fig 5.4 shows ricin to be the most toxic of the compunds tested, with an ID_{50} of 20 ng. The addition of lactose protects the cells to a large extent from the ricin toxicity, raising the ID_{50} to 800 ng/ml. The ricin A chain is much less toxic, having an ID_{50} of 10 µg/ml. That there is no protection of toxicity due to RTA by lactose shows that the RTA is not contaminated with any ricin, and hence the toxicity is due to a non-specific binding and internalisation. The RTA-MSH conjugate expresses a virtually negligible increase in toxicity towards the melanoma cells, with an ID_{50} of 5 µg/ml, however some of this toxicity is inhibitable by the addition of 6 µM MSH (10 µg/ml) decreasing the toxicity of the hybrid to an ID_{50} of approximately 20 µg/ml.

That the toxicity of the conjugate can be inhibited by the addition of free hormone (added at a 40 fold molar excess at the ID_{50} concentration) shows that some of the toxicity of the conjugate is indeed due to a specific receptor recognition and binding, but that at least 25% of it is due

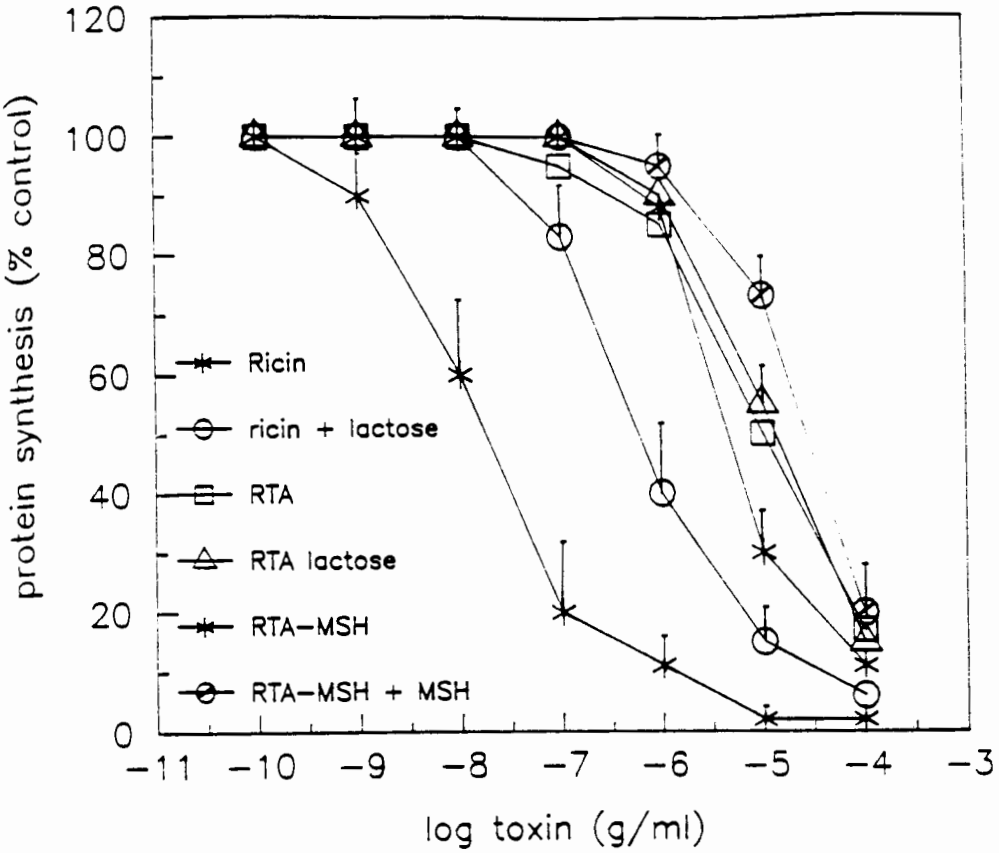

Fig 5.4: Inhibition of protein synthesis in B16 cells after exposure to ricin or ricin-A-chain hybrids for 48 hours. 10^4 cells in 0.1 ml were exposed to increasing concentrations of ricin, ricin + 50 mM lactose, ricin-A-chain, ricin-A-chain + 50 mM lactose, RTA-MSH, RTA-MSH + 6 μM MSH. Protein biosynthesis was assayed after 48 hours as described in methods. Data points are average of duplicates for ricin and ricin+lactose, and of quadruplicates for other data points. Where variance bars are not shown it is to prevent cluttering of the diagram, and variance is similar to other data points in the same experiment.

to non-specific binding. The fact that the conjugate in the presence of free hormone is half as toxic as the A-chain alone correlates well with the finding that the conjugated A-chain is only half as toxic in the cell-free protein biosynthesis assay. Since the toxicity of the A-chain has been decreased, the real increase in toxicity due to conjugation is in the order of four fold.

A four fold increase in toxicity is negligible considering the potent increases in toxicity which have been obtained using toxin-antibody conjugates by other authors. At the ID_{50} the concentration of conjugate is 1.5×10^{-7} M, at least ten fold greater than the expected K_D of the conjugate even allowing for the 85% decrease in biological activity, and hence the receptors should be significantly occupied even at ten fold lower concentrations. From this one can assume that specific binding is taking place even at lower concentrations, but that the internalisation of the conjugates A-chain into the cytoplasm is limiting. Several factors could cause this :

1) Specific binding to the receptor is occurring, but the receptor does not promote endocytosis.

2) Specific endocytosis of the conjugate is taking place but the conjugate is unable to gain access to the cytoplasm, either due to rapid degradation in the lysosomes, or an inability to cross the lysosomal membrane.

3) The low copy number of receptor, coupled with the phase dependant expression of the receptor result in a very low and slow uptake of the conjugate, possibly further complicated by the degradation of the conjugate in the medium, due both to proteolytic degradation of the hormone and thiol release of the hormone.

To determine to what extent the last possibility could be responsible, an aliquot of the conjugate was incubated with cells and full medium for 48 hours and then dialysed. 84% of the radioactivity was retained by the dialysis membrane, showing that some degradation of the conjugate had occurred, but not enough to account for the lack of specificity.

Considering the first two possibilities, if endocytosis of the conjugate is occurring, then the addition to the incubation medium of a substance which affects the endocytotic pathway will elicit a change in the toxicity of the conjugate.

Lysosomotropic agents such as NH_4Cl, chloroquin, monnensin and the carboxylic ionophore Br-X-537A, are known to alter the toxicity of toxic proteins such as abrin, ricin and diptheria toxin (Sandvig and Olsnes 1982) by altering either the pH of the lysosomes, or perturbing the vesicular traffic (Tartakoff 1983), and in the case of ricin, this results in an increased toxicity. Since the A-chain of ricin is thought to enter the cytoplasm from the trans-Golgi-network (van Deurs et al. 1988), it is likely that anything affecting the quantity of ricin-A-chain conjugate reaching this organelle will affect the efficacy of the toxin.

To investigate the effect of a lysosomotropic agent on the toxicity of RTA-MSH conjugates, cells were incubated with either ricin-A-chain or the conjugate in the presence and absence of 5 mM NH_4Cl, and the toxicity assayed.

As is shown in fig 5.5, when NH_4Cl is present the conjugate exhibits an ID_{50} of 0.2 µg/ml, a twenty fold increase in toxicity compared to incubation in the absence of NH_4Cl. The ricin-A-chain on the other hand does not exhibit any change in toxicity when incubated with NH_4Cl. Since the A-chain of

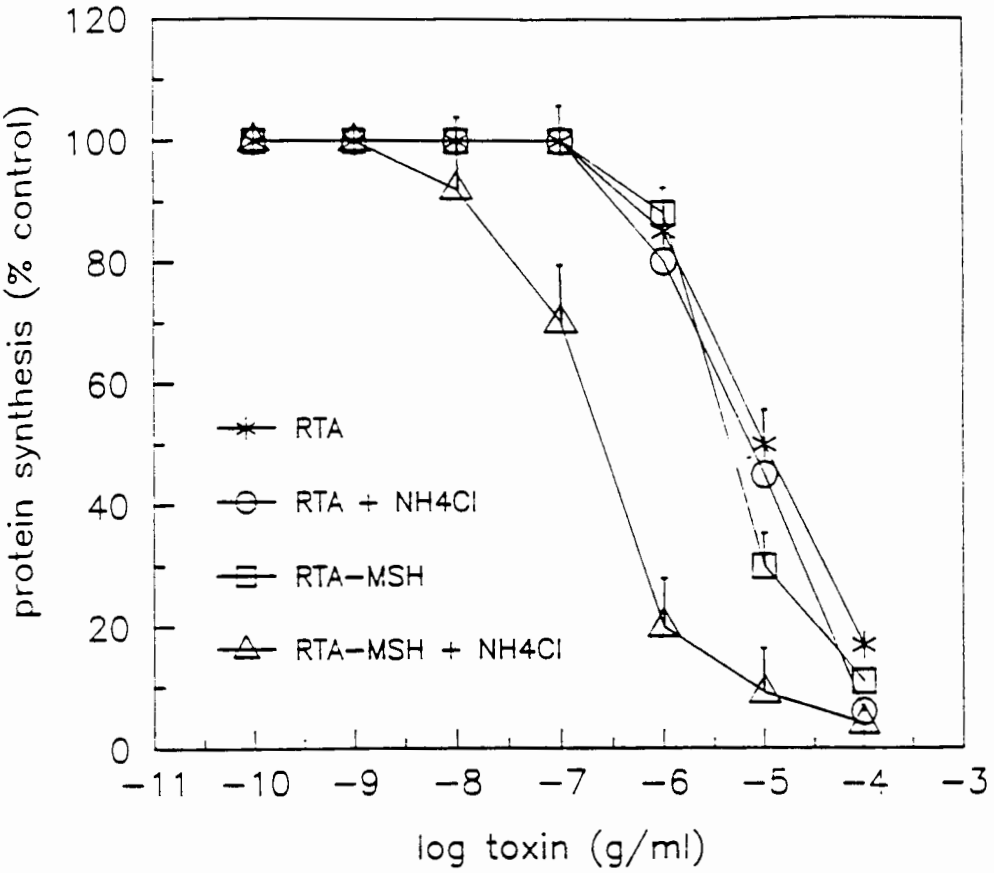

Fig 5.5: Effect of NH_4Cl on the toxicity of RTA and RTA-MSH towards B16 cells. Cells were incubated as for fig 5.4, except that the incubation was performed in the presence or absence of 5 mM NH_4Cl. Data points are average of quadruplicates.

the conjugate exhibits 50% less toxicity than the native A-chain, this represents a net 80 fold increase in toxicity of the conjugate compared to RTA. The effective concentration of MSH at the LD_{50} of 0.2 μg/ml conjugate is 6.4 X 10^{-9} M, slightly higher than that of native MSH, but lower than that of the RTA-MSH as anticipated from the biological activity assay.

This indicates that the MSH effectively targets the A-chain to the cell, and promotes its uptake into the cell, however the uptake is by an endocytotic pathway from which the A-chain is not readily able to escape into the cytosol. The addition of the lysosomotropic agent to the incubation medium alters the receptor-mediated endocytotic mechanism in some way, perhaps by raising the pH of the lysosomes, and facilitates the cytoplasmic release of intact A-chain. This is similar to the observed requirement for carboxylic ionophores to be present in order for anti-transferin receptor linked conjugates to be toxic (Raso et al. 1988), an effect thought to be due to either redirection of the intracellular distribution of the endocytosed conjugates, or due to facilitation of translocation of molecules through the vesicle membranes.

The toxicity of the A-chain alone is however unaffected by NH_4Cl, suggesting that it enters via a route from which it gains ready access to the cytosol. The entry of the A-chain into the cell is probably achieved by non-specific endocytosis, and hence the disparity of response to a lysosomotropic agent suggests that MSH-mediated endocytosis follows a different path to that of non-specific endocytosis. A close association of the MSH receptor with the Golgi complex has been demonstrated , and it has been suggested that receptor recycling occurs in this body (Varga et al 1976). Since A-chain entry to the cytoplasm is thought

to occur in the trans-Golgi-network (van Deurs et al. 1988), the recycling route may prevent normal access of the conjugate to the trans-Golgi-network hence limiting its toxicity. Raising the lysosomal pH may then permit escape to the cytoplasm at a site distinct from the Golgi apparatus.

Contrasting these results are the observation that lysosomotropic agents (NH_4Cl, chloroquine, methylamine) inhibit the specific toxicity of MSH-Daunomycin conjugates (Varga 1985), possibly because enzymatic cleavage of this complex is required for interaction of the daunomycin with DNA, and the enzymes responsible are less effective at the higher pH induced by the lysosomotropic agents. The ricin-A-chain conjugate on the other hand does not require such enzymes and probably escapes most readily from vesicles with a higher pH and less proteolytic activity.

The concentration of MSH-daunomycin conjugate required to mediate 50% killing of the cells was approximately 3×10^{-7} M (Varga et al. 1977), two orders of magnitude greater than the K_D of the hormone. In the case of the RTA-MSH conjugate however, the equivalent toxicity is achieved at a concentration of only 6.4×10^{-9} M, a consequence of using a potent enzyme toxin rather than a drug. The MSH-diptheria toxin conjugate prepared by Murphy et al. (1986) exhibits a K_D in the region of 6×10^{-11} M, two orders of magnitude lower than the toxicity of my MSH-RTA, possibly due to the higher biological activity of the genetically constructed toxin, and a greater ease of exit into the cytoplasm by the toxin component.

Conclusion :

MSH thiolated at Lys_{11} and conjugated to ricin-A-chain through a disulphide bond is able to promote the binding of the ricin-A-chain to B16 cells via the MSH receptor and to

promote its internalisation. The internalisation however follows a route not amenable to the translocation of the toxin into the cytoplasm. The addition of a lysosomotropic agent increases the ability of the toxin to escape into the cytoplasm, resulting in an 80 fold increase in toxicity of the conjugate compared with that of the untargeted A-chain.

Since MSH is able to promote the internalisation and subsequent toxicity of the ricin-A-chain, it is apparent that MSH does mediate endocytosis at the receptor, and hence its failure to achieve the parallel for methotrexate-containing liposomes needs to be reexamined.

Several explanations exist at this stage;

1) Internalisation of the bound liposomes is taking place, but the methotrexate is unable to gain access to the cytosol. This is unlikely because the influence of NH_4Cl on the toxicity of RTA-MSH suggests that an acidic vesicle is encountered during internalisation, and published reports on the effect of NH_4Cl on liposomally delivered methotrexate all point towards the requirement for an acidic environment for methotrexate delivery (Machy et al. 1982, Heath et al. 1983, Huang et al. 1983).

2) Internalisation and delivery are taking place, however, because of the low number of liposomes bound to the cell, the quantity of drug delivered is so low that it is not discernible above the background non-specific internalisation. This is possible, and made more probable by the cell-cycle dependant expression of the receptor which permits receptor-mediated internalisation to occur for only a fraction of the cell-cycle. If the cell experiences internalisation only during the G2 phase of the cell cycle, it may be able to overcome the THFR inhibition in the S- and

G1 phases. The non-specific binding however is not dependant on the cell-cycle and hence may become relatively more toxic.

3) No internalisation of specifically bound liposomes is taking place because the liposomes are either too large for internalisation via the receptor, or perhaps only some of the receptors mediate endocytosis, and the number of internalised liposomes is too low to be discernible.

At this stage it was considered that it would be convenient to have an alternative ligand by which to target liposomes, one which definitely did promote the binding and uptake of liposomes into cells, and which was not subject to the problems of cell cycle dependant expression nor a variable cell line. It was hoped that such a ligand would permit the elucidation of how many liposomes per cell were required for toxicity, and hence provide more insight into the parameters affecting liposomal delivery, and the apparent absence of MSH-mediated liposome delivery.

The ligand chosen for this purpose was the ricin-B-chain (RTB), chosen because it appears able to significantly promote the cellular internalisation of molecules bound to it (discussed in next chapter), interacts with galactosyl residues on cells and hence should be able to target to most cell types, has an exposed sulphydryl moeity making it convenient to attach to liposomes, and, after preparing the ricin-A-chain, I had a lot of the material available.

Chapter 6

RICIN-B-CHAIN TARGETING OF LIPOSOMES

The wide variety of lectins available, and their specificities for sugars, make them an interesting alternative to either antibodies or hormones. All cells exhibit glycoproteins on their cell surface, yet different cells frequently exhibit different sugars on the glycoproteins, a difference that could be exploited by lectin targeting.

A variety of lectins have been used to target either drugs or liposomes to cells. Wheat germ agglutinin, *Phaseolus vulgarus* phytohaemagglutinin and ricin agglutinin have been used to promote the attachment of sialoglycoprotein bearing liposomes to erythrocytes (Juliano and Stamp 1976) by first incubating the cells with the lectin, and then adding glycoprotein-bearing liposomes. No investigation of uptake of the liposomes was performed, however the ability of the free hapten sugar to release bound liposomes suggests that wheat germ agglutinin and phytohaemagglutinin promote internalisation, whereas ricin agglutinin does not. Liposomes have also been targeted to cells using concanavalin A (Con A) or *Phaseolus vulgarus* phytohaemagglutinin (Salame and Patel 1986), however most of the lectin-bound liposomes could be removed by treatment with trypsin, indicating that internalisation of the liposomes had not been promoted by the lectin. It must be noted however that in these experiments the liposomes used were multilammelar vesicles, and no selection for size was reported, hence the liposomes may have been too large for endocytosis.

Wheat germ agglutinin linked to SUV via a thioether linkage has been shown to promote the binding of liposomes to a glycophorin model surface, and the targeting efficiency dependant on the degree of coupling of the lectin to the liposomes (Hutchinson and Jones 1988), however, as in the above experiments, no functional delivery of liposomal contents to cells was demonstrated.

Daunomycin has been directly linked to Con-A and targeted to tumour cells in vivo. Significant increase in survival time demonstrates successful targeting towards the tumour (Kitao and Hattori 1977), and suggests that lectins may be useable as agents for promoting the uptake of other bound molecules or carriers.

The B-chain of the ricin toxin (RTB) is a lectin with a specificity for galactosyl residues, however apart from its lectin properties it appears to be able to significantly promote the internalisation of surface-bound ricin-A chain, and this ability is independent of its lectin property (Youle et al. 1982, Vitetta et al. 1983, Wawrzynczak et al. 1988). Ricin-B-chain has been conjugated to Insulin, and shown to promote the internalisation and a biological effect of insulin on cells which lack the insulin receptor (Hofmann et al. 1983), demonstrating apparent intracellular recognition of Insulin, and also the ability of RTB to promote the uptake of bound molecules. This experiment does not prove that the B-chain promoted cytosolic delivery, but does suggest that there was internalisation. Biological effect could be mediated from within the lysosome, or by a cleavage product leaking out of the lysosome.

Precisely what property of the B-chain it is that promotes the internalisation of the A-chain is not known. The B-chain has been shown to insert into membranes in the absence of any glycoprotein (Utsumi et al. 1984, Adair and Kornfeld

1974). Photolabelling experiments demonstrate that B-chain is able to insert into membranes with glycoprotein, but that binding of the B-chain to the glycoprotein significantly enhances its insertion (Ishida et al. 1983). This data suggests that there is a hydrophobic domain on the B-chain that might be responsible for promoting uptake, however the above experiments also demonstrate that the A-chain is also able to insert into membranes, and since the A-chain alone or conjugated to an antibody is less toxic than whole ricin, membrane insertion alone is insufficient for promoting cytosolic delivery. An alternative hypothesis is that the oligosacharide moeity of the B-chain is recognised by sacharide binding receptors on the cell, and that this determines the toxicity (Wawrzynczak et al. 1988).

Since most cells are likely to express galactosyl residues on their surfaces it is likely that RTB will be able to target a carrier to most cell lines, and further, since it appears to promote the internalisation of protein molecules attached to it, it may be able to function as a ligand by which to mediate the delivery of liposomal contents into most cells, and hence function as a model by which to compare other targeting systems.

RTB purification and characterisation

RTB was prepared by reduction of pure ricin (see methods) with DTT and affinity chromatography on Sepharose 4B. The absence of contaminating whole ricin was confirmed by the lack of toxicity of the RTB on Hepatoma cells on which an LD_{50} of greater than 1 mg/ml was obtained.

Binding of RTB to liposomes

The finding by Utsumi et al. (1984) that the carboxymethylated B-chain from ricin bound to phospholipid vesicles with a fairly high affinity (K_a = 14.5 X 10^5 M^{-1} for DPPC vesicles) led me to try to use this affinity of the B-chain for bilayers to bind RTB directly to liposomes.

^{125}I-B-chain or iodinated carboxymethyl B-chain (prepared as described in methods) were incubated at 2 mg/ml with liposomes (phosphatidylcholine / cholesterol / dicetyl phosphate 47.5:50:2.5 mol% ,10 µmol/ml) at 20 °C for 30 minutes. Liposomes were then separated from unbound protein by discontinuous density gradient centrifugation in Ficoll, and liposome-bound protein determined by counting the radioactivity.

Under these conditions approximately 5% of the radioactivity for both the native and carboxymethylated B-chains remained associated with the liposomes, representing 10 µg of protein bound per µmol lipid, significantly less than that expected from the data in the publication by Utsumi et al. although still representing approximately 160 lectin molecules bound per liposome.

Before such non-specifically bound ligand can be used for targeting, it is necessary to determine the stability of the association of the ligand and liposome. For this purpose the liposomes prepared and purified as above were incubated in 1.0 ml 0.1 mg/ml bovine serum albumin in PBS at 25°C for 2 hours and then repurifying the liposomes by gradient density centrifugation. Less than 10% of the initially associated radioactivity remained associated with the liposomes, indicating that significant dissociation of the bound lectin had taken place, and hence that this procedure was of no use in preparing targeted liposomes.

To overcome the problems of dissociatable non-specific binding of RTB to the liposomes I utilised the available sulphydryl of the B-chain to covalently bind the protein to liposomes through a thioether bond by the method of Martin and Papahadjopopoulos (1982) using the sulphydryl reactive phospholipid derivative N-[4-(p-maleimidophenyl) butyryl] phosphatidylethanolamine (MPB-PE). This reagent was used in preference to N-[3-(2-pyridyldithio)propionyl] phosphatidylethanolamine because the resulting thioether linkage is resistant to reduction and is hence likely to be more stable in serum than the disulphide link obtained using PDP-PE, and definitely more stable than using hydrophobic non-specific association.

Freshly reduced and dialysed RTB was incubated with liposomes (EYPC / Chol / DCP / MBP-PE, 9.5:10:0.25:0.25 mol%) containing 100 mM calcein for 8 hours at room temperature and unbound protein separated from the liposomes by gradient density centrifugation. Bound protein was quantitated by radioactivity measurement.

Increasing the initial concentration of RTB resulted in greater amounts of RTB becoming bound to the liposomes (fig 6.1). This shows a roughly linear relationship between initial concentration and the amount bound, however at high concentration of RTB (above 2 mg/ml) the vesicles tended to aggregate and separation of unbound protein on ficoll gradients became difficult. Using an RTB concentration of 1 mg/ml 270 µg of protein became bound to the liposomes representing 27% efficiency of binding and resulting in approximately 450 molecules becoming bound per vesicle.

Since other researchers have observed that RTB is able to bind to liposomes and to promote the phase transition

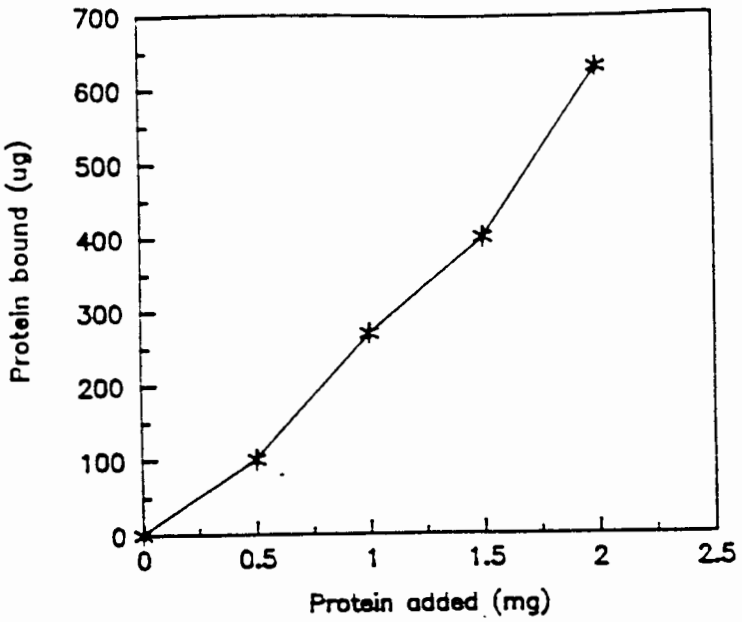

Fig 6.1: Binding of iodinated Ricin-B-chain to liposomes. Increasing quantities of freshly reduced iodinated RTB were added to freshly prepared liposomes and incubated for 8 hours at 25°C. Liposomes were separated from unbound protein by Ficoll gradient density centrifugation, and the amount of bound protein determined by counting the radioactivity.

release of encapsulated contents (Utsumi e al. 1984), and also to promote the fusion of SUV (Utsumi et al. 1989), it was neccesary to determine whether the covalent linkage of RTB to the liposomes had altered their integrity in any way.

Liposomes containing 100 mM calcein, with RTB bound, or as a control, without RTB bound, were incubated in either PBS or PBS with 10% fetal calf serum and the leakage after 24 hours determined by fluorescence. The results in table 6.1 demonstrate that there is no significant increase in the rate of leakage, implying that the RTB does not significantly perturb liquid crysteline membranes. These results are partially in conflict with those of Utsumi et al. (1984, 1989), however these authors used small unilammelar vesicles composed of DPPC, which is in a gel state at the temperature of incubation with the RTB, and monitored changes induced at the phase transition which are likely to be highly sensitive to contaminants within the membrane and also to curvature and torsional stress on the membrane. The liposomes I used were in a liquid-crysteline state at all times, and the membranes were not stressed.

	% Leakage	
	PBS	PBS+FCS
Lip	4.0	18.0
Lip-RTB	5.0	20.0

Table 6.1 : % efflux of calcein over a 24 hour period from liposomes with or without MSH bound to the surface in PBS with or without 10% fetal calf serum. 10 nmoles lipid was incubated in 1.0 ml buffer or buffer with FCS. Fluorescence after 24 hours was compared to fluorescence after adding 10 µl of 10% Triton X-100 which lysed the liposomes and resulted in 100% leakage.

Thus RTB has been covalently bound to liposomes without significant perturbation of the bilayer structure. It remained to be ascertained whether the galactose binding property remained intact and was able to mediate the binding of liposomes to cells.

Binding to cells

To determine the interaction of RTB targeted liposomes with cells, Hepatoma cells were used instead of Melanoma cells. The reasoning behind this was that the B16 melanoma line used for the previous experiments appeared to be polyclonal and also abnormally resistant to methotrexate. It was felt that interpretation of the results of liposome targeting towards hepatoma cells, which I knew from earlier experiments to be highly sensitive to both methotrexate and ricin, would be less subject to variables within the cell line.

Incubating increasing quantities of fluorescent targeted liposomes, and, as controls, untargeted liposomes, and targeted liposomes in the presence of 10 mM lactose which should compete with the galactosyl residues for the lectin binding site, with hepatoma cells in suspension, resulted in significantly increased binding of targeted liposomes compared to that of the controls (fig 6.2).

The number of targeted liposomes binding to the cells reaches a plateau of 11000 liposomes per cell at a phospholipid concentration of 100 μM. Untargeted liposomes at the same concentration bind only 90 liposomes per cell, hardly noticeable on the graph, hence the ricin-B-chain mediates a 110 fold increase in the binding of liposomes to cells.

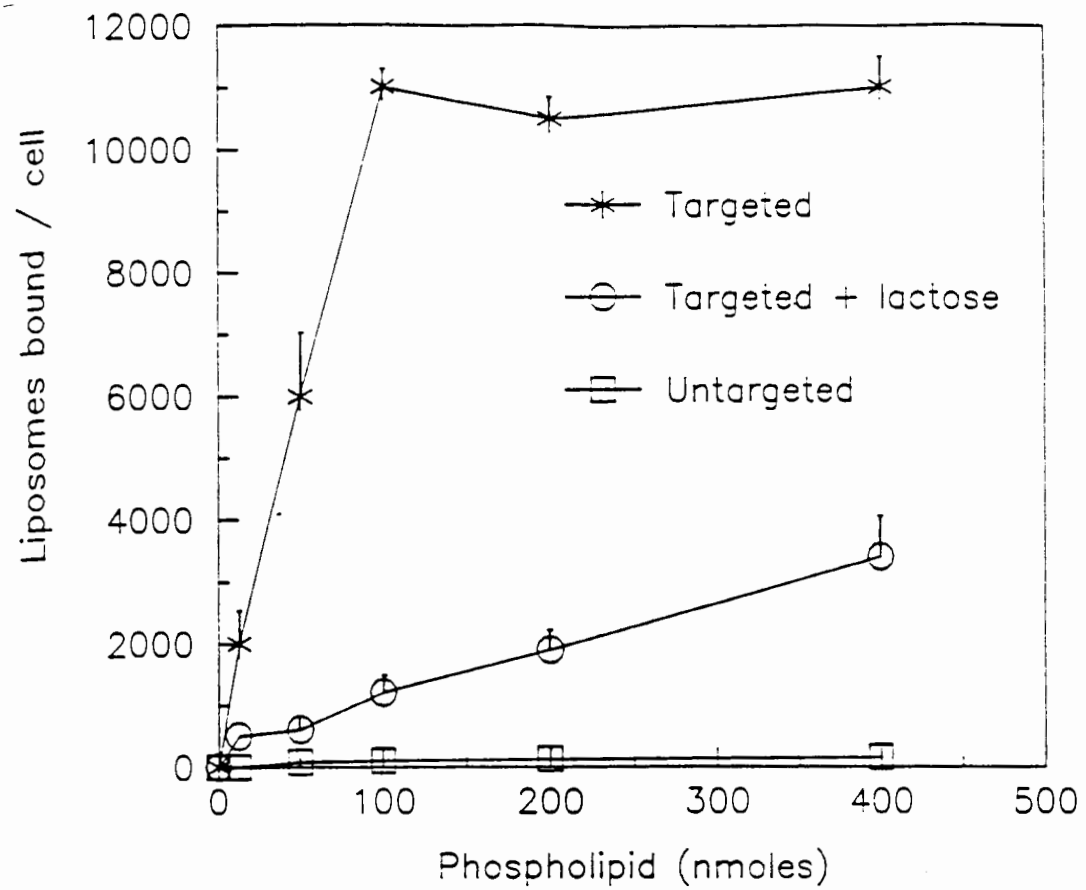

Fig 6.2: Binding of calcein containing RTB targeted liposomes to Hepatoma cells in suspension. 2.5 X 10^5 cells in 1.0 ml PBS were incubated with increasing concentrations of either RTB-targeted liposomes, RTB-targeted liposomes in the presence of 10 mM lactose, or untargeted liposomes. Incubations were performed at 4°C for 1 hour after which the cells were washed three times in PBS, lysed with 1% triton X-100, and fluorescence determined. The cells had been released from monolayer growth with EDTA only, as trypsin might decrease the total surface glycoprotein and hence the number of RTB binding sites. Data points are average of triplicate. Variation betwen experiments performed on different days was within the variation shown on the graph.

Not all of the increase is due to the galactose binding properties of the lectin, as demonstrated by the binding of RTB-targeted liposomes in the presence of lactose. This sugar is able to compete for the galactose binding site on ricin, and hence the binding of 1000 liposomes per cell at 100 μM lipid in the presence of lactose indicates that 10% of the RTB-mediated binding is due to an alternative association of the ricin-B-chain with the cells. This is similar to the inability of lactose to completely abolish the binding of ricin or RTB to Newcastle Disease Virus membranes (Ishida et al. 1982) and could be due to non-specific association, or to receptors on the cells recognising sacharide chains on the protein, or possibly via the weak glucose binding site on RTB (Villafranca and Robertus 1981).

Taking the average diameter of the liposomes to be 200 nm, and an average cell diameter of 15 μm, and assuming that the liposomes pack as solid spheres and do not exhibit significant repulsion, one can expect to pack at most 20000 liposomes on the surface of the cell. The influence of a hydration sphere around the vesicle or mutual repulsion will rapidly decrease this figure, hence the observed plateau of 11000 liposomes per cell is likely to be a function of the available space, not of receptors. (The number of receptors for ricin on Hepatoma cells I calculated to be 2.5×10^7 - see chapter 4). This figure is similar to that obtained by Fraley et al. (1981) who used negatively charged liposomes and obtained a binding of approximately 30 nmol lipid / 5×10^6 cells, which is equivalent to 7000 liposomes per cell.

RTB mediated binding reaches saturation at a much lower concentration of lipid than that reported for negatively charged liposomes (> 1mM lipid Fraley et al. 1981).

The maximal level of binding was reached at a concentration of liposomes representing 1.2 X 10^{11} liposomes present in the incubation medium. There were 2.5 X 10^5 cells present hence a total of 2% of the added liposomes were bound to the cells. This figure is lower than that reported for the optimal association of negatively charged liposomes with cells (Fraley et al. 1981), however these authors used 20 times as many cells per incubation, allowing many more liposomes to bind, hence a comparison of binding efficiency is not really informative. A better comparison is the concentration of lipid required to achieve maximal binding. For the case of RTB-mediated binding this is achieved at 100 μM, however the association of negatively charged lipid with cells approaches saturation only at concentrations above 1 mM. The concentration of RTB at 100 μM lipid is 8 X 10^{-8} M, approximately the same as K_D of ricin. Since some of the RTB is unavailable for interaction with the cells, due to its physical orientation on the liposomes, this implies that the multimeric association of the ligand that is available for interaction is greater than that of the free ligand.

Fluorescence Microscopy

To further elucidate the events occurring, fluorescence microscopy of targeted liposomes containing 100 mM calcein, interacting with Hepatoma cells was performed (fig 6.3). At high concentrations of liposomes (fig 6.3B) the binding is so extensive that the cell is lost in a haze of fluorescence, however when the incubations are performed at lower concentrations (fig 6.3A) the fluorescence is seen to be associated mainly with the periphery of the cell and appears to be punctate. There is some apparently diffuse fluorescence, but whether this is due to delivery of calcein into the cytoplasm, or artifactual, resulting from

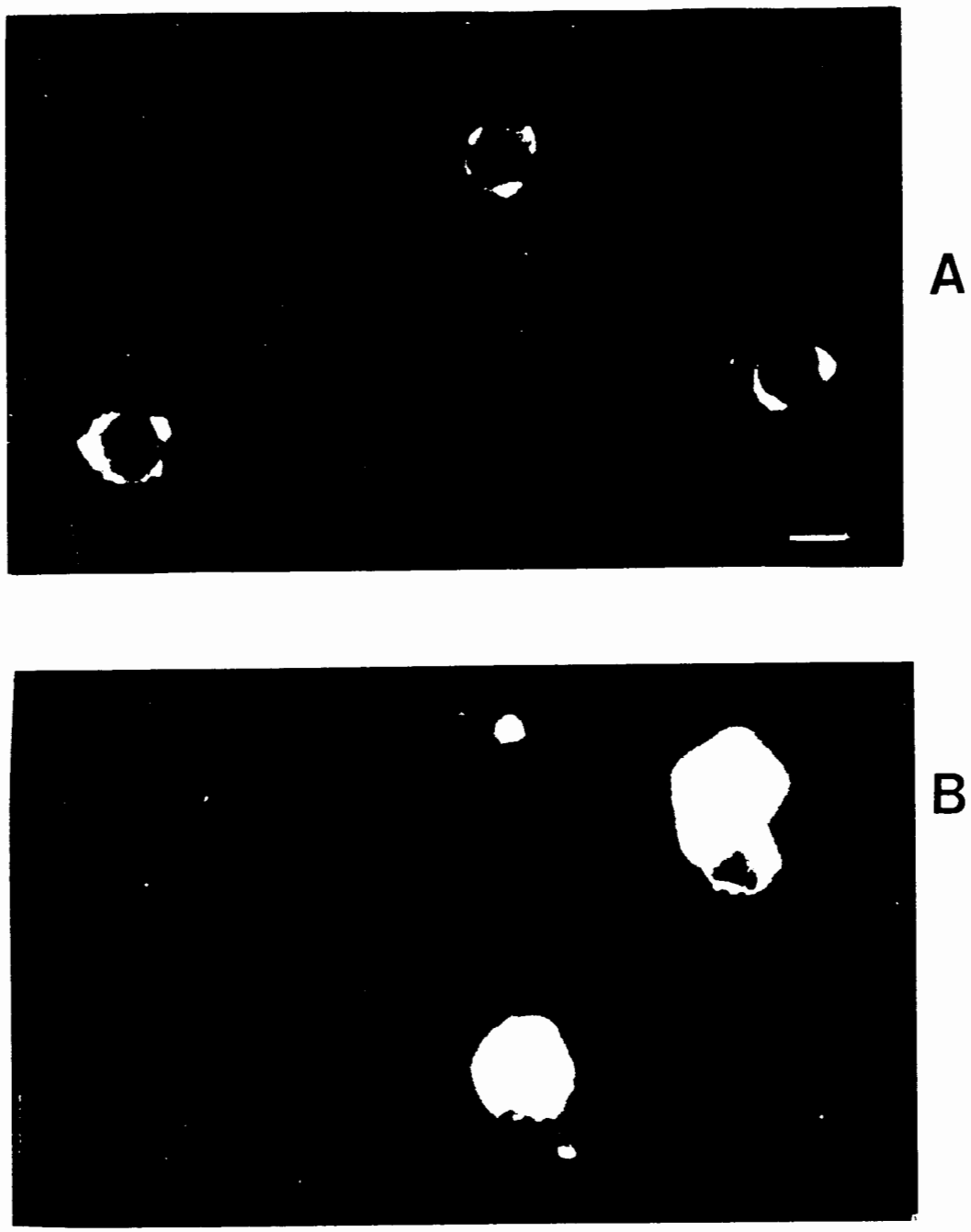

Fig 6.3: Fluorescence microscopy of interaction of RTB targeted liposomes with hepatoma cells. 2.5 X 10^5 cells in suspension in PBS were incubated with either 10 nmoles (**A**) or 100 nmoles (**B**) targeted liposomes containing 100 mM calcein. Bar = 10 μm.

fluorescence of liposomes out of the field of focus, could not be determined from this experiment.

Scanning electron microscopy

Scanning electron microscopy was used to give some idea of the morphology and arrangement of bound liposomes on the cells.

Liposomes were first examined on 0.2 μm polycarbonate membranes (fig 6.4A) to provide a control of the conditions required for fixing and staining the liposomes, as well as to size the liposomes and to visualise what non-cell-associated liposomes resembled. The conditions used for fixing and staining are described under methods. Liposomes are seen on a polycarbonate membrane as discreet spheres with a diameter of 0.2 μm.

The binding of untargeted liposomes to hepatoma cells is shown in fig 6.4B. A few liposomes are discernible, however far fewer than in fig 6.4C and 6.4D which show the binding of targeted liposomes and demonstrate the very high degree of binding achievable by targeted liposomes. The cell surface in fig 6.4B is smoother than that in 6.4C in which ruffling of the plasma membrane appears to be taking place, indicating that the binding of the multimeric lectin promotes a change in the cytoskeletal structure, possibly associated with an endocytotic mechanism. Similar ruffling is observed in other cells in response to glycerol treatment (Fraley et al 1981) which is thought to promote endocytosis, and hence its presence here may indicate that the ricin-B-chain liposomes, unlike the control liposomes, not only bind to the cell, but also mediate endocytosis.

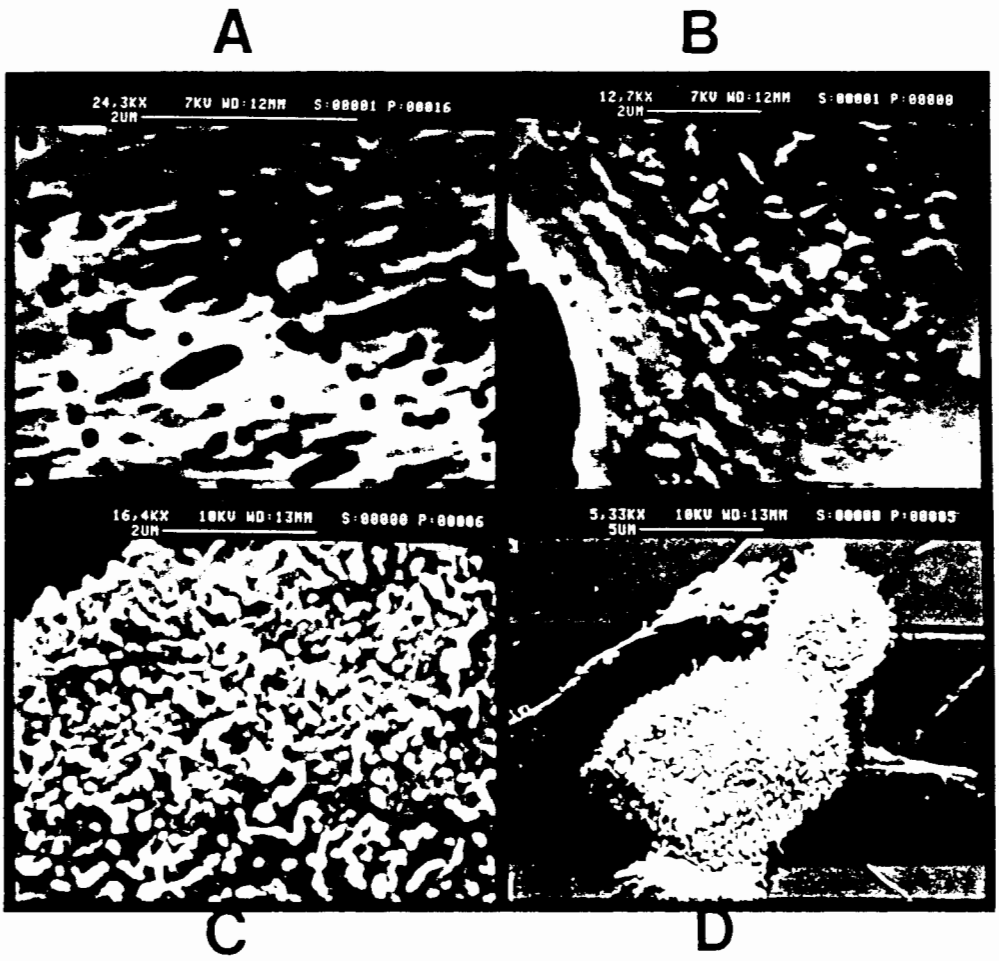

Fig 6.4: Scanning electron microscopy of liposomes and cells. Liposomes associated with either 0.2 μm polycarbonate membranes (A) or hepatoma cells growing on glass coverslips (B,C,D) were fixed with osmium tetroxide and formaldehyde and processed for electron microscopy as described in methods. Liposomes were added to cells at 100 nmoles lipid / ml and incubated at 37°C for 1 hour. Untargeted liposomes (B), ricin-B-chain targeted liposomes (C,D).

Some of the liposomes have become long sausage-like structures. This is possibly a result of the fixation procedure in which adjacent liposomes may become bound to one-another.

The diffuse fluorescence in the fluorescence microscopy, and the ruffled surface in scanning electron microscopy suggest that there may be internalisation of the liposomes taking place. To investigate whether there is an energy dependant endocytotic process taking place, the differences in binding of targeted liposomes to cells at 4°C and at 37°C were investigated. RTB-targeted liposomes were incubated with cells at either 4°C or 37°C for 1 hour, and then the ability of lactose to dissociate the liposomes from the cells measured.

	Incubation Temperature	
	4°C	37°C
no lactose	11000 (270)	11500 (320) lip/cell
lactose	2100 (100)	3400 (180) lip/cell
% lactose resistant	19	29

Table 6.2: Incubation temperature dependence of lactose mediated dissociation of ricin-B-chain targeted liposomes from hepatoma cells. 2.5 X 10^5 cells in 1.0 ml PBS were incubated with 100 nmoles targeted liposomes for 1 hour at 4°C or 37°C. Aliquots were either washed immediately or incubated with 50 mM lactose in PBS for 10 minutes at 4°C and then washed. Cells were then lysed with 1% triton X-100 and cell associated fluorescence determined. (Standard deviation shown in brackets).

This demonstrates that incubation at 37°C results in a statistically insignificant (500 liposomes per cell) increased association of liposomes with cells compared with that at 4°C.

The ability of lactose to remove bound liposomes appears to be lower than its ability to inhibit binding. Thus when present during the incubation at 4 degrees in the previous experiment to determine concentration dependence of binding, 900 liposomes were bound per cell, however when added after incubation, 2100 liposomes remained bound. This difference could be due to any or all of the following effects :
1) Steric hindrance of bound liposomes preventing lactose competition for binding.
2) Increased non-specific interaction as a result of being brought into close proximety by the specific interaction.
3) Uptake, probably by a fusion process since endocytosis is unlikely to occur at 4°C, of the bound liposomes into the cell.

The number of liposomes associated with cells following lactose treatment after a 37°C incubation is significantly greater than after the 4°C incubation. The difference of 1300 liposomes per cell reflects events occurring at 37°C which result in decreased susceptibility to release of liposomes that do not occur at 4°C. The most likely event to cause this is endocytosis, however it is possible that other events such as glycoprotein aggregation are temperature dependant processes and may mask the liposome-cell interaction site from lactose, decreasing the effectiveness of the sugar to compete for the binding.

The discrepancy between the binding at 4 °C and at 37 °C of 1300 liposome/cell with lactose treatmentcompared with the negligible difference without lactose treatment, reflects

simply that not all events that inhibit the release of liposomes permit the binding of new liposomes. For example inhibition of release due to an active masking process will not expose new binding sites.

This data therefore suggests, though not conclusively, that the ricin-B-chain is able to promote the uptake of liposomes into the cell, and that the uptake is in the order of 10 to 20% of the liposomes bound. To confirm that internalisation of the liposomal contents was occurring, the ability of RTB to promote the internalisation and toxicity of methotrexate containing liposomes was investigated.

Cytotoxicity

The intracellular delivery of the liposomal contents was monitored by measuring the ability of methotrexate, free or encapsulated in targeted or control liposomes, to inhibit the incorporation of ^{3}H-thymidine into cells after exposure of the cells to the drug for 24 hours. The results are shown in fig 6.5, which shows that the free drug exhibits an ID_{50} of 60 nM. In contrast to this, the drug when encapsulated in untargeted liposomes has an ID_{50} of 800 nM, which probably represents leakage of the drug from the liposomes into the medium and the subsequent entry as free drug into the cell. The rate of leakage in medium containing 10% fetal calf serum was shown to be 19% over 24 hours, and hence by the end of the incubation period the concentration of free drug will be 160 nM, above the ID_{50}, although it will not have been at this level the entire time. Targeted liposomes on the other hand exhibit an ID_{50} of 80 nM, marginally less toxic than that of the free drug but 10 times greater toxicity than that of untargeted liposomes, indicating that the ricin-B-chain has been able to promote the intracellular delivery of the liposomal contents.

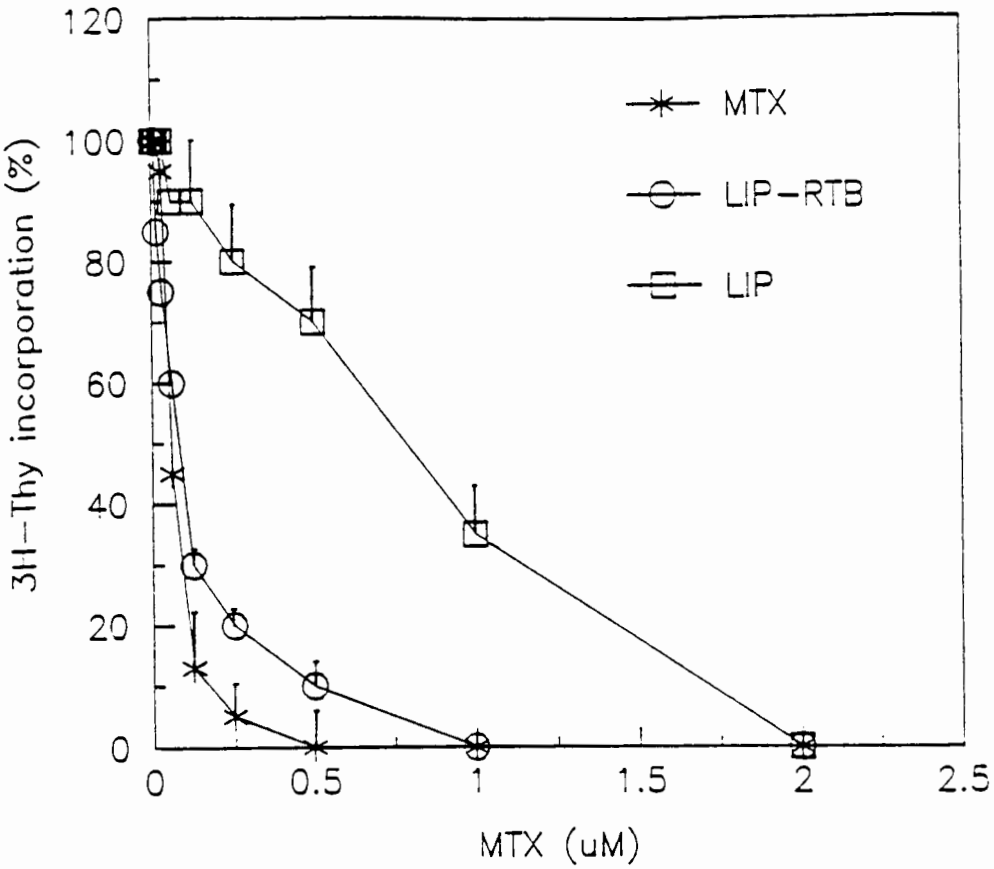

Fig 6.5: Growth inhibition by methotrexate in targeted liposomes. 1 mM MTX-containing Liposomes targeted with ricin-B-chain or untargeted, or free MTX were incubated at the drug concentration shown with hepatoma cells for 24 hours. ^{3}H-Thy incorporation into cellular DNA was assayed as described in methods. The measurements are averages of duplicates.

While the significantly higher toxicity of targeted liposomes compared to that of untargeted liposomes is suggestive of liposomal internalisation, and this is further supported by the fluorescence binding data, the possibility that the toxicity is due to leakage of bound liposomes, with subsequent high concentration of drug at the cell membrane leading to internalisation of the free drug, cannot, at this stage, be excluded. This demonstrates the problems of using a marker for liposomal delivery which can by itself gain access to the cell.

Because of the possibility of toxicity due to leakage, any assessment of how many liposomes have delivered their contents to the cytosol is difficult, however assuming that an uptake process has occurred, the following arguments present themselves:

At the ID_{50} of 80 nM for targeted liposomes, the lipid concentration is 20 μM. This concentration corresponds to 20 nmoles/ml, which from fig 6.1 corresponds to 2500 liposomes binding to the cell within 1 hour. By fluorescence (table 6.2) approximately 10% of bound liposomes are internalised within 1 hour. Therefore at this concentration approximately 250 liposomes are taken up into the cell within the first hour of incubation. By comparison the calculations presented previously indicate that the contents of only 20 to 30 liposomes need enter the cytosol for toxicity. Even assuming that no further binding or uptake occurs after the first hour, a discrepancy between these two arguments exists. Several explanations exist;

1) Only 10% of the liposomes internalised release their contents to the cytoplasm, the rest are vesicularised. Or,

2) The calculations for the amount of methotrexate required in the cytoplasm are incorrect due to compartmentalisation within the cell, Or,

3) The assessment by lactose release of bound liposomes is overestimating the uptake.

In order to demonstrate unequivocally whether or not the liposomes deliver their contents directly into the cell, and to obtain a more accurate assessment of how many liposomes deliver their contents, the material contained within the liposomes must not be able to cross the plasma membrane in the free form. In following this philosophy, the ricin-B-chain targeting of liposomes to cells was followed by two alternative assays;

1) Transformation of cells by liposomes containing foreign DNA, and,

2) The digestion of cellular DNA by liposome mediated delivery of nuclease into the cell.

The results of these two routes of investigation are given in the next two chapters.

Chapter 7

Liposome mediated transformation

Because of the problems experienced in interpreting the results from experiments in which the liposomal contents were able to gain access to the cytoplasm in the free form, liposomes containing foreign DNA, which is by itself unable to enter the cell, were incubated with cells, and the subsequent transformation monitored. Liposome mediated transformation of eukaryotic cells thus provides both an unequivocal demonstration of the intracellular delivery of liposomal contents, and may also provide a method by which to transform cells which are refractory to other transformation procedures.

Gene transfer

Apart from the use of viral vectors, a variety of methods exist for introducing foreign genetic material into mammalian cells, each method differing in efficiency of transformation, applicability to cell types, technical difficulty and reproducibility.

Precipitation of complexes of the DNA with calcium phosphate (Graham and Van der Eb, 1973) is technically simple although requires a carefully controlled pH, and many cells are refractory to this procedure as the uptake is an essentially phagocytotic process. Becoming more widely used is the method of electroporation (Potter et al. 1984), in which the cells in the presence of DNA are exposed to a short but intense electric field, inducing transient pores in the plasma membrane through which the DNA can diffuse. This procedure is widely applicable, however requires fine-tuning for each cell type used.

Alternative, though less frequently used techniques include scrape loading (Fechheimer et al. 1987) , poly-ornithine mediated transformation(Bond and Wold 1987), and laser micropuncture (Tao et al. 1987). While the latter is extremely efficient, it requires highly specialised equipment, and is not applicable to large numbers of cells. The same is true of microinjection, which while yielding high frequencies of gene transfer is technically demanding and not applicable to large cell numbers.
Targeted delivery of DNA to hepatoma cells has been achieved by binding DNA to a poly-lysine-asialorosmomucoid complex. The resulting complex was highly efficient at transfecting hepatoma cells which express receptors for asialoproteins, but less infective for other cell types.

Liposome mediated transformation

Liposomes have been used to transform mammalian cells, and a wide variety of efficiencies have been reported (reviewed Lurquin 1984);

Untargeted phosphatidylserine liposomes carrying the thymidine kinase gene have been reported to stably transform FM3A*tk*$^-$ cells (mouse mammary carcinoma) with an efficiency of 0.02 (Itani et al. 1987). The liposomes used were prepared by the calcium induced fusion method. REV liposome mediated transfer of the same plasmid to mouse L*tk*$^-$ cells was far less efficient (Schaefer-Ridder et al. 1982), yielding only 200 colonies per 10^6 cells, an efficiency of 0.0002. In both cases the liposomes were prepared from PS, although in the latter, 50% cholesterol was present, and the final difference in DNA concentration is only two fold (Itani et al. used 1000 ng DNA per 10^6 cells, Schaeffer-Ridder et al used 500 ng.). The 100 fold difference observed must be due to either the nature of the liposome preparation

or the cell types or fine detail in the experimental protocol.

Transformation of L*tk*$^-$ cells with pSV2-neo in LUV prepared by calcium induced fusion also yielded an efficiency of 0.02 (Nakatsuji et al. 1987), suggesting that the cell line is not limiting the transformation efficiency. For further comparison, calcium phosphate transformation of the L*tk*$^-$ cells yielded an efficiency of 0.0005, approximately the same as the REV mediated transformation. There is however a danger in trying to draw conclusions by comparisons of transformation frequencies from different reports, as the same method applied to the same cell line may yield vastly differing results depending on how soon after transformation the selective agent was added (Itani et al. 1987).

SV40 DNA in REV liposomes composed of PS has been found to be more infective than the free DNA, and the addition of glycerol or polyethylen glycol increased the infectivity, however only to values comparable to calcium phosphate mediated infectivity (Fraley et al. 1981).

All of the above experiments have utilised the affinity of some cells for negatively charged lipid. The applicability of targeted liposomes to gene transfer has been investigated (Machy et al. 1988) using large (0.4 μm) REV liposomes to which protein A had been coupled. Targeting was achieved by incubation of the cells with anti-HLA monoclonal antibodies and subsequent incubation with the liposomes. While successful binding of these liposomes to the target cells was achieved, in the absence of electroporation, no significant transformation occurred. Electroporation of the cells subsequent to liposome binding did however promote transformation, indicating effective delivery of the liposomal contents. In the absence of targeting negligible binding of liposomes to cells took place, however

electroporation still promoted transformation albeit at a frequency five fold less than for targeted liposomes. This data suggests that liposomes targeted via antibodies to HLA do not promote the nuclear delivery of their contents, although significant binding does take place. It is argued in the paper that the transformation due to targeted liposomes is representative of only those liposomes actually bound to the cell, in which case the efficiency of transformation is improved several hundred fold over untargeted liposomes, however the methods section of the paper indicates that the total number of liposomes present is identical in both the targeted and control experiments. In this case the further argument that transfection depends on the electroporation mediated generation of either a pore between the cell and bound liposomes or fusion of the bound liposomes with the cell membrane is open to debate since the relatively high (compared to non-electroporated) transformation with control liposomes represents DNA delivery from liposomes not bound. That transformation occurs even with control liposomes argues that the transformation is not specific. One can provide an alternative explanation by postulating that electroporation causes perturbation of the cell membrane and induces long-lived fusogenic states (Sowers 1987) which are able to fuse with liposomes and the binding of liposomes to the cell surface simply increases the probability of contact with such sites within the life time of the state. Since the fusogenic state is not laterally mobile (Sowers 1987), only a few of the bound liposomes can be in contact with it, possibly explaining why the targeting does not cause more of an increase in transformation frequency. Bates et al. (1987) point out that electrical fusion of small liposomes with cells is unlikely because the field induced membrane potential is directly proportional to the particle radius, and hence the vastly differing radii of liposomes and cells

may make it impossible to form pores in the liposomes without totally lysing the cells.

The development of a cationic lipid DOTMA (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethyl-ammonium) has provided a novel method of liposome mediated transformation. An aliquot of the liposomes prepared in the absence of DNA, will bind to DNA in solution by virtue of the tertiary amine on the lipid,resulting in surface bound DNA. Subsequent electrostatic interaction with the cells promotes the delivery of the DNA into the cells (Felgner et al. 1987). The frequency of stable transformation with the pSV2neo plasmid was 15 times greater using the liposomes than was obtained using calcium phosphate.

Liposomes have also been used to promote the delivery of foreign genes *in vivo* (Cudd and Nicolau 1984, Wang and Huang 1987, Nandi et al. 1986), a process which is only achievable by liposome technology.

Thus liposomes are able to promote the delivery of transfecting DNA into cells, however the efficiency is highly dependant on the technique used. Since DNA alone is in most cases unable to transform cells, any transformation that occurs indicates a liposome mediated delivery, and hence a potentially better assay for liposome uptake than toxicity due to drugs.

Transforming DNA.

Assaying stable transformation or transfection of a cell line is most readily achieved by the expression of a gene conferring resistance to an inhibitor of growth, or expression of an essential gene in a cell line deficient in that gene. The latter case requires the availability of mutants deficient in a gene, typically the thymidine kinase

gene. The former case obviates this necessity, and is as such more widely applicable.

A variety of genes conferring drug resistance on mammalian cells are available (reviewed by Esser and Dohmen 1987), amongst them are resistance genes to aminoglycoside antibiotics, metallo-glycopeptide antibiotics, heavy metals and fungicides.
One of the most versatile of these is the bacterial phosphotransferase gene conferring resistance to neomycin antibiotics (neo). The aminoglycoside G418 (Geneticin) which interferes with the function of the 80S ribosomes, and blocks protein synthesis in eukaryotes is similar in structure to the bacterial antibiotics neomycin and kanamycin, and can be inactivated by the bacterial gene products, hence expression of the neomycin resistance gene in mammalian cells confers resistance of these cells to G418. The pSV2neo plasmid contains the bacterial gene for neomycin resistance under the control of the SV40 early region promoter (Southern and Berg 1982). This plasmid can be maintained in bacterial cells in the presence of neomycin, and when introduced into mammalian cells is able to insert into the genome and be expressed conferring resistance to G418. The frequency of calcium phosphate induced stable transformation varying between 7 X 10^{-6} to 3 X 10^{-4} depending on the cell type.

Linear Vs Circular DNA

The comparable transformation frequencies with linear or circular plasmids have been investigated (Potter et al. 1984, Machy et al. 1988), with a finding that linearised plasmid DNA is significantly more efficient than circular DNA at transforming cells, presumably due to increased ease of integration into the genome. The linearisation site is also important (Johnson and Phelps 1988), with optimal sites

for linearisation being far away from either end of the resistance genes.

Results and discussion

The following experiments were undertaken to establish whether ricin-B-chain targeted liposomes could promote the delivery of pSV2neo DNA into cells, and if so, whether subsequent stable transformation was a valid assay of liposomal delivery.

Liposomal encapsulation of DNA

The pSV2neo plasmid was maintained in E.coli and isolated and purified by standard methodology. After purification on caesium chloride gradients the plasmid was subject to restriction enzyme digestion and shown to exhibit the correct cleavage sites, confirming that it was the correct plasmid. The DNA was then cleaved with EcoR1 to linearise it. This site was chosen because it is well removed from the neo gene and from the SV40 promoter, and because fairly large quantities were required, and EcoR1 is relatively cheap.

The linearised plasmid (1 mg/ml)was encapsulated in liposomes composed of EYPC/CHOL/PS/PE-MBP (9.5:10:2.5:2.5) by a modification of the reverse phase procedure as described in methods. Since the normal procedure requires sonication, and I observed that even at the lowest setting of the probe sonicator significant degradation of the linear DNA occurred within the time period required for emulsification of the organic and aqueous phases, the procedure was modified so that sonication was not required. This was achieved by vortexing the two phases together and applying a vacuum while continuing vortexing. Using this

procedure no degradation was discernible, and after extrusion through 0.2 μm membranes and separation from unencapsulated DNA by ficoll gradient density centrifugation, the encapsulation of the DNA was 15%. This is equivalent to 5 μg of DNA per μmol phospholipid, which corresponds to approximately one plasmid molecule per vesicle.
Since 85% of the DNA is not incorporated within the liposomes, this represents a significant wastage of precious material. Attempts to recover the unentrapped DNA for reuse were unsuccessful as the ficoll interfered with ethanol precipitation and could not be dialysed out. Alternative density gradient media compatible with ethanol or propanol precipitation or dialysable (eg. metrizamide) may overcome this problem.
Liposomes were targeted with ricin B chain as described previously.

Sensitivity of Hepatoma cells to G418

In order for the transformation assay to be effective, the cells must be sensitive to the antibiotic, and the antibiotic must be used at a concentration where it exerts an effect. The determination of the required concentration is shown in fig 7.1.

This demonstrates that below 400 μg/ml (real G418 conc 200 μg/ml because the G418 used was only 50% by weight antibiotic) there is inhibition of growth, however the time period required is large. Furthermore at low concentration of antibiotic more cell divisions occurred, and after several days the cells reached confluence. Once confluent cell death did occur, however leaving patches which by visual inspection resembled colonies. These 'colonies' died later and detached from the plates, but could result in overestimation of the transformation frequency if

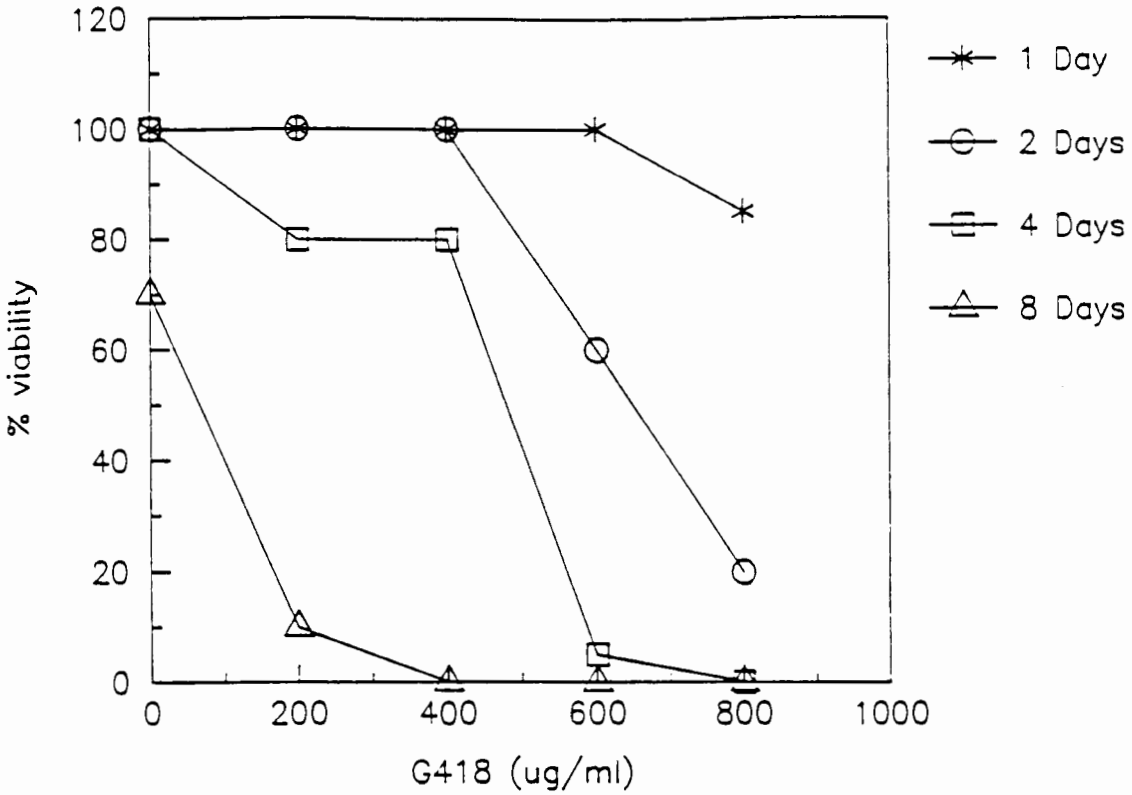

Fig 7.1: G418 sensitivity of hepatoma cells. Hepatoma cells in mid-log phase were replated at 10^5 cells per well in six well dishes in complete medium with 10% FCS. G418 at various concentrations was added and cell viability assayed after 1,2 4 and 8 days by trypan blue exclusion. The G418 used was only 50% by weight antibiotic. Single data points were used.

insufficient time was allowed for them to die. Subsequent experiments used 800 µg/ml G418. Care was taken to ensure that at this concentration the G418, which is acidic, was well buffered by the addition of HEPES buffer.

Targeted liposome delivery of DNA

Hepatoma cells were incubated at 37°C in suspension with increasing concentrations of targeted or untargeted DNA-containing liposomes, and then plated out and selected for G418 resistance as described in methods. After 10 days the number of resistant colonies in each well was scored, and the resistant frequency expressed as the number of colonies per 10^5 cells plated. The results (fig 7.2) show that untargeted liposomes do not promote the uptake and expression of their DNA, whereas targeted liposomes are able to promote the transformation of the cells, resulting in the appearance of colonies resistant to the antibiotic. Naked DNA, at comparable concentrations does not result in transformation, indicating that DNA alone is unable to enter the cells, and hence the liposome mediated transformation is indicative of internalisation of the liposomes.
This is in agreement with the observation (previous chapter) that untargeted liposomes do not bind to the cells to a significant extent, and targeted liposomes are able to bind to the cells and to deliver some of their contents into the cells.
The maximal frequency of transformation is 0.012%, or 12 colonies per 10^5 cells exposed to the liposomes. This frequency is attained with 2 µg of plasmid, corresponding to 400 nmol of lipid, and remains at a plateau, decaying slightly by 10 µg plasmid (2000 nmol lipid). Data on fluorescent liposome binding (previous chapter) show that maximal binding occurs with 100 nmoles of lipid, and since binding is probably a prerequisite for internalisation, one would not expect any increase in internalisation once

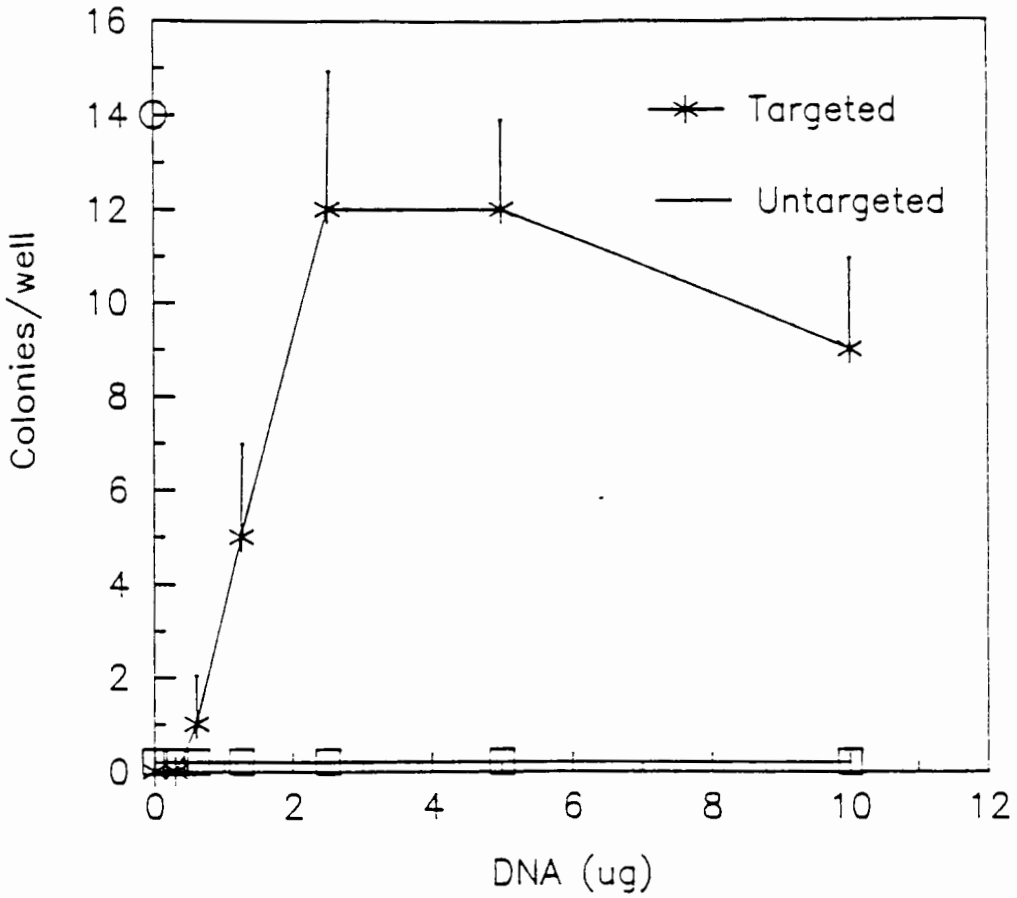

Fig 7.2: RTB-targeted liposome mediated transformation of hepatoma cells. Increasing quantities of pSV2-neo plasmid DNA in liposomes either targeted with ricin-B-chain or not targeted, was added to 10^5 cells and transformation detected as described in methods. Data points for untargeted liposomes are single experiments, for targeted liposomes the average of triplicates performed on a single day.

maximal binding has been obtained. When the data for transformation is plotted on the same graph as that for binding (fig 7.3), it appears that the liposomes which mediate transformation have a lower affinity for the cells than those carrying calcein. While a comparison of the two experiments is hampered by the different time periods used for the assays, since both liposomes were targeted with the same ligand, and the binding of the ligand to the liposomes was the same (data not shown), the presence of two different affinities suggests that either;

1) There are sites on the cell to which liposomes bind with a lower affinity than to other sites, and it is only from these sites that uptake leading to transformation is possible (because of internalisation avoiding a degradative pathway). Such sites may exist as areas sparsely populated with glycoproteins, and if the number of liposomes which bind to these is low, their binding will not be noticed by fluorescence above the very high background binding, hence it will not be seen in the fluorescence binding assay. Or,
2) Only a subpopulation of the liposomes are able to mediate DNA delivery because of their size, and likewise, because of their size, exhibit a slightly lower affinity for the cell. Thus the size of the liposome determines not only whether it is internalised or not, but also the route of internalisation, possibly avoiding lysosomes and hence delivering intact DNA to the nucleus.

Since all the cells bind targeted liposomes (as confirmed by fluorescence microscopy) and at least 5-10000 liposomes are bound per cell, representing 5-10000 plasmid molecules on the surface of every cell. However only 12 cells out of 10^5 actually incorporate the DNA into the genome and express it. This low frequency of transformation must be due to either;
1) Only a small population of the cells internalise the liposomal contents, or,

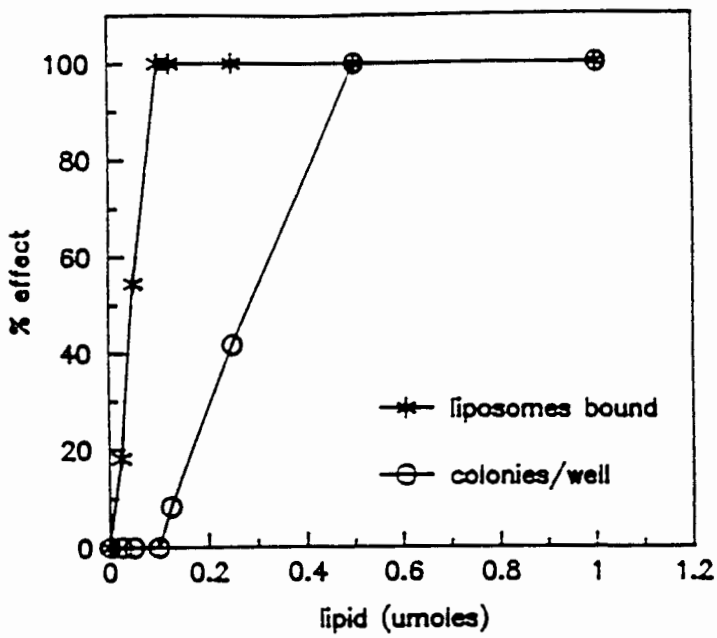

Fig 7.3: Comparison of binding and transformation data. The data for the binding of targeted fluorescent liposomes (fig 6.3) and for transformation mediated by targeted liposomes containing DNA (fig 7.2) expressed in terms of lipid present.

2) All of the cells internalise some of the liposomal contents, however only a small population are competent to stably insert and express the plasmid, or,
3) Insufficient DNA is being delivered into the cells, and in only a small proportion is the small amount delivered able to evade the activity of intracellular nucleases, and to reach the nucleus.

In order to ascertain whether the limiting frequency of 0.012% was due to insufficient DNA being delivered into the cytoplasm, two other transformation protocols were investigated with the same cells and the same DNA for comparative purposes.

Calcium phosphate precipitation

pSV2neo was transferred in Hepatoma cells using the method of Wigler et al. (1977) as modified and described in Current Protocols (1986). Contrary to claims in the literature that this is a technically simple technique, I found it to be highly variable, occasionally yielding no transformants. While no detailed investigation of the parameters was undertaken it appeared to depend on the batch of calcium chloride used, how rapidly the cells were dividing prior to transformation, and of course how long the precipitate was allowed to form.
The data presented in fig 7.5 represent the transformation frequency with increasing concentrations of DNA. This shows that the calcium phosphate technique yields fewer transformants than the targeted liposomes, and requires more DNA. Transformation mediated by liposomes is therefore superior in this case to calcium phosphate mediated transformation. It appears that there is no plateau, and

hence the limiting factor is likely to be the cytosolic delivery of the DNA.

Electroporation

Electroporation of cells makes them transiently permeable to macromolecules, permitting DNA to diffuse into the cell, and since such diffusion is independent of metabolic processes, is therefore a good indicator of the parameters limiting liposome mediated DNA transfer. The electroporation itself is subject to a variety of parameters, including cell diameter and resistivity of the medium, and hence the conditions for optimal electroporation need to be established for each cell line used. Fig 7.4 shows the effect of increasing the pulse voltage on the transformation frequency of the pSV2neo plasmid on the hepatoma cells. The optimal pulse voltage was 1200 V, and hence this was used for subsequent electroporation.

The efficiency of transformation by electroporation is shown in fig 7.5, which demonstrates that electroporation is able to transform the cells with a higher frequency than that achievable by targeted liposomes, yet still reaches a plateau, in this case 33 colonies per 10^5 cells transformed. This plateau could be due to limited competence of the cells, or to limited quantities of DNA entering the cell. Evident is that more transformations have occurred than for targeted liposomes, indicating that the cells are capable of being transformed at a higher frequency than that achieved by liposomes, and hence the plateau observed with liposomes probably represents a limit in the amount of DNA being delivered. Interestingly, at low concentrations of DNA, the liposomes mediate transformation at a slightly higher frequency than by electroporation, indicating that below

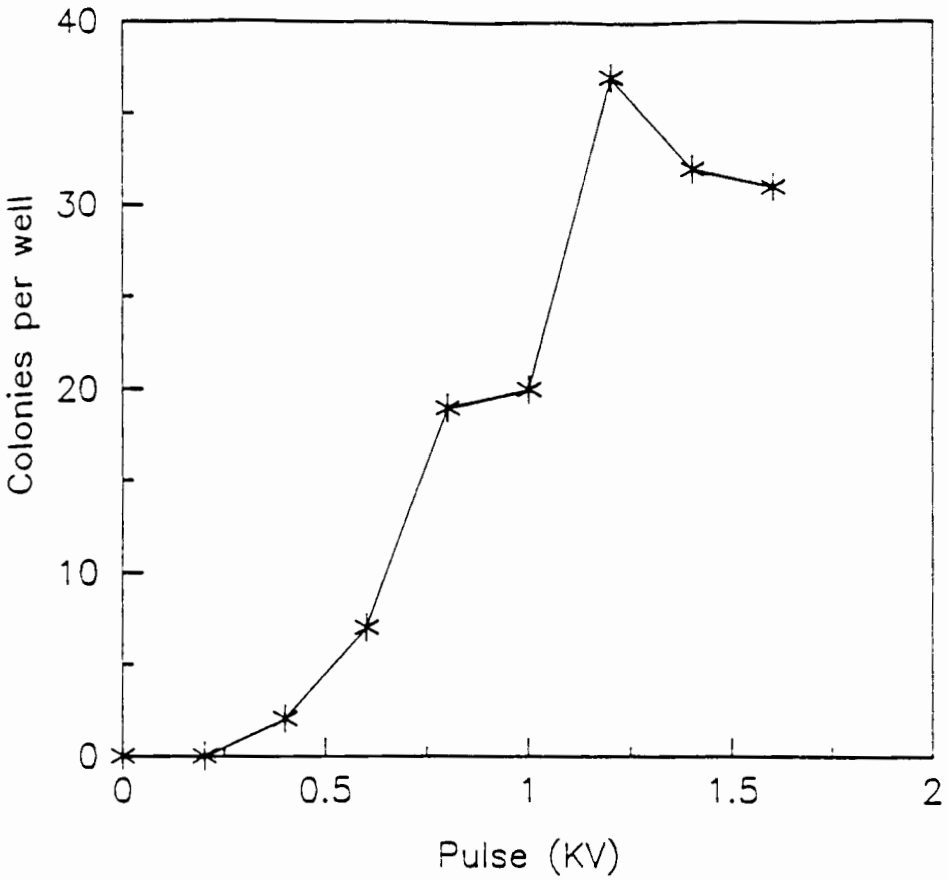

Fig 7.4: Effect of pulse voltage on the transformation frequency of pSV2neo on hepatoma cells. 40 µg of linearised plasmid was added to 5 X 10^6 cells in PBS at 4°C and pulsed once at the voltage shown. The conditions of electroporation and selection for transformants are described in methods. Data points are from single experiments.

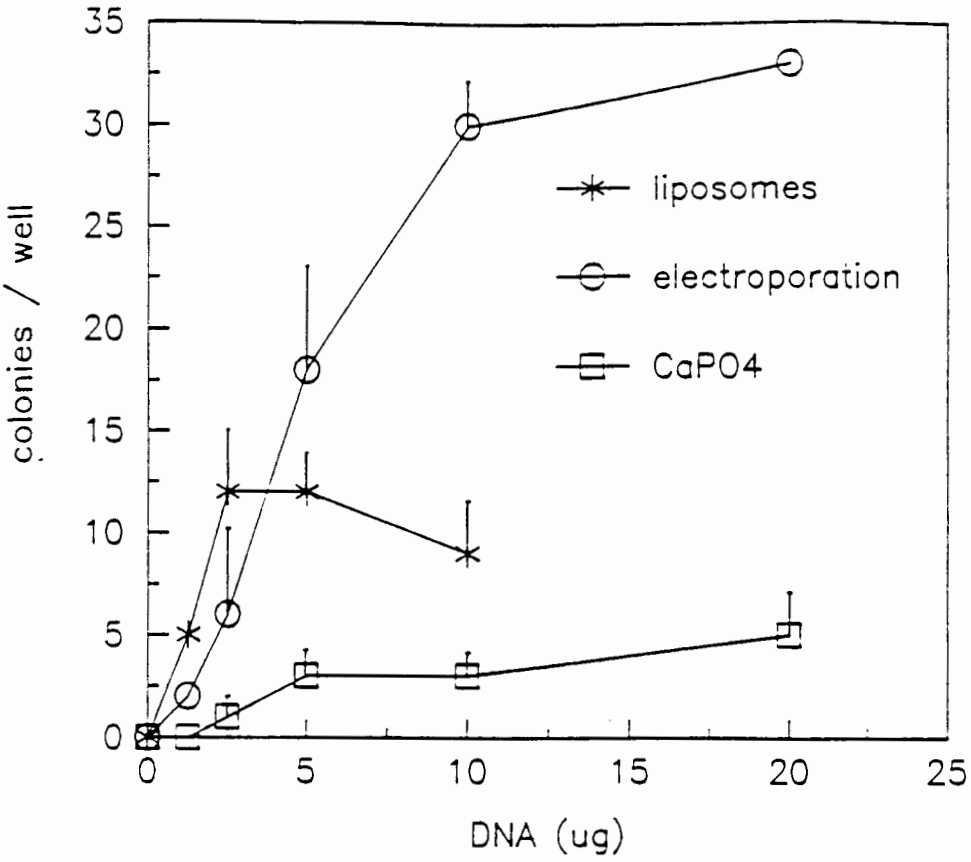

Fig 7.5: Comparison of transformation frequencies for liposome mediated transformation, electroporation, and calcium phosphate mediated transformation. Data points are average of triplicates.

saturating levels, the delivery by targeted liposomes is more efficient than by electroporation.

Comparison

The same plasmid has been delivered into the same cells by three different techniques, and the results are widely differing, probably representative of the different modes of uptake of the DNA in the three methods. While electroporation mediated delivery is independent of the cells metabolism and promotes the delivery of macromolecules directly into the cytoplasm, cellular uptake of calcium phosphate precipitates requires an endocytotic process which probably results in much of the DNA being degraded by lysosomal enzymes, resulting in a much lower frequency of transformation. The liposomal delivery is probably due also to an endocytotic mechanism, although fusion of some the bound liposomes with the plasma membrane is a possibility. That it is more efficient than calcium phosphate could be due to an alternative endocytotic route, and also protection of the DNA in liposomes from nucleases, and its better efficiency at low concentrations than electroporation reflects the concentrating effect of binding the liposomes to the cells, effectively increasing the local concentration.

That transformation has occurred demonstrates conclusively that there has been liposome mediated transfer of the DNA into the cells. The absence of transformation with untargeted liposomes further demonstrates the requirement of strong interaction of the liposomes with the cells. However while it demonstrates delivery, it is not clear that it is either 1) an efficient method of transformation, or,

2) a quantitatively accurate assessment .

Efficiency : While optimal transformation frequency was observed with 2 µg of DNA for the liposome mediated transformation, whereas 4 µg were required for the same frequency by the electroporation procedure, it must be remembered that the preparation of the 2 µg sample of liposomal DNA required 10 µg of DNA to start with. Furthermore the DNA for electroporation can be stored indefinitely in the frozen state. Liposomes require to be frozen in the presence of cryoprotectants (Machy and Leserman 1984) and even then undergo some leakage on freezing. Finally, the skill and number of steps required in preparing targeted liposomal DNA is far greater than for the electroporation procedure. Hence the liposomal procedure is not more efficient than the electroporation procedure.

Quantitative validity : Greassmann and Graessmann (1975) have demonstrated that the infectivity of microinjected DNA is directly proportional to the quantity of injected DNA. This would then suggest that the plateau of transformation frequency observed for both liposomal and electroporation mediated transformation is due to a limit being reached in the number of DNA molecules entering the cells. This is more readily apparent for the liposomal transformation, where the plateau probably correlates with a maximum internalisation rate being reached, however the plateau for electroporation is not readily explained. Since electroporation yields the cells transiently permeable to macromolecules, the quantity of DNA entering the cell should be proportional to the concentration of DNA outside the cell, in which case the plateau is due to a limitation being imposed on the stable insertion and expression of the DNA. This suggests that factors other than total quantity of intracellularly delivered DNA affect transformation frequency, hence quantitative assessment of liposomal delivery based on transformation frequencies is only valid within a linear

range established by alternative techniques such as electroporation or microinjection.

Such a condition does apply to the above experiments, so a quantitative assessment may be valid:

Assuming that electroporation makes the cell transiently totally permeable to the DNA, then after diffusion the concentration of DNA in the cell will be identical to the concentration in the medium. The concentration of DNA which would yield 0.012% transformation in the electroporation method is approximately 4 μg of DNA per ml. If the diameter of a Hepatoma cell in suspension is 15 μm (see micrographs of liposomes bound to cells), then the volume of the cell is 1766 μm^3. If the concentration of DNA in the cell is 4 μg/ml, then 7 X 10^{-15}g of DNA is present in the cell, representing approximately 800 molecules. Since there is roughly one plasmid per vesicle, this transformation efficiency (which is the optimal for liposomal delivery) represents the cytosolic delivery of 800 liposomes. This is a large number of liposomes, and yet only 12 transformants occurred, indicting the very low probability of foreign DNA becoming stably inserted and expressed in the genome.

The assumption made that after electroporation the concentration inside is equal to that outside is empirical, based on the report that at critical voltage the increase in membrane conductance corresponds to loss of up to 0.1% of the membrane area (Kinosita et al. 1988) which accounts for a large area of pores, and such pores persist almost indefinitely at 4°C (Kinosita and Tsong, 1977). Since the cells were kept at 4°C for 10 minutes after poration the pores will have persisted for that time, allowing diffusion of DNA into the cell. Taking the diffusion coefficient of the plasmid as being 2 X 10^{-7} cm^2s^{-1} (based on BMV Cantor and Schimmel), in water at 20°C it will diffuse 6.3 μm in 1

second, roughly the radius of the cell. Hence the limitation to the DNA entering the cell is imposed by the size and number of the pores. From fig 7.4 it is evident that increasing the pulse voltage beyond 1.2 KV does not increase the transformation frequency. Since a higher voltage should increase the pore size or number, and hence the rate of diffusion, it is likely that maximal DNA internalisation has been achieved by a pulse of 1.2 KV, and that this represents equilibration with the external medium.

A further complication in the assessment is that 30 - 40% of the cells electroporated died due to electroporation, not G418 sensitivity. Had these cells not died they would have contributed towards an increased frequency of transformation, lowering the estimation of liposome internalisation.

Conclusion:

The ricin-B-chain is able to promote the binding and internalisation of liposomes to hepatoma cells, mediating the delivery of foreign DNA to the nucleus where it becomes expressed. As a procedure for delivering foreign DNA it is superior to calcium phosphate precipitation, but inferior to electroporation. Further, the wastage of material during liposome preparation and storage, as well as the technical difficulties, make electroporation in this case a far superior method.

Maximal transformation was obtained when the lipid concentration was 400 μM, at which concentration an estimated maximum of 800 liposomes per cell had delivered their contents into the cytoplasm. In comparison to this, methotrexate delivery was optimal at 50 μM lipid (previous chapter). At 50 μM lipid no transformation could be detected, therefore although this data demonstrates that at

high concentrations internalisation of the liposomal contents does occur, it is still not possible to determine whether the toxicity of methotrexate in targeted liposomes was due to liposomal delivery or leakage at the membrane.

Thus, liposome mediated transformation unequivocally demonstrates the internalisation of the liposomal contents, however as an assay for liposomal delivery it is hampered by the very low probability of scoring a 'hit', due to the very low probability of the internalised DNA becoming inserted in the genome and being expressed. The low frequency of positive responses could very well lead to situations in which liposomal uptake is not detected, and hence as a routine assay for liposomal delivery it is not ideal. Furthermore it is hampered by technical details, requiring that all solutions are DNase free, the liposomes are sterile at all times, and ten to fourteen days are required to select for transformants.

Because of these problems I tried to develop an alternative assay for liposome mediated delivery which;
1) Was more sensitive than transformation assays and less dependant on unknown factors such as insertion and expression.
2) Was more rapid, ideally giving results the same day.
3) Did not require such strict maintenance of sterility, freeing laminar flow hoods for other research.
4) Did not use such large quantities of precious materials such as plasmids.
5) Would provide unequivocal and hopefully quantitative demonstration of liposomal delivery.

The development of such an assay is described in the next chapter.

Chapter 8

TARGETED LIPOSOME DELIVERY OF DNASE1 INTO CELLS

The methodologies available for ascertaining the successful intracellular delivery of liposomal contents often present problems ;

1) Fluorescence microscopy is a good indicator of whether liposomes have bound, but in my experience it has been difficult to differentiate between surface bound liposomes and liposomes entrapped within intracellular vesicles, and the appearance of diffuse fluorescence, normally associated with cytoplasmic delivery, I have found can be confused with out-of-focus punctate fluorescence, especially at high magnification (narrow depth of focus) and when a large number of liposomes have been bound.

2) The encapsulation of small drugs such as methotrexate, and monitoring the resulting toxicity is not ideal because the drug is able to enter the cell unaided, hence leakage from the liposomes can present difficulties in interpretation of the results, and if extensive exposure of the cells to the liposomes is required, the time period magnifies this difficulty. Modifying the drug so that the free drug is unable to enter the cells (eg methotrexate-gamma-aspartate Heath et al. 1983) improves this, however since small drugs are often resistant to lysosomal inactivation, successful delivery of a small drug is not correlateable to that of a macromolecule.

3) The delivery of transfecting DNA into cells, while overcoming the above drawbacks, is hampered by the apparent lack of direct correlation between cytosolic delivery and transformation efficiency, the latter being affected by a

variety of factors controlling the ability of the transforming DNA to be delivered into the nucleus, insert into the genome, to remain there, and to be expressed. A slight technical drawback is the length of time required for positive selection of the transformed cells (typically 10 to 14 days). Transient expression of exogenous DNA is likewise affected by factors other than the total cytosolic delivery, including its topology (Weintraub et al. 1986) and its association with factors such as nuclear proteins (Kaneda et al. 1989).

An ideal assay system for liposomal delivery would involve the delivery of a macromolecule that was unable to enter the cell unaided, and that exerted an enzymatic effect on a substrate within the cell, rapidly producing an easily identifiable product. Such a system would hopefully permit the detection of low numbers of liposomes and within a time frame permitting an analysis of the kinetics of uptake.

An example of such a molecule is DNase1. It is unlikely to be able to enter the cell by itself, hence leakage from liposomes will not interfere with the results, and once in the cell is small enough to gain access to the inside of the nucleus where a large quantity of substrate in the form of the genomic DNA is available, and the product, smaller DNA, should be readily discernible from the starting material. Furthermore, unlike toxicity or transformation assays, the assay would not require the cell to be actively metabolising or dividing, hence avian erythrocytes or similar non-dividing nucleated cells could be used.

DNase1

The interaction of bovine pancreatic DNase (DNase 1, EC 3.1.4.5) with isolated chromatin or with intact nuclei has been investigated in detail since it is one of the principle probes for the determination of chromatin structure (Lutter 1978, reviewed Felsenfeld 1978). Partial digestion of either isolated chromatin or intact nuclei with DNase1 and analysis of the resulting fragments by gel electrophoresis yields a 'ladder' of discreet bands corresponding to multiples of monomers of approximately 200 base pairs of DNA. This arises from the preferential digestion of the unprotected DNA in the spacer region between nucleosomes, with the DNA of the nucleosome being less susceptible to attack. Continued digestion results in the dissapearance of higher multiples and a dominance of monomers or nucleosome cores, and finally even those are digested away.

While minute quantities of DNase 1 are able to achieve digestion of chromatin in isolated nuclei, the presence of inhibitors of DNase 1 present in eukaryotic cells is likely to significantly reduce the activity of cytoplasmically delivered enzyme. Actin has been shown to have a very high affinity (K_D = 2 X 10^{-7} M) for bovine DNase 1 (Mannherz et al. 1980), forming a 1:1 complex with the enzyme and inhibiting its activity (Hitchcock et al. 1976). It is unlikely that such an interaction is of significant physiological importance because DNase 1 from rat tissue is not inhibited by actin (Lacks 1981). Because there is a finite amount of actin within a cell it may be necessary to deliver enough DNase to bind to the actin before any digestion of the DNA is able to take place, this may significantly decrease the sensitivity of the assay.

DNase1 has previously been encapsulated in liposomes and shown to exert an intracellular effect by inducing dose-dependant cytotoxicity and somatic mutations in cells exposed to the liposomes (Zajak-Kaye and Ts'o 1984). While this experiment demonstrates that liposome mediated delivery of DNase1 into cells is possible, and the delivered enzyme is able to digest the cellular DNA, the assays used required extensive incubation times. Toxicity was measured after 7 days, and phenotypic expression of the neoplastic transformation required incubation for 28 days. These authors did note that at low concentrations of liposomes there was no noticeable effect on the cells, a phenomenon ascribed to scavenging of the DNase by actin. While it would be useful to gauge from this paper how much enzyme was delivered and by how many liposomes, very severe inconsistencies within the report concerning the ratio of enzyme to lipid, the percentage encapsulation and even the molecular weight of the enzyme preclude this. Nonetheless, since these authors used LUV vesicles with a reported low (2%) efficiency of encapsulation, and binding of the liposomes was mediated by the negative charge on the lipid, it is possible that lectin mediated targeting of REV vesicles may result in superior delivery.

The experiments described below were initiated to ascertain whether lectin-targeted liposomes could mediated the delivery of DNase1 into cells, and whether such delivery could be assayed by monitoring the appearance of short DNA fragments on agarose gels, and if so, whether this technique could be used as a rapid, unequivocal assay for liposomal delivery.

DNase 1 entrapment

The entrapment of DNase1 into reverse phase evaporation liposomes without losing activity, and the subsequent analysis of the activity of the entrapped material presented several problems which required the development of new techniques. One of the main problems was that release of the entrapped enzyme by detergents resulted in inactivation of its enzymatic activity. This was overcome by using melittin, a lytic peptide from bee venom, to lyse the liposomes. The techniques used and the results obtained are described in detail in appendix B.

It was found that entrapment of biologically active DNase 1 in REV liposomes was possible only if the aqueous phase contained 20% glycerol, and that under these conditions, the process of encapsulation significantly increased the activity of the enzyme. The increase in activity appeared to be due to the presence of the lipid, hence an exact determination of encapsulation was not possible, but it appeared that at least 20% of the DNase 1 activity became associated with the liposomes, corresponding to 50 Kunitz units of enzyme per μmol lipid.

Liposome mediated delivery of DNase1 into cells

In order to ascertain whether functional delivery of the DNase 1 could be achieved by the liposomes, and the time period required to detect the appearance of DNA fragments, cells were incubated with either free DNase 1, or DNase1 entrapped within RTB-targeted liposomes. Incubation was performed at 37°C for increasing time periods, after which the reaction was terminated by the addition of EDTA and phenol. The EDTA served to chelate any divalent ions present which effectively stops all DNase 1 activity, and the phenol

served to solubilise the cells and liposomes, and to immediately denature the proteins, providing protection from further nuclease activity, and also releasing DNA from the chromatin for subsequent isolation. The DNA was isolated and purified from the cells as described in methods, and then applied to an agarose gel to ascertain whether any cleavage of the chromatin had occurred. The results are shown in fig 8.1.

This demonstrates that free DNaseI is unable to enter the cell, whereas liposomally targeted DNaseI is able to enter the cell and pass into the nucleus where it digests some of the high molecular weight chromosomal DNA into shorter pieces, appearing as a smear on the gel. Thus, functional delivery of the liposomal contents is unequivocally demonstrated.

It is interesting that the digestion of the DNA only becomes noticeable after 60 minutes of incubation. The virtual absence of digestion after 30 minutes is due presumably to the complex kinetics of liposome internalisation or fusion, saturation of possible inhibitors such as actin, diffusion into the nucleus, and the kinetics of DNase 1 activity on DNA. The latter is likely to introduce a lag period in the appearance of DNA fragments due to its mechanism of action; DNase 1 cleaves DNA by nicking single strands, hence, at low concentrations of the enzyme there is a time lag before both strands get nicked at the same site, producing a fragment.

Although fig 8.1 shows that the internalisation of the DNase 1 has been mediated by the liposomes, it is possible that this internalisation is not a function of the targeting of liposomes, but simply due to association of the enzyme with liposomes, enhancing its activity. Likewise the absence of digestion with free DNase 1 is not directly comparable because the encapsulated enzyme has a much higher activity.

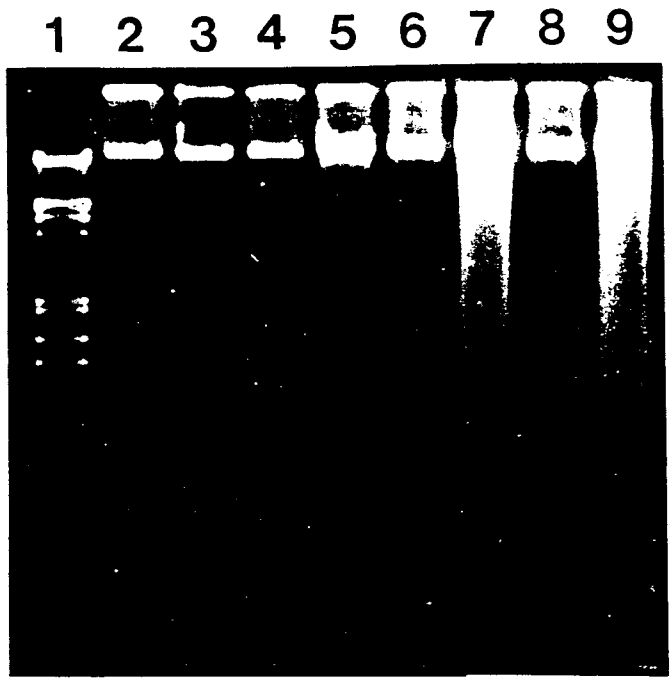

Fig 8.1:
1% agarose gel of DNA isolated from hepatoma cells incubated with either DNase1 or RTB targeted liposomal DNase1. Hepatoma cells in mid-log phase were released from monolayer growth with EDTA and resuspended in PBS/Ca/Mg at 2.5 X 10^5 cells/ml. The liposome concentration was 500 nmol/ml which corresponds to a DNase 1 concentration of 20 µg/ml. Free Dnase 1 was 20 µg/ml.
Lane 1 : Lambda standard. Lanes 2,4,6,8 ; cells incubated with free enzyme, reaction terminated after 0, 30, 60, 120 minutes respectively. Lanes 3,5,7,9 ; cells incubated with liposomaly entrapped enzyme and reactions terminated after 0, 30, 60, 120 minutes respectively.

To confirm that the digestion above was due to release of the liposomal contents into the cell, and that this release was due to the targeting of the liposomes, cells were interacted with either targeted liposomes, targeted liposomes and lactose, or untargeted liposomes. In all cases the liposomes contained DNasel. Free DNase 1 at a ten fold higher concentration than previously was also incubated with cells. The results are shown in fig 8.2.

This demonstrates that neither untargeted liposomes, nor RTB targeted liposomes in the presence of lactose, are able to significantly promote the delivery of DNasel into the cell. It also further confirms that free DNasel is unable to enter the cell within the time period of the experiment, and hence the liposomal delivery must be due to liposome mediated internalisation of the enzyme.

Since it has been shown (chapter 5) that there is some binding of RTB-liposomes to cells in the presence of lactose, an event ascribed to increased non-specific interaction of the liposomes with the cells, the absence of delivery of DNase 1 in the presence of lactose (fig 2 lanes 3,6) suggest that the internalisation of the contents of the RTB targeted liposomes is dependant on the sugar binding properties of the lectin. There are two components within this argument: the first is that in the presence of competing sugar the total binding is significantly less (10% of the binding in the absence of lactose) and hence there is less DNase 1 available for internalisation; and the second is that the binding is mediated via an alternative mechanism which may not be capable of mediating uptake.

Since DNase 1 is normally stored in the lysosomal apparatus, from which its escape would be detrimental to the cell (Sablina 1978), it is unlikely that any lysosomally delivered DNase 1 is subsequently able to escape into the cytoplasm. Hence the nuclear delivery of DNase 1 suggests that internalisation of the liposomal contents via a route other than the lysosomal pathway has occurred. The event most likely to mediate such delivery is fusion of the bound liposomes with the plasma membrane.

Although fusion of phosphatidylcholine vesicles with cells has not previously been demonstrated (as discussed in chapter 1), it is conceivable that targeting via the ricin-B-chain is able to mediate fusogenic events as well as endocytosis. Such an effect may be a result of both the structure of the ricin-B-chain, and also the widespread distribution of galactosyl residues on the cell surface, potentially resulting in multimeric binding of a single liposomes which would then undergo distortion and concomitant thermodynamic stress, making fusion of the liposome with the plasma membrane a more likely event.

Liposome-liposome fusion has been shown to be significantly enhanced when the liposomes are brought into close proximety by agglutination of glycolipid containing vesicles with *Riccinus communis* agglutinin (Sundler and Wijkander 1983), indicating that this lectin is capable of establishing interbilayer contact. It is therefore quite conceivable that the ricin-B-chain, a very similar lectin from the same source and with the same sugar specificity, is able to mediate a similar effect. To this extent it is worth considering that lectin mediated targeting is capable of binding the liposomes directly to glycolipids within the plasma membrane, potentially establishing much closer proximety of the two membranes than antibody mediated targeting, which binds the liposomes to proteins on the cell

surface. Hence lectin mediated targeting may be able to promote fusion to a far greater extent than antibody mediated targeting of liposomes.

Such a mechanism may also explain the disparity between binding and transformation in the previous chapter. If cytoplasmic internalisation of the bound liposomes is mediated primarily by binding close to the membrane surface, since such sites are less available than large surface glycoproteins, the binding will be of a lower affinity and hence more liposomes will be required to achieve maximal internalisation than maximal binding.

It would be interesting to obtain an estimate of the quantity of enzyme that has been delivered to the nucleus. Since a simple radioactive labelling of the enzyme and subsequent isolation of the nuclei and assessment of associated radioactivity would be hampered by leakage out of the nucleus during nuclei isolation, and also the binding of DNase 1 to proteins such actin which will co-purify with the nuclei, the best method is to perform parallel digestions under conditions in which the concentration of enzyme in the nucleus is known. For this reason nuclei isolated from hepatoma cells were incubated with increasing quantities of DNase1 for 120 minutes at 37°C, and the extent of digestion of the chromosomal DNA determined by the same method as for whole cells. The nuclei for this experiment were isolated by the method of Smith et al. (1988) which avoids the use of detergents and harsh conditions, and produces nuclei transcriptionally active nuclei.

Fig 8.3 shows that increasing the DNase1 concentration results in increasing quantities of digestion until all of the large molecular weight DNA is degraded. While a quantitative comparison of this gel with figs 8.1 and 8.2 is

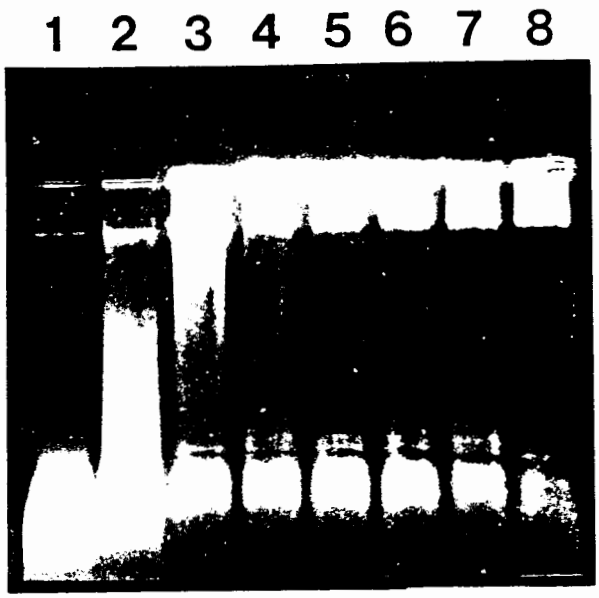

Fig 8.3:

1% agarose gel of DNA from isolated hepatoma nuclei incubated with increasing quantities of DNasel at 37 degrees for 120 minutes. 2.5 X 10^5 nuclei were resuspended in 100 µl of nuclei storage buffer containing 10 mM Mg^{++} and various quantities of DNase 1 expressed as Kunitz units;

Lane 1; 4 Units.

Lane 2; 2 Units.

Lane 3; 1 Units.

Lane 4; 0.5 Units.

Lane 5; 0.2 Units.

Lane 6; 0.1 Units.

Lane 7; 0 Unit.

Lane 8; 0 Unit.

not possible, a qualitative assessment shows that digestion of the nuclei with 1 unit of enzyme yields similar digestion to that obtained by targeted liposomes in the same time period. Assuming that in the nuclei digestion the concentration of enzyme in the nuclei is equal to that in the solution, then a concentration of 10 U/ml achieves digestion similar to that experienced by the nuclei in the cells. It can then be further argued that the concentration of DNase 1 in the cells after targeting is at least 10 U/ml. Since the cell has a diameter of 15 μm, its volume is 1.7×10^{-12} l, which at 10 U/ml is equivalent to an intracellular 1.7×10^{-8} U of enzyme. It is possibly greater than this since the potential inhibition of the enzyme by actin may mask some activity. This corresponds to approximately 400 of the liposomes having delivered their contents into the cytoplasm. Interestingly this figure is within the same order of magnitude as that estimated by liposomal mediated delivery of transfecting DNA (chapter 7), and yet is two orders of magnitude lower than that quoted by Zajac-Kaye and Ts'o (1984). As mentioned previously, the report by these authors does contain contradictions preventing any numerical assessment of the binding, but they do quote a figure of 10^7 molecules of DNase 1 having been internalised per cell. The RTB mediated delivery of 400 liposomes per cell corresponds to 1.2×10^5 molecules per cell, significantly less than that found by these authors.

As a comparison to assess whether this technique worked with other cell types, and also to determine the dependence of the digestion on liposomal concentration, Friend erythroleukemia cells were incubated for 120 minutes with increasing quantities of targeted liposomes containing DNase1. Analysis of the digestion of the DNA is shown in fig 8.4.

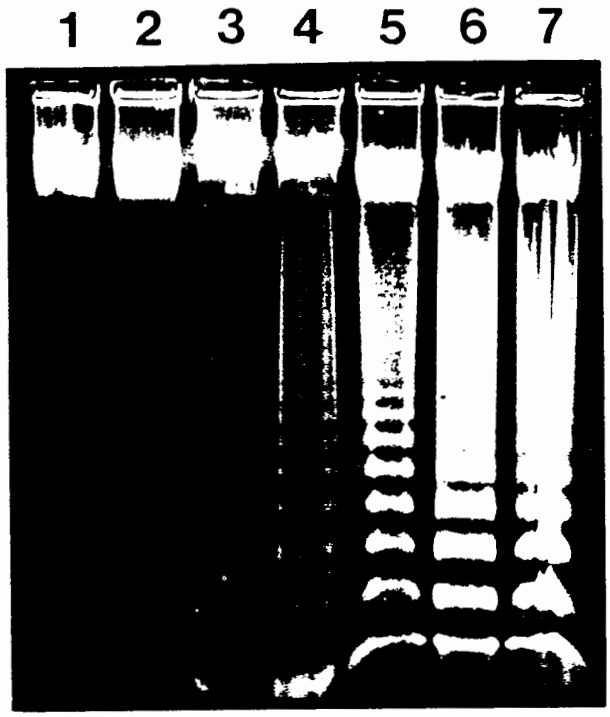

Fig 8.4:
1% agarose gel of DNA isolated from Friend cells incubated with increasing quantities of targeted liposomal DNase1 at 37 degrees for 120 minutes. 2.5 X 10^5 cells in 1.0 ml RPMI medium containing 10% FCS were incubated with:
Lane 1; No liposomes added.
Lane 2; 30 nmoles liposomes
Lane 3; 60 nmoles liposomes
Lane 4; 125 nmoles liposomes
Lane 5; 250 nmoles liposomes
Lane 6; 500 nmoles liposomes
Lane 7; 1000 nmoles liposomes.
DNA was isolated and analysed as described in methods.

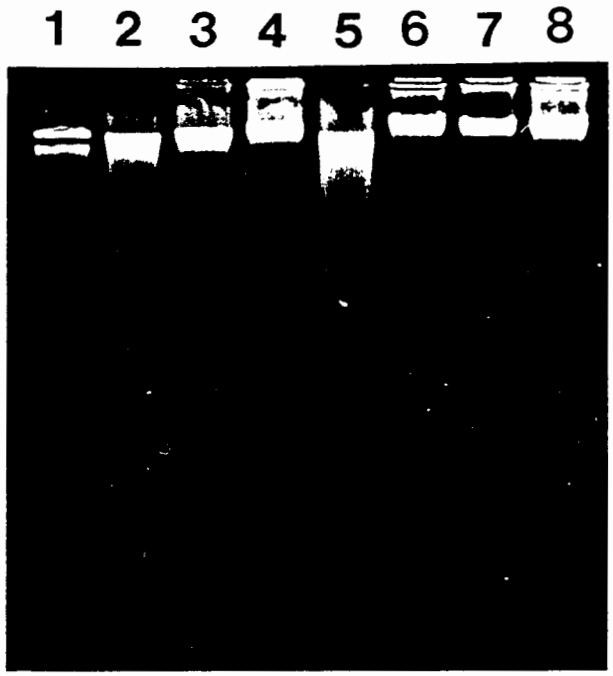

Fig 8.2:
1% agarose gel of DNA isolated from hepatoma cells incubated with 500 nmol of liposomes (containing 20 µg DNase1) or 200 µg of free DNase 1. Incubation was performed in EMEM medium with 10% FCS for 60 or 120 minutes at 37°C.
Lane 1: Lambda standard.
Lanes 2,3,4 ; 60 minutes incubation. Lanes 5,6,7,8; 120 minute incubation.
Lanes 2,5 ; RTB targeted liposomes.
Lanes 3,6 ; Targeted liposomes in presence of 50 mM lactose.
Lane 4,7 ; Untargeted liposomes.
Lane 8 ; Free DNase1 .

There is slight digestion of DNA in the absence of liposomes (lane 1), due probably to the presence of some dead cells and cell debris in the sample of Friend cells which grow in suspension. This does not occur with the Hepatoma cells where any dead cells are removed when rinsing the monolayer with medium prior to harvesting the cells.

30 nmoles of liposomes (lane 2) does not promote a significant increase in digestion over the control, indicating that insufficient enzyme reaches the nucleus to exert an effect. This could be due either to no liposomes delivering their contents into the cell, or to the binding of any delivered enzyme by actin. It is difficult to differentiate between the two possibilities. Since binding occurs at 30 nmoles of lipid, failure of the bound liposomes to mediate internalisation may represent the location of binding not being permissive for uptake. To this extent it is conceivable that initially the binding of the liposomes to the cells is via an exposed glycoprotein, which may result in the liposome being kept away from the plasma membrane hence minimising the chance of internalisation. Higher concentrations possibly result in binding to the less exposed glycoproteins and glycolipids decreasing the intermembrane distance and increasing the probability of internalisation through either an endocytotic or fusion mechanism.

Increasing the concentration of liposomes results in significant increases intensity of the degraded DNA, pointing to increased nuclear fate of the liposomal contents. The digestion appears to plateau at 500 nmoles of phospholipid added, similar to the plateau observed for the delivery of transforming DNA, and may indicate a saturation of binding being reached. Since the binding to Friend cells should be similar to that for hepatoma cells, the apparent plateau of delivery at 500 nmoles tends to confirm the

previous postulate that the binding that mediates cytosolic delivery of the liposomal contents occurs with a lower affinity than the bulk binding.

Fig 8.4 shows very clearly a 'ladder' of digestion, resulting from preferential digestion of the linker DNA between nucleosomes. Such a digestion pattern is expected from DNA packaged into nucleosomes (Felsenfeld 1978), hence its absence in the digestion of hepatoma cells is intriguing. That there is no evidence of a repeat length in the digestion of isolated hepatoma nuclei demonstrates that this is not an artifact of liposome mediated delivery of nuclease, and points therefore to the possibility that the difference between the digestion pattern of the two cell types is a function of the DNA packaging. To this extent the Friend erythroleukemia cell, which is almost fully differentiated, is likely to have less DNA actively transcribing than the hepatoma cell, and concomitantly, a more tightly packaged chromatin.

Comment on the methods

Several different methods were compared for their use in stopping the reaction of the DNaseI with the DNA and subsequent isolation of the DNA for size analysis on agarose gels. The method finally employed is described in detail in the methods section. It comprises stopping the reaction with phenol, followed by two phenol extractions, ether extraction to remove the phenol, RNase digestion to degrade the RNA which otherwise interferes significantly, and an optional ethanol extraction, depending on the concentration of the DNA. This method was chosen because it is rapid, the entire procedure including running the gel achievable in a day, however it is difficult to obtain reproducible quantities of

DNA during the phenol extractions because the solution is so viscous and the DNA is not readily separated from the protein interface. An alternative approach (also described in methods) more suited to analysing large numbers of samples is to stop the reaction with an EDTA/SDS/proteinase K solution at 50 degrees, and to incubate at this temperature for 16 hours followed by RNase digestion. Subsequent phenol extraction and ethanol precipitation is not interfered with by large quantities of protein. The disadvantage of this approach is that it requires at least 2 days, and since larger volumes are used, ethanol precipitation is mandatory. Quantitative resuspension of the precipitated DNA is dependant on the DNA being very clean and being only air-dried after precipitation.

Conclusion

The entrapment of DNase1 in liposomes, and the isolation of DNA from cells interacted with the liposomes provides an unequivocal and sensitive assay of cellular delivery of the liposomal contents. The assay is rapid, permitting many variables to be investigated within a short time period and furthermore the time period of the assay obviates the necessity for ultra-sterile working conditions and the concomitant expense.

The ability to deliver nucleases directly into living cells may have great impact on the field of chromatin structure, where isolated nuclei are at present the best substitutes available as models of the living cell. Since the process of isolating the nuclei may produce rearrangement of the chromatin, a process in which the enzymatic digestions are performed *in vivo* will provide a less artifactual assessment of DNA arrangement. Further, the ability to digest the DNA while it is actively transcribing within the cell may result in a more detailed understanding of the process of packaging

and expression of active genes. To this extent targeted liposome mediated delivery of DNase 1 provides not only an assay of liposome effectiveness, but also a powerful tool for molecular biologists.

Chapter 9

CONCLUSIONS

It has been demonstrated that melanocyte stimulating hormone is able to mediate the binding of liposomes to melanoma cells via the receptor for the hormone, and yet although binding occurs, there was no evidence of internalisation of the liposomal contents. Since the hormone was able to mediate both the binding and internalisation of ricin-A-chain resulting in increased toxicity towards melanoma cells, it is apparent that binding of the hormone to the receptor does mediate endocytosis. Hence the absence of functional delivery of the liposomal contents could be due to either too few liposomes being internalised and the assay being insufficiently sensitive to detect such levels, or to the large size of the liposomes preventing their internalisation via the MSH receptor.

To provide a comparison to this, liposomes were targeted with ricin-B-chain, a lectin known to mediate the internalisation of molecules to which it is bound. This ligand was able to mediate both the binding and internalisation of the liposomes, functional delivery being demonstrated by the cellular uptake of liposomally entrapped methotrexate, transfecting DNA, and also DNase 1.

Whereas MSH mediated targeting resulted in the specific binding of at most 1000 liposomes per cell, the lectin mediated targeting yielded much greater binding, with saturation at 11000 liposomes per cell. The saturation levels for both are far below the receptor number, and for the lectin mediated binding appear to be due to a physical limit being reached beyond which no more liposomes are able to pack on the cell surface. In contrast, the binding via the MSH receptor appears to be limited due to the localised

distribution of the receptor, and the subsequent masking of binding sites by liposomes.

The ability of the two ligands to mediate the internalisation of liposomally entrapped methotrexate is vastly different. Even when the specific binding via MSH is saturated, at which point those cells expressing the receptor bind approximately 1000 liposomes, no cellular toxicity occurs. Lectin mediated targeting on the other hand results in the delivery, and concomitant toxicity, of the drug far below the saturation level. Half maximal toxicity occurs at a liposome concentration which results in 2500 liposomes binding per cell, and represents a ten fold increase in toxicity over untargeted liposomes. Although this indicates functional targeting, it indicates that a large number of liposomes do need to be attached to the cell for toxicity to occur, and hence the absence of toxicity of MSH targeted liposomes could be due to insufficient liposomes binding to the cell. This explanation becomes more credible when one considers that the MSH mediated binding occurs for only 30% of the cells life cycle and that at any moment the cells bind on average only 300 liposomes per cell. The concentration of ricin-B-chain targeted liposomes that mediate a comparable binding (300 liposome per cell), does not result in any toxicity.

It is therefore concluded that melanocyte stimulating hormone is not a good ligand by which to target liposomes to cells even though the receptor expresses a high affinity for the hormone, does mediate endocytosis of the hormone, and the hormone can be linked to liposomes without concomitant loss of recognition by the receptor. It is also concluded as a general rule that receptor expression and density will play a critical role in functional delivery of targeted liposomes. While the MSH receptor is expressed at a density of approximately 10^4 per cell, which should mediate binding

comparable to that for ricin-B-chain, its localised distribution masks most of the receptors, making them unavailable for liposome binding, and its cell-cycle dependant expression results in only a fraction of the cells at any moment binding the liposomes.

In contrast to the delivery of a small drug such as methotrexate, the ricin-B-chain mediated delivery of liposomally entrapped nuclease or transfecting DNA requires saturation of the binding sites. Since maximal delivery of the liposomal contents occurs at a concentration of liposomes higher than that which results in maximal binding, it is concluded that the binding that mediates the nuclear delivery of the liposomal contents occurs with a lower affinity than the bulk binding. It is possible that nuclear delivery of the liposomal DNA or nuclease results primarily from fusion between the liposomes and the plasma membrane, in which case only the binding events which bring the two membranes into close proximity will result in functional delivery of the contents. To this extent the binding of the lectin to glycolipids in the plasma membrane may be the primary route of delivery of liposomal contents which would probably be degraded within an endocytotic route, and such binding may occur with a lower affinity than that to the more exposed glycoproteins.

It is concluded therefore that the ricin-B-chain is a useful ligand ~~by~~ which to promote the binding and rapid cytosolic or nuclear internalisation of liposomes, and as such may prove to be a valuable tool in experiments requiring intracellular delivery of biological agents.

Chapter 10

MATERIALS AND METHODS

Materials

The reagents used were supplied as follows :

Merck (Darmstadt) - Acrylamide, DMF, TEA, TFA, Hydrogen Peroxide, Silica F 254 plates for TLC.

Sigma (St. Louis, Mis., USA) Calcein, DPPC, DPPS, DPPE, DSPC, EYPS, SPDP, Melittin, MSH, Methotrexate, Geneticin

Pierce (Rockford, Ill. USA) DTBP, EDC, DTNB, DTT

Pharmacia Fine Chemicals (Uppsala, Sweden) Sephadex G-25, Sepharose 4B, Ficoll 400.

Carlo Erba (Milan, Italy) HPLC grade acetonitrile

Amersham ^{3}H-Tyrosine, ^{3}H-Leucine, ^{3}H-Thymidine, ^{125}-I, cAMP assay kit.

Flow laboratories Tissue culture media.

State vaccine (Cape Town, S.Africa) Foetal calf serum

All other reagents were of analytical grade, and obtained from commercial sources.

Purification of reagents

Many of the reagents used were found to be of insufficient purity and were purified prior to use. Their purification procedure is outlined :

Diethyl Ether was found to contain peroxides which are potentially damaging to lipids and to proteins. The peroxides were removed by shaking the ether with an equal volume of 10% sodium sulphite and then washing the ether several times with water. Storage of the ether resulted in regeneration of peroxides, so this procedure was performed immediately prior to all uses of ether.

Triethylamine (TEA) was distilled from a solution with ninhydrin and used within several weeks. Storage for longer periods, even at -20 degrees, resulted in the formation of primary amines.

Dimethylformamide (DMF) was refluxed over phosphorous pentoxide and then distilled off, retaining the fraction distilling between 151 and 154 °C. It was stored over molecular sieve which had been dehydrated and washed with distilled DMF.

MSH

The MSH purchased from Sigma contained only 80% MSH by weight. It was purified on HPLC using a C18 column and a 0 to 70% (v/v) acetonitrile in 0,05% (v/v) TFA. The resulting single peak was dried and stored as the TFA salt after quantitation by amino acid analysis.

Melittin

Melittin was found to contain only 50% by weight peptide, and was contaminated with phospholipase activity. It was purified on HPLC, quantitated by amino acid anylysis, and tested for phospholipase activity (Smith et al. 1988). It was found to have less than 0.01 units of phospholipase A2 activity per mg peptide.

Lipids

DPPE, DPPS, DPPC, DSPC were shown to be pure by TLC and by microcalorimetry, and were used without further purification. Cholesterol was recrystalised twice from hot methanol before use.

Egg yolk lecithin was isolated from fresh egg yolks by the procedure of Singleton et al. (1965) on alumina. TLC showed it to be contaminated with lysolecithin so it was further purified on silicic acid by applying the lecithin (4g) in 10 ml of chloroform to a column (200 X 25 mm) of silicic acid (Merck) which had been heated to 110 degrees for 1 hr and then suspended in chloroform. The column was eluted with 2 column volumes each of 0, 10, 20, 30, 50% methanol in chloroform. The lecithin eluted with 30% methanol. TLC showed it to be homogenous with no trace of lysolecithin, nor any colouration. The lecithin was stored under nitrogen at -20 degrees.

TLC of lipids was performed on Silica F_{254} plates (Merck) and development was in chloroform / methanol / water (65:25:4). Detection was by iodine vapour. The presence of amino groups for PS and PE was achieved by spraying with a 1% solution of ninhydrin in acetone and heating the plate at 80 degrees for 10 minutes.

Calcein was recrystallised from hot ethanol (Allen 1984), made up to 100 mM in 25 mM sodium phosphate, the pH adjusted to 7.4 and the osmolarity adjusted to 280 mosM. After filter sterilisation the calcein solution was stored frozen.

General methods :

HPLC : All HPLC analytical and preparative separation was performed on a Millipore-Waters HPLC equiped with two model 6000A pumps, a model U6K injector, a model 441 absorbance detector equiped for monitoring absorbance at 230 nm, and a model 680 gradient controller. The column used was a μ-Bondapak C-18 (end-capped) 4 X 300 mm operated at a flow rate of 1.0 ml/min. Samples were eluted using a 0-70% acetonitrile gradient in 0.05% trifluoracetic acid. The shape of the gradient is shown for each application in results.

Tissue culture

All cell lines were grown at 37 degrees, 5% CO_2, 95% RH.

B16 F1 mouse melanoma cells were grown in McCoys 5A media with 10% FCS. No antibiotics were used. Cells were subcultured 1:10 weekly.
Hepatoma cells were grown in DMEM with 10% FCS and penecillin and streptomycin.
Friend erythroleukemia cells were grown in RPMI media with 10% FCS and penecillin and streptomycin.

Microscopy

Fluoresence photographs were taken on Pan F (Ilford) film with a Nikon inverted microscope equiped with fluoresence optics.

Freeze fracture electron microscopy: A sample of the liposomes without any cryoprotectant was frozen between two gold discs by immersion in nitrogen slush (prepared by placing a container of liquid nitrogen under vacuum), and immediately placed into a Balzers freeze fracture unit maintained at -170°C. A vacuum of 10^{-6} T was immediately established and the sample fractured by separation of the two gold discs. The sample was shadowed with platinum by evaporation for 10 seconds, followed by carbon coating by gradual evaporation of the carbon rods for 1 minute. The sample was removed and the replica floated off the discs onto 1% sodium hypochlorite. The replica was picked up with copper grids coated with formvar and viwed on a Zeiss TEM.

Scanning electron microscopy: Cells were grown overnight on glass coverslips in six-well tissue culture dishes. After treatment with liposomes for the required time period the coverslips were rinsed three times with PBS. Fixation was achieved by immersing the coverslips sequentially in :
1% osmium tetroxide in PBS pH 7.5 280 mOsm for 30 minutes,
2% glutaraldehyde, 2% formaldehyde in PBS pH 7.5 280 mOsm for 30 minutes,
30% ethanol in water for 2 min,
50% ethanol in water for 2 min,
70% ethanol in water for 2 min,
90% ethanol in water for 2 min,
100% ethanol.

The critical point drying and gold coating were performed by Morven Griffiths. Details are outlined briefly:
The samples were dried in a Polaron critical point dryer for 90 minutes, after which the coverslips were stuck onto aluminium stubs and covered with gold-palladium in a Balzers evaporation unit. Samples were viewed on a Cambridge 200 SEM at a 30° tilt.

To visualise liposomes on polycarbonate membranes, liposomes which had already been extruded through a polycarbonate membrane (0.2 um) were extruded through a fresh membrane and the membrane immediately removed from the filter holder, fixed and stained as above.

MSH biological activity assays

Adenylate cyclase: Cells from a mid-log phase culture were collected with EDTA (0.1% in PBS) and were seeded into 24-well plates at 10^5 cells per well in 1.0 ml McCoys 5A medium supplemented with 10% fetal calf serum. After 24 h incubation the cells were rinsed with PBS-Ca-Mg (Phosphate bufferd saline containing Ca^{++} and Mg^{++}). Hormone or conjugate was added in PBS and the cells incubated at 37°C for 30 minutes. The medium was decanted and the 1.0 ml 0.2 M HCl in ethanol at 4°C added. The cAMP was extracted into this by overnight incubation at -20°C. The ethanol was aspirated into eppendorf vials and evaporated extensively *in vacuo*. The residue was dissolved in 0.1 ml 50 mM Tris-HCl containing 1 mM EDTA and the cAMP determined using the Ammersham ^{3}H-cAMP/cAMP binding protein kit. Occasionally the protein content was determined using the method of Lowry et al. (1951) however this was found to be highly reproducible accross the plate and hence data is presented as pmoles cAMP

per well averaged accross duplicate wells. It was found that if the cells had reached stationary phase prior to seeding into the 24-well dish, the magnitude of the hormonal response was significantly decreased.

Tyrosinase: Cells from a mid-log phase culture were collected with EDTA (0.1% in PBS) and were seeded into 24-well plates at 10^5 cells per well in 1.0 ml McCoys 5A medium supplemented with 10% fetal calf serum. After 24 h hormone was added in sterile PBS, and incubation continued for a further 24 hours at which time 0.5 uCi L-[3,5-^{3}H]tyrosine was added and incubated for 24 hrs. The amount of ^3H_2O in the medium was then determined by a modified method of Pomerantz(1965). The medium (1ml) was applied to a column comprising a pasteur pipette containing 0.5 ml Dowex AG 50W, above which was a glass wool plug, and 0.5 ml activated charcoal. The medium was washed through this column with a further 2 ml of water, and a 1.0 ml aliquot of the combined eluant mixed with scintillation fluid and counted.

Preparative procedures :

MSH-SH: MSH was thiolated by interaction of MSH in 100 mM sodium phosphate pH 7.5 with a 20 fold molar excess of dithio-bis-propionimidate (DTBP) for two hours at 25°C. A 100 fold excess of dithiothreitol (DTT) was then added and reduction allowed to proceed for a further two hours. If the material was not needed immediately it was stored at -20°C in this form. Prior to use the reaction mixture was separated on HPLC by running isocratically in 0.05% TFA for 20 minutes to remove the vast excess of reducing agent, and

then running a gentle gradient from 30 to 45% of eluant B (70% acetonitrile in 0.05% TFA) to separate the modified and unmodified peptide.
The modified peptide was dried on a spin-vap apparatus, redissolved in the desired buffer and used immediately.

CM-MSH : Carboxymethylated MSH was prepared by incubating freshly prepared MSH-SH with a five fold molar excess of iodoacetic acid in 100 mM sodium phosphate pH 7.5 for four hours followed by separation on HPLC as described for MSH-SH. For the preparation of radiolabelled CM-MSH ^{3}H-iodoacetic acid was used.

^{125}I-MSH: Radio-iodinated MSH was prepared by lactoperoxidase mediated iodination of the peptide and subsequent purification on HPLC.
To 10 ug of MSH in 100 ul 100 mM phosphate buffer (pH 7.5) was added 1 ul of a 100 ug/ml freshly prepared lactoperoxidase solution, 25 ul of a 4.5 mCi/ml Na^{125}I (carrier free in phosphate buffer), and finally, after mixing, 10 ul of a freshly prepared 10 uM hydrogen peroxide solution. The reaction was allowed to proceed at 4°C for 1 hour. 100 ul of a 1M DTT solution was then added to reduce any methionine sulphoxide (Lambert et al. 1982), and incubation continued at 24°C overnight. The products were isolated on HPLC. Since the iodinated MSH is slightly retarded on HPLC compared to the non-iodinated species, and the iodinated form comprises only a minute proportion of the sample, the iodo-MSH can not be detected by absorbance at 230 nm, and hence must be detected by counting the radioactivity. Lacking on-line gamma detection facilities this was achieved by collecting the eluate of the HPLC in fractions and counting those for radioactivity. A typical elution profile is shown in fig 9.1. The iodo-MSH was identified as the main radioactive peak, eluting just after

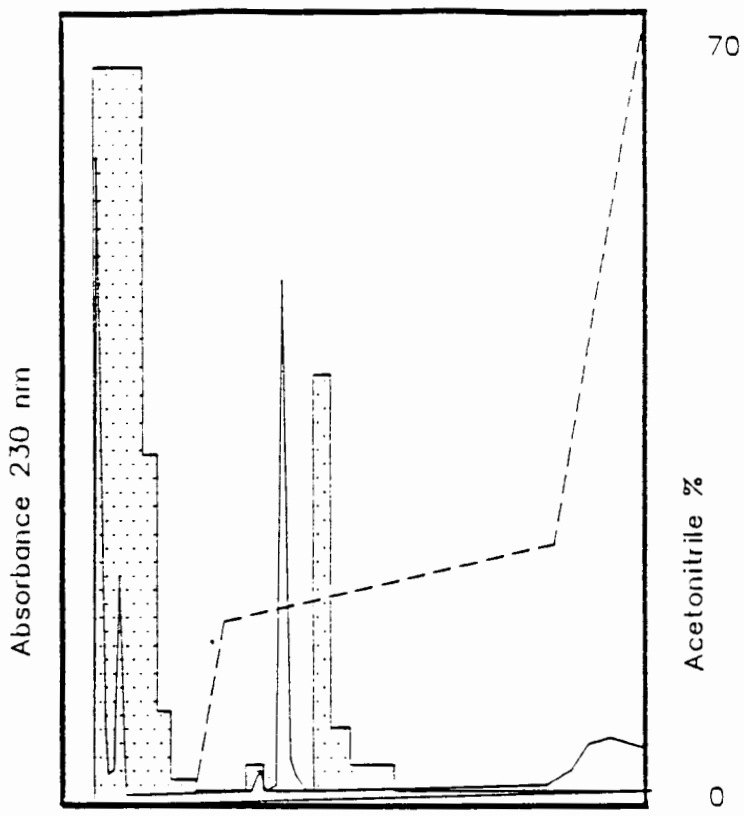

Fig 9.1: Purification of ^{125}I-MSH. MSH iodinated by the lactoperoxidase method was purified on HPLC using a C-18 column (Waters) and eluted in an acetonitrile gradient in 0.05% trifluoracetic acid. Dashed line indicates acetonitrile gradient, solid line absorbance at 230 nm, shaded area radioactivity.

The test-tube was then attached to a venturi vacuum pump, and the contents exposed to a vacuum of 0.2 Atm. In the case of DNA encapsulation vortexing continued during this period. In other cases the tube was vortexed intermitently untill the emulsion had formed a hard gel. Vortexing was then performed continously untill the gel liquidised. The vacuum was then increased for 5 minutes to remove any traces of ether.
The aqueous phase was either :
100 mM Calcein; 1 mM methotrexate; 1 mg/ml pSV2neo plasmid in PBS containing 1 mM EDTA; 3 mg/ml DNase1 in 20% glycerol in PBS/Ca/Mg.

Immediately after liposome formation the liposomes were extruded through sterile 0.2 um polycarbonate membranes and separated from unentrapped material by centrifugation of Ficoll gradients.

Ficoll centrifugation : The separation method was based on the method of Fraley et al. (1981). Liposomes were mixed with an equal volume of 20% Ficoll in a centrifuge tube. This was then overlayed with 2.0 ml of 5% Ficoll and 0.5 ml PBS. After centrifugation for 20 minutes the liposomes banded at the 5% Ficoll- PBS interface and could be aspirated in a volume of approximately 200 ul. This process removed 95-98% of the unentrapped material, a small quantity being carried between liposome aggregates. If greater purity was required the process was repeated.

Precautions were taken to ensure that the Ficoll did not impose osmotic imbalance on the liposomes. To ensure that the Ficoll solutions had the same osmotic pressure as the liposomes the solutions were dialysed against PBS.

Leakage assesment : Liposomes (10 nmoles) prepared to contain 100 mM calcein were incubated in buffer or in medium as described for 24 hours. The fluorescence of the sample was then determined on an Aminco spectrofluorometer (excitation at 490 nm, emmision at 520 nm). 10 ul of 10% Triton X-100 was then added which lysed the liposomes completely. The resulting fluorescence yielded a figure for 100% leakage. 0% leakage was assesed from a freshly dialysed sample of the liposomes.

Toxicity assays

The effect of cytotoxic drugs and toxins on cells was determined by incubating the drug, or liposomally entrapped drug with cells, and monitoring the incorporation of either a radiolabelled nucleotide into the DNA for methotrexate, or a radiolabelled amino acid into protein for ricin and ricin conjugates. The time period of incubation of toxin or drug with the cells is dependant on the experiment.

Methotrexate:

The effect of MTX or liposomally entrapped MTX on B16 melanoma cells was determined by seeding the cells into 24-well plates at 5 X 10^4 cells/well. After 24 hours in medium with 10% FCS, MTX or liposomes containing MTX were added. 24 hours later 0.5 uCi ^{3}H-thymidine was added to each well and incubation continued for a further 24 hours. The incorporation of the ^{3}H-thy into the cellular DNA was assayed by harvesting the cells onto glass fibre filter paper strips using a cell harvester (Titertek cell harvester, Flow laboratories, Rockville,MD) with an attachment for 24 well plates. The filters were submerged in 5% trichloracetic acid for 5 minutes, washed in ethanol, dried and counted in a scintillation counter.

Ricin chains

The crude lectins from Ricinus communis were prepared by a modification of the method of Kornfeld et al. (1974). Castor bean seeds (100 g) were homogenised in a Waring blender with 500 ml PBS , and left to extract for 2 hours at 4 degrees. The homogenate was poured through cheese cloth and the filtrate centrifuged at 10000g for 30 mins. The supernatant was brought to 75% saturation with solid ammonium sulphate, centrifuged at 10000g for 30 mins. The precipitate was dissolved in PBS, dialysed extensivly, recentrifuged , and applied to a Sepharose 4B column (Pharmacia, Sweden). After extensive washing with PBS the crude lectins were eluted with 10% lactose.

After extensive dialysis the lectins were purified to yield the agglutinin and toxin components by affinity chromatography on alpha-L-rhamnosyl polyacrylamide (Horejsi,V. 1979).

The affinity column was prepared by the method of Horejsi (Horejsi,V., Kocourek J., 1974). 10 g of Rhamnose (Merck) was refluxed with 20 ml allyl alchohol into which dry HCl gas had been bubbled until the weight of the alchohol increased by 0.6 g. Refluxing was at 80 degrees for 4 hours with stirring. The dark brown solution was neutralised with 25% NH_4 and the alchohol removed by vacuum distillation at 70 degrees. The resulting allyl rhamnose was copolymerised with acrylamide by mixing ;

10 g acrylamide, 1g bis-acrylamide, 4g allyl rhamnose, in 100 ml water. 0.1 g ammonium persulphate was added and 10 ul TEMED. The solution polymerised within 10 minutes.

The polymerised gel was then cut into small pieces, homogenised in a dounce homogeniser, and filtered through a 200 mesh gauze. After decanting the fines, this material was poured into a column (250 X 25) and washed with PBS.

Crude lectins were applied to the column and eluted with PBS. Only ricin toxin eluted. Subsequent elution of ricin aglutinin was achieved by washing the column with 10% lactose.

SDS polyacrylamide gel electrophoresis showed the crude lectins to have been separated out into pure agglutinin, yielding a single band with molecular weight of 120000, and ricin toxin, yielding two close bands of molecular weight 60000 and 61000. The toxin component was not contaminated by any agglutinin.

RTA

Ricin A chain was purified from ricin toxin by applying ricin toxin to a Sepharose 4B column, washing extensivly with TBS, and then eluting the RTA with 1% DTT in TBS. The A-chain which was shown to be pure by SDS PAGE was sterilised by filtration and stored at 4 degrees in 20 % glycerol, 1% DTT in TBS. Quantitation was achieved by monitoring the absorbance at 280 nm and taking the published figure of $A_{280} = 7.65$ (Olsnes 1984).
Immediately prior to use the required amount was dialysed against several changes of PBS to remove reducing agent. Toxicity was determined by inhibition of protein biosynthesis in a cell-free assay.

RTB

Ricin B chain was purified from ricin toxin by incubating the toxin with 1% DTT in TBS overnight at 4 degrees, applying the toxin to a Sepharose 4B column, and after washing with seven column volumes of TBS, eluting the B-chain with 10% lactose in TBS. The B-chain was sterilised by filtration and stored in 10% lactose, 1% DTT in PBS.

RTA-MSH

The conjugate between MSH and RTA was prepared by a modification of the method of Youlle and Neville (1982). 100 ug of freshly prepared MSH-SH containing a trace of ^{125}I-MSH-SH (yielding a final specific activity of 30 mCi/mmol) was dissolved in 200 ul 50 mM sodium borate, 1 mM EDTA, pH 8.5. 60 ul of 0.1 M 5,5'-dithiobis(2-nitro benzoic acid). After 2 hours at 25°C the modified peptide was separated from unreacted DTNB on HPLC as described for MSH-SH purification. The modified peptide was not evaporated to dryness because difficulty was experienced getting it back into solution. Most of the solvent was removed by evaporation with a gentle stream of nitrogen, leaving approximately 0.5 ml to which was added 0.5 ml 100 mM phophate buffer pH 8.0. The yield of peptide judged by radioactivity was 80%. There did appear to be loss of peptide due to the formation of some insoluble material. This was added to 1.0 ml freshly reduced RTA (1 mg/ml) in PBS, representing a 1.5 fold excess of peptide over toxin, and incubated at 4°C for 16 hours. Unbound peptide was separated from the toxin by dialysis against several changes PBS at 4°C, and the remaining radioactivity determined.

FITC-RTA-MSH: 100 ug of RTA-MSH in 200 ul PBS was added to 20 ug FITC in 100 ul 0.2 M $NaHCO_3$ pH 8.5. After incubation for 1 hour at 4°C the mixture was dialysed against several changes of PBS at 4°C.

Cell-free protein biosynthesis : The ability of ricin A-chain (RTA) and of conjugates of the A-chain to inhibit protein biosynthesis in a cell-free system was assayed by the method of Olsnes (1981). A commercial rabbit reticulosyte lysate assay system (BRL) was used. Ten fold dilutions of the A-chain or toxin conjugate in 50 mM DTT

were made into eppendorf vials in 10 ul PBS. Control was PBS without toxin. To each vial was added;
4 ul 2M potassium acetate,
4 ul 1 mM cold amino acids mixture (leucine deficient),
2 ul TMV-RNA (from kit),
2 ul human placental RNase inhibitor,
6 ul ^{3}H-Leucine,
52 ul lysate.

Incubation was performed at 30°C. After 10, 30 and 60 minutes 20 ul aliquots were withdrawn and added to tubes containing 1.0 ml 0.1 M pottasium hydroxide. 30 minutes after the last aliquot was added the protein was precipitated by adding 0.2 ml 50% trichloracetic acid. The precipitated protein was collected onto glass fibre filter disks and washed with 10 ml of 5% trichloracetic acid. After drying, the filters were counted by scintillation counting. The toxicity is measured as DPM per minute during the linear part of the curve. Graphically it is expressed as a percentage of the velocity (DPM/min) of the control (no toxin present).

Cellular protein biosynthesis :

The *in vitro* inhibition of protein biosynthesis in whole cells by ricin, ricin-A-chain or RTA conjugates was performed by a slight modification of the method of Youle and Neville (1982). 10^{4} cells in 0.1 ml medium were added to each well of a 96 well plate and incubated for 24 hours. Toxin which had been sterilised by filtration through 0.2 um membranes was added at the highest concentration required in 10 ul volumes, and the dilutions performed by making serial 10 fold dilutions accross the tissue culture plate. Incubation was continued for 48 hours at which time 0.1 uCi of ^{3}H-Leucine was and incubation continued for 6 hours. The medium was aspirated and the cells detached with 0.2 ml 1 mM

EDTA, 0.01 mg/ml trypsin in PBS. The cells were then collected onto glass fibre filter paper with a titertek cell harvester. The filters were submerged in 5% trichloracetic acid for 5 minutes, washed in ethanol, dried and counted in a scintillation counter.

Transformation

The plasmid pSV2neo was obtained from Dr I Parker, and grown up in E.coli (strain JM109) in the presence of neomycin using standard techniques for plasmid preparation and isolation (Maneatis 1984). Care was taken to ensure that the plasmid was very pure. The integrity of the plasmid was verified by restriction enzyme mapping. For transformation studies the plasmid was linearised with EcoR1 followed by two phenol extractions, two chloroform extractions and ethanol precipitation.

Liposome mediated transformation:

Hepatoma cells growing in mid-log phase were harvested with 1mM EDTA in PBS and resuspended in PBS/Ca/Mg at 10^5 cells / ml. Liposomes containing DNA, or free DNA was added, and the cells incubated at 37°C for 1 hour. 3 ml of DMEM containing 10% FCS was added and the cells poured into 1 well of a six well dish. Incubation was continued for 48 hours at which time fresh medium containing 800 ug/ml G418 was added and incubation continued for 12 days. The number of colonies was then counted.

Calcium Phosphate Transfection :

The calcium phosphate transfection of cells was performed according to the method of Wigler et al. (1978) as modified and reported by Kingston (Current protocols).
The cells were replated at 1 X 10^5 cells / well 48 hours after addition of the DNA, and G418 (800 ug/ml) added. Medium was replaced every fourth day.
Colonies were counted after 12 days by washing the cells with PBS, fixing with 70% ethanol and staining with crystal violet. Only colonies clearly visible to the naked eye were counted.

Electroporation :

Electroporation was performed using a Bio-rad Gene Pulser electroporation apparatus.
Hepatoma cells were grown to 50% confluence in DMEM medium, harvested with 1 mM EDTA, washed twice with ice cold PBS and suspended in PBS at 4 degrees at 5 X 10^6 cells / ml.
0 to 20 ug of linearised pSV2neo plasmid was added to 1 ml of cell suspension in disposable electroporation cuvettes (Bio-Rad) with an electrode gap of 4 mm. The cells were incubated with the DNA for 10 minutes on ice prior to electroporation.
Electroporation conditions were established by varying the voltage of the pulse from 0 to 2000 V with 40 ug of plasmid present. The capacitance of the apparatus was set at 25 uF. Only single pulses were employed. The optimal setting was found to be 1200 V which was the setting used for all subsequent experimentation.
After electroporation the cells were incubated on ice for 10 minutes. The cells were then plated at 1 X 10^5 cells / well in six well dishes and incubated in medium for 48 hrs prior to adding G418 at 800 ug/ml. Colonies were selected for and counted as described for calcium phosphate.

DNasel delivery

Hepatoma cells were grown to 50% confluence, harvested with 1 mM EDTA in PBS, and resuspended in prewarmed complete medium (DMEM with 10% FCS) at 1 X 10^6 cells / ml. Friend erythroleukemia cells were harvested from mid log phase growth by centrifugation and resuspended in complete medium (RPMI with 10% FCS) at 1 X 10^6 cells / ml.

Liposomes or free DNasel were added at various concentrations to 250 ul aliquots of the cells and the reaction terminated at various times as described below.

Method 1: This method is fast but requires care during the phenol extractions because of the small volumes and the large amount of protein at the interface.

The reaction mixture in an eppendorf vial is microfuged for 30 seconds, the supernatant decanted, and the pellet vortexed vigorously with 200 ul of phenol and 200 ul of 100 mM EDTA. This is then centrifuged for 10 minutes in a microfuge, the supernatant aspirated and phenol extracted with a further 200 ul. The supernatant from this is extracted twice with 400 ul of fresh diethyl ether. 10 ug of DNase free RNase (1 mg/ml, prepared by heating RNase solution at 100 degrees for 10 minutes) is added and incubated at 50 degrees for 30 minutes. The material can be analysed on a gel at this stage, 20 ul yielding visible bands, however a further phenol extraction and ether wash resulted in less material sticking in the wells.

Method 2 : This method yields identicle results to the above procedure, but was found to be preferable when a large

number of samples had to be processed and processing time was not a consideration.

The reaction was terminated by centrifuging the reaction mixture in an eppendorf vial, and resuspending the pellet in 1 ml of (100 mM EDTA / 10 mM Tris pH 8 / 1% SDS / 0.1% Proteinase K) at 50 degrees, and incubating for 16 hours at 50 degrees. 50 ul of DNase free RNase was added and incubation continued for a further 30 minutes. The solution was then extracted twice with 1 ml of phenol, twice with 1 ml of ether, and ethanol precipitated with 3 ml 100 ethanol. After washing with 80% ethanol the DNA was air dried by inverting the tubes over tissue paper. It was found that vacuum drying invariably over-dried some of the samples, and subsequent solubilising of the DNA was impaired, resulting in variable concentrations of DNA. The DNA was dissolved in 100 ul of TE and applied to the gel.

Analysis by agarose gels

Samples were applied to 1% agarose gels in TBE and run for 4-5 hours at 150 V. DNA was visualised by ultraviolet light and photographed using Technical Pan film.

Nuclei isolation:

Nuclei were isolated from cells by the method of Smith et al. 1988. Briefly: After harvesting, the cells were suspended in 0.34 M sucrose in Buffer A (15 mM NaCl, 65 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris, pH 7.4, 15 mM β-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 0.2 mM EDTA, 0.2 mM EGTA). Melittin (1 ug per 10^6 cells) was added and the cells briefly homogenised in a Dounce homogeniser. The homogenate was centrifuged at 1500 g for 15 minutes and resuspended in a small volume of the above 0.34 M sucrose in Buffer A. Nine volumes of 2.3 M

sucrose were added and the mixture centrifuged at 50000g for 45 minutes. The pellet was resuspended in nuclei storage buffer (12.5% v/v glycerol in Buffer A).

Nuclei digestion:

2.5 X 10^5 Nuclei in 50 ul of nuclei storage buffer were added to 50 ul nuclei storage buffer containing 10 mM Mg^{++} and DNase 1 at the concentrations shown in the legends. Incubation was performed at 37°C for 120 minutes at which time the reaction was terminated by the addition of 100 ul phenol. DNA was isolated and analysed as desribed above.

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APPENDIX 1:

During the course of this thesis many interesting observations were made which were investigated, but turned out to have no immediate bearing on the main topic of receptor mediated targeting. Some led to publication, others were not persued in sufficient depth to permit any firm conclusions. Some of these are outlined briefly for the record.

MELITTIN AS AN ANCHOR FOR LIGANDS ON LIPOSOMES

Melittin from bee venom is a 26 amino acid long peptide, of which the first twenty amino acids are predominantly hydrophobic, and the rest hydrophilic and predominantly positively charged. The sequence is ; Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-$CONH_2$ (Habermann and Jentsch 1967). The sequence thus confers on the peptide a structure resembling that of an ionic detergent, and to a large extent the peptide is able to behave simmilarly to a detergent, being able to lyse erythrocytes and liposomes (Sessa et al. 1969).

The mode of action of melittin on lipid membranes has been reviewed (Bernheimer and Rudy, 1986). On interaction with lipid bilayers melittin inserts into the bilayer and causes massive perturbation of the bilayer structure resulting initially in the leakage out of the contents of the vesicle or cell, and at higher concentrations in the disruption and fragmentation of the bilayer. That its haemolytic ability is not due simply to its behaviour as a cationic surface active but that other structural features of the peptide are critical for its action is demonstrated by investigations of

fragments and modifications of melittin (Schroder et al. 1971). These authors also observed that the 1-20 hydrophobic fragment of the peptide had full surface activity but was however unable to lyse erythrocytes, indicating a requirement of the C-terminal hexapeptide for lytic activity.

On the basis of melittins ability to insert spontaneously and with high affinity into lipid bilayers, it was investigated as a candidate for an anchor by which to bind ligands to liposomes subsequent to liposome preparation and purification. Since whole melittin perturbs liposomes resulting in the leakage of the contents it was reasoned that the 1-20 fragment which has been reported to have no haemolytic ability may fulfill the function. For this purpose the 1-20 fragment was prepared from whole melittin by enzymatic digestion and HPLC purification, and assayed for its ability to insert into bilayers and to induce leakage of the liposomal contents.

Materials and methods:

Preparation of 1-20 fragment (MEL_{1-20}): 10 mg Melittin (Sigma) in 1.0 ml 10 mM Tris/HCl (pH 8.0) 1 mM $CaCl_2$ and 1 mM DTE was digested with Clostripain (50 ug) at 37°C for 12 hours followed by the addition of Carboxypeptidase B (100 ug) and continuing digestion for a further 12 hours at 37°C. The insoluble hydrophobic core fragment was isolated by centrifugation for 10 minutes at 3000g. The pellet was redissolved in 20% acetonitrile in 0.05% trifluoracetic acid and purified by HPLC (Waters) on a u-Bondapak C18 reverse phase column using a 20-100% acetonitrile in 0.05% TFA gradient. The MEL_{1-20} fragment eluted well after any remaining melittin and was quantitated by amino acid analysis. To test for the presence of contaminating whole

melittin 100 nmol of the purified fragment was hydrolysed and the absence of Arginine and Glutamic acid (hydrolytic product of Glutamine) confirmed by amino acid analysis which would detect 0.1 nmoles of the amino acids.

Membrane insertion: The ability of the peptides (melittin and MEL_{1-20}) to insert into bilayers was monitored by the intrinsic fluorescence of the $tryptophan_{19}$ residue (Dufourcq and Faucon 1977). Phospholipid vesicles were prepared using egg yolk lecithin by the reverse phase evaporation procedure (Szoka and Papahadjopopoulos 1981) in PBS. Increasing quantities of liposomes were added to 10 ug of melittin or 13 ug of MEL_{1-20} in 0.5 ml PBS in an Aminco spectrofluorometer and the emmision spectra of the peptide scanned after incubation for 5 minutes at 25°C (excitation at 280 nm). Fluorescence titration curves were analysed according to the method of Bashford et al. (1979).

Leakage from vesicles:
1) The lytic effect of the peptides was assayed by monitoring the release of calcein from the liposomes. Liposomes were prepared containing 100 mM calcein and separated from unencapsulated calcein by Ficoll gradient density centrifugation. Peptide at the desired concentration was added to 10 nmoles of liposomes in 0.5 ml PBS. After 20 minutes at 25°C the fluorescence was measured (Excitation was at 490 nm, emmision at 520 nm) and compared against the fluorescence after adding triton X-100 to a final concentration of 0.1% which yields 100% lysis. At 100 mM calcein is highly quenched, and as it leaks out of hte liposomes becomes fluorescent providing a very sensetive assay for liposome disruption.

Results:

MEL_{1-20} purification: Amino acid analysis showed the dominant peak from HPLC to have a composition corresponding to its assignment as the 1-20 fragment. When 100 nmoles was hydrolysed and applied to the analyser less than 0.2 nmoles of arginine was detected indicating that the contamination of the fragment with whole melittin is less than 0.2%.

Membrane interaction: Fig 1 shows the emmision spectra resulting from the interaction of 13 ug of MEL_{1-20} with increasing quantities of liposomes containing PBS. The enhancement of the relative fluorescent intensity and the shift of the emmision maxima to shorter wavelengths reflects the decreased polarity of the solvent environment of the tryptophan residue, and indicates the incorporation of the tryptophan residue into the bilayer.

The shift in the emmision maxima is taken as a parameter of the amount of peptide that has become associated with the lipid. When this is plotted against the amount of lipid present (Fig 2) for both MEL_{1-20} and melittin it is apparent that at large lipid concentrations there is no further change in the emmision, which is expected since all the peptide will have become associated with lipid. The advantage of using emmision maxima rather than emmision intensity in this experiment is that the intensity, unlike the wavelength, is affected by the scattering properties of the liposomes. Corrections for liposome light scattering by controls without melittin are not possible since at low lipid concentrations the melittin significantly perturbs the liposomes and hence the light scattering properties.

A comparison of the data for the two peptides is not readily obtainable from fig 2 since the final (at infinite lipid concentration) spectral properties of the two peptides

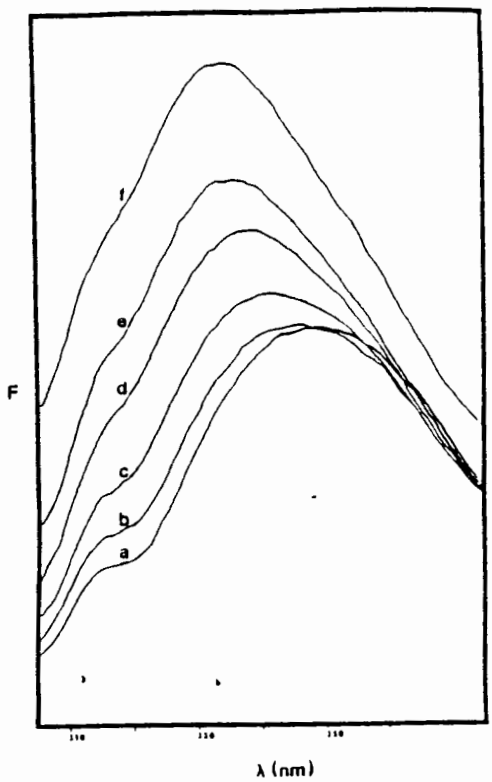

Fig 1: Fluorescence spectra of MEL_{1-20} (10 ug in 0.5 ml PBS) in the presence of increasing quantities of liposomes. Excitation at 280 nm. A; control, B; 15 nmole lipid, C; 30 nmole lipid, D; 45 nmol lipid, E; 60 nmol lipid, F; 90 nmol lipid.

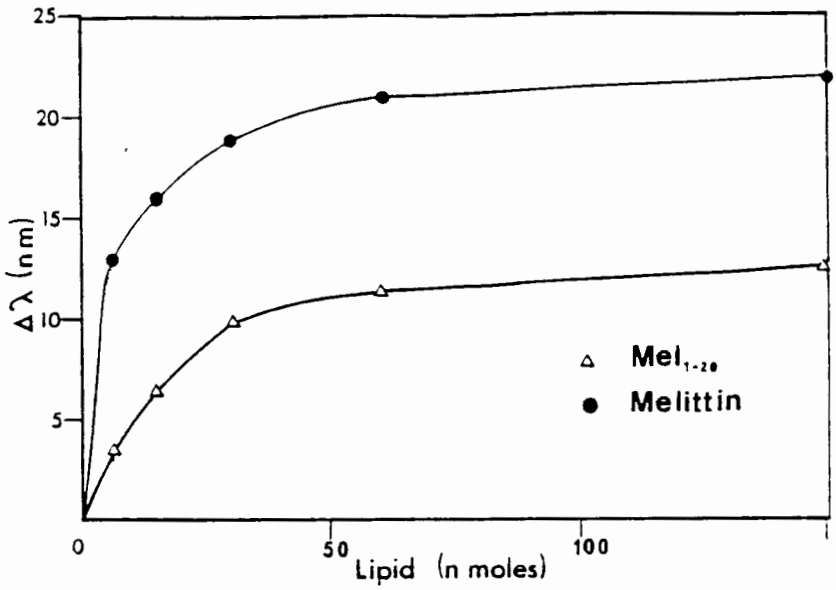

Fig 2: Shift in emmision maxima with increasing lipid concentrations for melittin and MEL_{1-20}.

differ. A more informative comparison is obtained by the formula (Basford et al. 1979);

$$e-1 = (e_b-1)-K_d.(e-1)/n.m$$

where e = optical paramater observed / initial
e_b = characteristic enhancement of optical parameter
K_d = dissociation constant
m = membrane concentration
n = number of binding sites per unit of membrane.

This equation predicts that a plot of e-1 vs e-1/m will yield a straight line with an ordinate intercept of e_b-1, and a slope of K_d/n. The value of K_d/n provides a good indication of the relative affinities of the two peptides for lipid membranes (Fig 3).

From figure 3 it is evident that although the two peptides have different characterisitic enhancements, determined from the intercept, the slopes are nearly identical for the two peptides. This suggests that the peptides have very simmilar affinities for lipid bilayers and therefore the 1-20 fragment will behave simmilarly to melittin in its ability to insert spontaneously and quantitatively into liposomes.

An interesting conclusion which can be drawn from fig 2 is that for both peptides there is no further change in spectral properties when more than 60 nmoles of lipid are present, and hence 60 nmoles of lipid, which represents a 15 fold molar excess over the peptide, is sufficient to adsorb all of the peptide out of solution. Therefore under conditions in which the lipid quantity exceeds the peptide quantity by more than 15 fold, the binding of the peptide to the membrane will be quantitative.

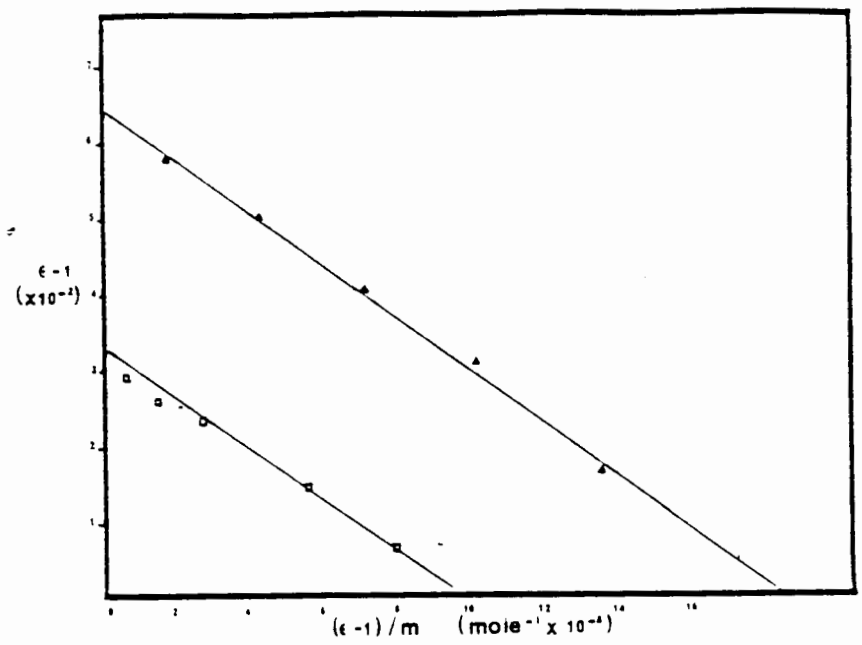

Fig 3: Comparison of affinities of melittin and MEL_{1-20} for lipid bilayers by method of Bashford et al. (1979). The ordinates are ; (Observed emmision maxima / initial maxima) - 1 vs (Observed / initial -1)/ membrane concentration. The slope has gradient K_d/n.

The two peptides were then compared for their ability to promote the leakage of calcein out of liposomes (Fig 4).

From fig 4 it can be seen that the fragment has significantly less lytic activity than whole melittin, yet it is still able to promote the leakage of calcein from the liposomes. Comparing the concentrations required to yield 50% lysis in 20 minutes shows that melittin is 50 to 100 fold more potent than the fragment. If the lytic property of the fragment was due to the presence of contaminating whole melittin, the contamination would have to be in the order of 1 to 2%, yet amino acid analysis shows less than 0.2% of the fragment to be whole melittin. Therefore the lytic effect observed for the fragment is a property of the hydrophobic core of melittin.

From this it can be concluded that not all of the detergent-like properties of melittin are due to its structural similarities to ionic detergents, but that the hydrophobic core alone is able to perturb the bilayer to a sufficient extent to promote lysis. This has not previously been demonstrated, possibly because the haemolysis assay employed by most investigators is less sensitive than the assay described here or because the erythrocytes have a different lipid composition to the liposomes.

There is disagreement within the literature regarding the mechanism of action of melittin (for review see Bernheimer and Rudy 1985) and specifically regarding the orientation of melittin within the bilayer. One hypothesis is that melittin exists as a tetramer spanning the bilayer (Vogel and Jahnig 1986) and forming a hydrophilic pore through the membrane.

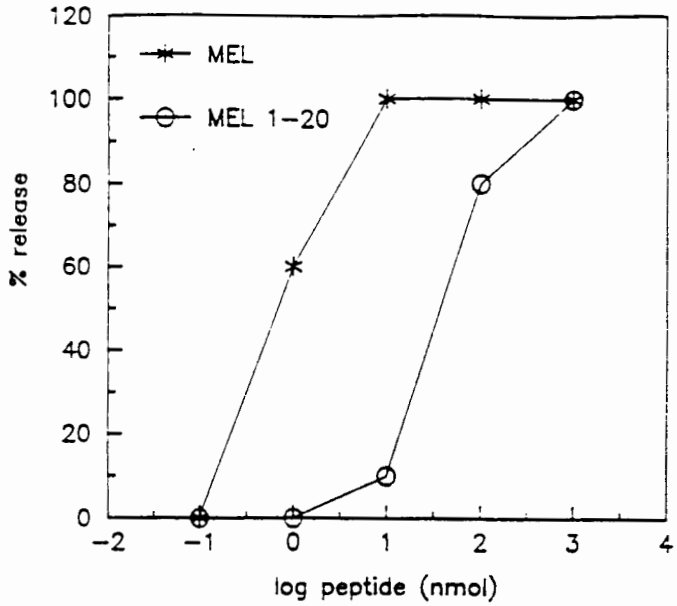

Fig 4: Peptide induced leakage of calcein from liposomes. Melittin or MEL_{1-20} were added to liposomes containing 100 mM calcein and the fluorescence monitored after 20 minutes. The fluorescence was compared against fluorescence after adding triton X-100 which induced 100% lysis.

The other principle hypothesis proposes that melittin lies in the membrane with the helix axis approximately parallel to the plane of the bilayer and with the hydrophilic surface exposed to the aqueous phase (Terwilliger et al. 1982). The ability of the hydrophobic core to promote lysis can be interpreted in terms of both hypotheses;

If the peptide lies horizontally in the membrane as a ridgid bent rod which is expected from its crystal structure (Teerwilliger and Eisenberg 1982) this will probably cause disruption of the bilayer and may also cause a surface area difference across the bilayer which may lead to lysis. On the other hand if the peptide spans the bilayer and forms tetramers with a pore in the middle, the 1-20 fragment will also be capable of doing this. In this case the absence of the positively charged C-terminal hexapeptide may result in closer association of the peptides and hence a more compact tetramer and also smaller pores. This could account for the present but diminished lytic activity.

A ramification of this result is that using the 1-20 fragment of melittin as an anchor by which to attach proteins to liposomes will probably result in lysis of the liposomes and loss of the contents, hence this avenue of research was not persued further.

These experiments did however themselves produce interesting observations that were persued further. These were ;

1) Since melittin can lyse cells, the observation (fig 2) that melittin can be quantitatively adsorbed from solution by small quantities of lipid, suggests that in lysing cells, all of the melittin becomes adsorbed from solution. To this extent melittin behaves very differently from normal detergents. This led to a novel and superior method for

isolating intracellular organelles described in the next chapter.

2) That melittin could cause the rapid and complete release of BSA from within liposomes, it was used to lyse liposomes where detergent would have inactivated the material being released. This application is described later.

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Appendix 2:

ISOLATION OF NUCLEI FROM MELITTIN-DESTABILISED CELLS

From the data shown in fig 2 in the previous chapter, and also based on the published report (Sessa et al. 1969) that the haemolytic property of melittin could be abolished by prior interaction with liposomes, I reasoned that the lytic action of the peptide would allow lysis and release of the intracellular contents of the cell under conditions in which all of the lytic agent had been adsorbed by the plasma membrane, and thus the intracellular contents would not become exposed to any detergent or lytic agent. To this extent the detergent-like lytic property of melittin differs from conventional detergents.

To investigate whether this property could be used to provide a tool whereby intracellular organelles were isolated without exposure to lytic agent, and whether such a technique was preferable to conventional detergent solubilisation, nuclei were isolated from cells by three different techniques, and the quality of the nuclei determined by monitoring the transcriptional activity.

This work was done in collaboration with collegues in my laboratory and is described in the publication reproduced here.

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Isolation of Nuclei from Melittin-Destabilized Cells[1]

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Melittin, the hydrophobic polypeptide from bee venom, sufficiently destabilizes the plasma membrane of cultured cells to allow cell disruption in the absence of detergents with minimal homogenization. Nuclei are thus isolated in high yield with intact nuclear membranes and high transcriptional activity.

KEY WORDS: melittin; detergent; nuclei isolation; tissue culture; nuclear membrane.

The isolation in high yield of nuclei from cultured cells requires efficient disruption of the cell envelope. Whereas cell–cell adhesion in tissue enhances the shearing forces during homogenization and thus facilitates the disintegration of the cell envelope, this assisting effect is absent in cultured cells. To achieve effective lysis in isolated cells, homogenization in the presence of a nonionic detergent such as Triton X-100 has become the method of choice (1). Nonionic detergents disrupt the plasma membrane primarily through the solubilization of membrane phospholipid (2). The detergent, however, also disrupts all intracellular membranes, including the nuclear membrane (3), making the isolation and purification of the latter by sucrose gradient density centrifugation impossible. In addition the detergent may affect intranuclear biological activities. We have therefore examined alternatives to the use of detergents in order to isolate intact nuclei from cultured cells.

Melittin from bee venom is an effective lytic agent. Its mode of action has been recently reviewed (4). Melittin is an amphiphilic peptide of which the first 20 amino acids are predominantly hydrophobic. The remaining residues are all hydrophilic, four being positively charged. This amino acid sequence confers on the peptide a structure resembling that of an ionic detergent (5). Melittin is able to lyse both, erythrocytes and artificial bilayer membranes (liposomes), at very low concentrations. In inducing lysis melittin binds to the plasma membrane and is absorbed into the lipid phase, from which it is not released to further interact with other membranes (6). If proteins are present in the membrane melittin may also be capable of interacting with them (7).

We reasoned that the ability of melittin to perturb membranes and to be readily absorbed from solution into the lipid phase would not only facilitate cell lysis but also result in only minimal exposure of the cellular contents to melittin. The latter would hopefully partition essentially into the plasma membrane.

MATERIALS AND METHODS

Purification of melittin and preparation of liposomes. Commercially available melittin may contain phospholipase(s) A2. The hydrophobic characteristics of phospholipase A2 suggest that they may be efficiently re-

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moved by chromatography on a hydrophobic column (8). We have purified melittin (Sigma) by high pressure liquid chromatography (HPLC) on a μBondapak C18 reverse phase column (Waters) using a 0–100% acetonitrile gradient in 0.05% trifluoroacetic acid (TFA).[3] Phospholipase A2 activity was determined by the method of Hayaishi (9). Less than 0.005 unit of phospholipase A2 was present per milligram of purified melittin.

Calcein (Sigma) was purified by gel filtration on Sephadex G25. Large unilamellar phosphatidylcholine vesicles (15 μmol phospholipid/ml) were prepared by the reverse phase evaporation procedure (10) with an aqueous solution of 100 mM calcein. The vesicles were extruded through 0.2-μm pore diameter polycarbonate membranes (Nucleopore) and separated from unencapsulated calcein by gel filtration on Sepharose 4B. Fluorescence measurements were performed on an Aminco spectrofluorometer. Egg yolk phosphatidylcholine (Sigma) formed a single spot on thin layer chromatography (chloroform:methanol:acetic acid:water (25:15:4:2)).

The lytic effects of melittin on liposomes were monitored by following the increase in fluorescence of calcein as it leaked out of the liposomes and was no longer self-quenching (11). Melittin or Triton X-100 was added to 0.05 μmol of calcein-containing liposomes at a concentration of 0–10 μM (melittin) or 0–10 mM (Triton X-100). Fluorescence intensity was measured after 20 min and compared to the intensity after incubation of the liposomes with 30 mM Triton X-100, which was taken as 100% lysis.

In order to monitor the absorption of melittin from solution by isolated plasma membranes, the minimum concentration of melittin required to elicit 100% release of calcein from calcein-loaded liposomes was established. Increasing amounts of rat liver plasma membrane were incubated for 20 min at that concentration of melittin. Liposomes containing calcein (0.05 μmol lipid) in 0.1 ml of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, were then added and incubated for 20 min, after which the percentage of lysis that had occurred was measured.

Isolation of nuclei. Friend erythroleukemia cells were grown in suspension culture in Iscove's Modification of Dulbecco's medium at 37°C, 5% CO_2, 95% humidity. Cells were harvested by centrifugation at 1500*g* for 5 min, resuspended in Earle's balanced salt solution, and again pelleted. The cells were resuspended in 0.34 M sucrose in Buffer A essentially as described by Hewish and Burgoyne (12) (15 mM NaCl, 65 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris, pH 7.4, 15 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 0.2 mM EGTA). At this point melittin or Triton X-100 was added to the desired concentration. The cells were centrifuged to remove excess melittin or detergent and resuspended in 0.34 M sucrose in Buffer A to a concentration of 10^8 cells/ml. The cells were homogenized at 700 rpm using a Pierce 1.0-ml rotary homogenizer with a clearance of 0.10–0.15 mm or with a 1-ml tight-fitting Dounce homogenizer (Pierce). Breakage of cells and nuclei was monitored by light microscopy. The homogenate was centrifuged at 1500*g* for 15 min and resuspended in a small volume of 0.34 M sucrose in Buffer A. Nine volumes of 2.3 M sucrose in Buffer A were added and the mixture centrifuged at 50,000*g* for 45 min. The pellet comprising purified nuclei was resuspended in storage buffer (12.5% (v/v) glycerol in Buffer A) to a DNA concentration of 1 mg/ml. DNA was determined at OD_{260} in 4 M NaCl.

Transcription. The transcription assay on isolated nuclei was carried out essentially as described by Marzluff using [α-^{32}P]GTP (1.77 Ci/mmol) (13). Transcription was monitored by assaying for the incorporation of [^{32}P]GMP into TCA-precipitable material retained on Whatman GF/C filters.

[3] Abbreviation used: TFA, trifluoroacetic acid.

Isolation of nuclear envelopes. Nuclear envelopes were isolated from purified nuclei according to the method of Bornens and Courvalin (14). The envelopes were purified by centrifugation on a discontinuous sucrose gradient and collected at the 45/50% interface and stored in liquid nitrogen until used.

RESULTS AND DISCUSSION

Triton X-100 is a nonionic detergent often used to lyse cultured cells (1). We initially compared the lytic effects of melittin and Triton X-100 on liposomes. Melittin was found to induce complete lysis at almost 1000-fold lower molar concentration than Triton X-100 (Fig. 1).

In order to establish how effectively melittin would partition into plasma membrane and be removed from solution, 40 pmol of melittin, the minimum quantity necessary to induce 100% lysis of a standard aliquot of calcein-loaded liposomes, was incubated with increasing amounts of rat liver plasma membrane. Preincubation of melittin with as little as 70 ng (protein) of plasma membrane completely inhibited lysis of the calcein-loaded liposomes. Thus all free melittin had been removed from solution (Fig. 2). However, in the case of Triton X-100, the minimum concentration needed to lyse a standard aliquot of calcein-loaded lipsosomes still induced at least 80% lysis even after preincubation with as much as 100 μg of plasma membrane (Fig. 2). These results indicate that not only is melittin a better lytic agent than Triton X-100 but it is able to partition quantitatively into the plasma membrane and thus be effectively removed from solution.

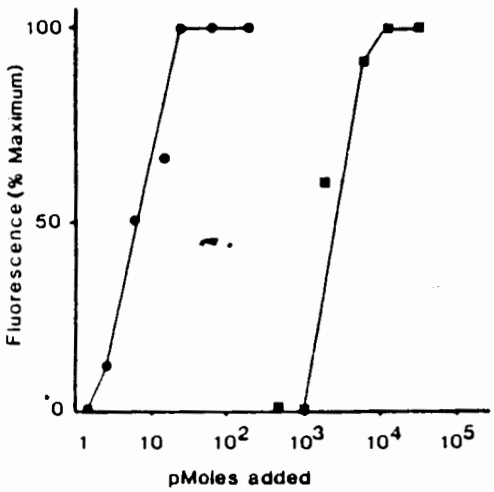

FIG. 1. Increasing amounts of melittin (●) and Triton X-100 (■) were added to a standard aliquot of calcein-loaded liposomes and calcein release was monitored after 20 min of incubation. Liposomes were 50 nmol of phosphatidyl choline in 100 μl of phosphate-buffered saline. The temperature of incubation was 25°C.

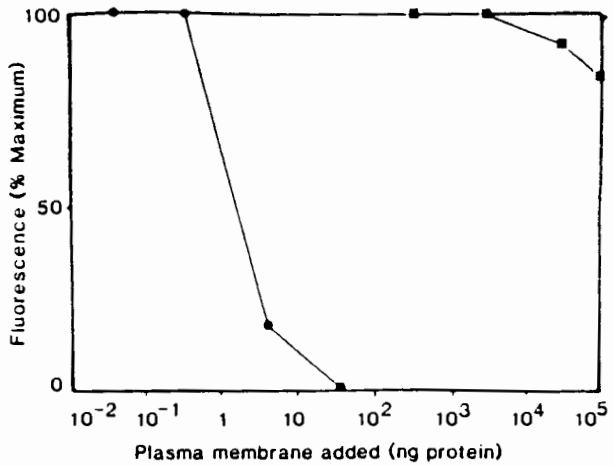

FIG. 2. Removal of melittin from solution by plasma membrane. Fifteen picomoles of melittin (●) or 10 nmol of Triton X-100 (■) (the minimum lytic concentrations for a standard aliquot of calcein-loaded liposomes) was preincubated with varying amounts of rat liver plasma membrane prior to addition to 100 μl of phosphate-buffered saline containing 50 nmol (lipid) of calcein-containing liposomes.

We incubated Friend erythroleukemia cells with various amounts of melittin. We found that amounts greater than 2 μg (720 pmol) per 10^6 cells began to induce spontaneous lysis of the cells. When melittin was added at a ratio of 1 μg (360 pmol) per 10^6 cells, no lysis occurred but the cells could be easily broken by very brief homogenization (one to five strokes), leaving the nuclei intact. If lower amounts of melittin were used, increased homogenization was needed to break the cells, resulting in damage to the nuclei. The yield of nuclei obtained using 1 μg of melittin/10^6 cells was routinely between 95 and 100%, the same as that obtained when

TABLE 1

Nuclei isolation method	Yield: Nuclei (%)	Yield: Nuclear membrane (μg protein/10^8 nuclei)
Homogenization only	<25	49
Melittin	>95	56
Melittin[a]		48
Triton X-100	>95	<1

Note. The yield of nuclei represents that obtained after the cells are homogenized with five strokes of a Dounce homogenizer. The yield of nuclear membrane represents material recovered from equal amounts of intact nuclei after purification of the membrane by sucrose gradient centrifugation.

[a] Purified nuclear envelope incubated with excess melittin (100 μg/mg protein) and rerun on a sucrose gradient.

0.5% (v/v; 7.5 μmol/10^6 cells) Triton X-100 was used to lyse the cells (Table 1). When attempts were made to break the cells without the use of detergent or melittin, extensive homogenization—up to 20 strokes—was needed to achieve 80% lysis. This resulted in progressive damage to the nuclei as evidenced by breakage and clumping. Briefer homogenization (five strokes) in the absence of detergent or melittin yielded intact nuclei but only 20% lysis of cells (Table 1).

In order to assess the effect of melittin on the yield of nuclear membranes, equal amounts of intact nuclei isolated in the presence of melittin and Triton X-100 and in the absence of additives were used as starting material for the isolation of nuclear membranes.

Nuclei obtained from cells broken by homogenization only yielded membranes banding typically at the 45/50% interface with a buoyant density of 1.2 after sucrose gradient centrifugation (Fig. 3). The yield of nuclear membrane, expressed as protein, was 49 μg per 10^8 nuclei (Table 1). Nuclei isolated from cells broken using melittin yielded membranes with the same buoyant density (Fig. 3). Fifty-six micrograms nuclear membrane protein was obtained per 10^8 cells (Table 1). Thus the yield of nuclear membrane protein isolated from cells disrupted using melittin is almost identical to that obtained from cells broken by homogenization only, although the yield of nuclei by the latter method is considerably lower. Furthermore if isolated nuclear membranes were exposed to excess melittin (100 μg/mg membrane protein) virtually no solubilization of protein occurred (Table 1) and the buoyant density of the membranes remained the same, indicating no significant loss of phospholipid. On the other hand attempts to isolate envelopes from nuclei of Triton X-100 lysed cells yielded no material at the 45–50% interface (Fig. 3, Table 1), indicating the absence of intact nuclear envelopes. Lowering the concentration of Triton X-100 in the isolation buffers resulted in reduced lysis of cells but identical damage to nuclear envelopes. This result was expected given the known ability of Triton X-100 to extract membrane phospholipid (2,3).

Transcriptional activity in nuclei from cells broken using melittin was indistinguishable from that of nuclei isolated from cells broken by homogenization only (Fig. 4). However, the latter could be isolated only in low yield when minimal homogenization

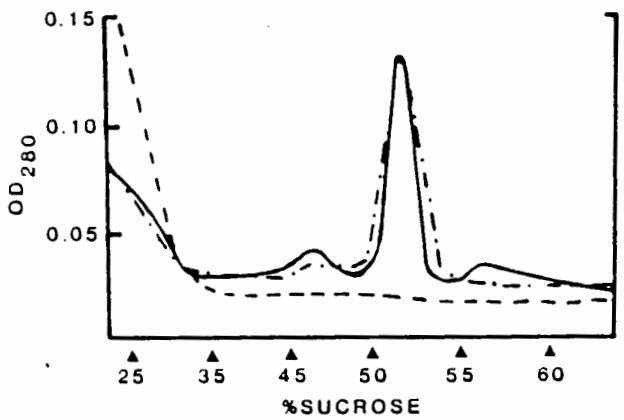

FIG. 3. Purification of nuclear envelopes on a stepwise sucrose gradient from nuclei isolated using homogenization only (·-·-), melittin (—), and Triton X-100 (- - -). The gradient was fractionated continuously using an Isco density gradient fractionator. The yield of the envelopes is reflected in Table 1.

was applied to avoid damage. Nuclei from cells lysed with Triton X-100 exhibited at best 40% of the transcriptional activity of nuclei isolated using melittin (Fig. 4). This result is not surprising as penetration of the nucleus by the detergent and breakdown of the chromatin structure is well documented and histones have been shown to be the major species of nuclear protein leaching from nuclei of cells lysed with Triton X-100 (2).

We have shown that melittin, at the very low amount of 360 pmol/10^6 cells, destabilizes cultured cells, allowing cell breakage with minimal homogenization. Nuclei are recovered in high yield with intact nuclear membranes and a transcriptional activity over two times greater than that found in nuclei from cells lysed using Triton X-100.

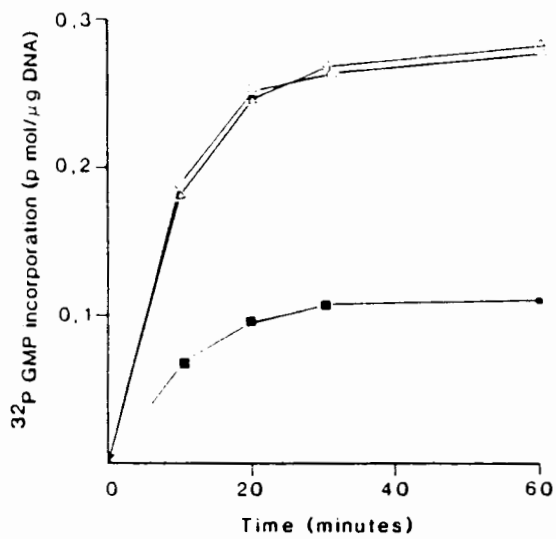

FIG. 4. Transcriptional activity of nuclei isolated using homogenization only (△), melittin (○), and Triton X-100 (■). Transcription was monitored by incorporation of [^{32}P]GMP into RNA. Each incubation contained nuclei corresponding to 150 μg DNA.

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Appendix 3

Following the arguments for the rapid and quantitative adsorbtion of lytic concentrations of melittin by lipid bilayers, melittin was used to release the contents of liposomes where conventional detergent mediated lysis resulted in inactivation of the liposommal contents.

THE ENCAPSULATION AND ASSESMENT OF ENZYMATIC ACTIVITY OF DNASE 1 IN LIPOSOMES.

Prior to attempting to deliver DNase 1 into cells by encapsulation within targeted REV liposomes, it was necessary to determine what proportion of the enzyme had been encapsulated, and whether the process of encapsulation of the enzyme had significantly altered the enzymatic activity of the DNase 1. For this reason a technique had to be developed whereby the enzymatic activity of the encapsulated enzyme could be monitored. This required that the enzyme be released from the inside of the liposomes, but that the method of disrupting the liposomes, and hence releasing the enzyme, did not itself inactivate the enzyme.

The release of liposomal contents, whether for assesment of enzymatic activity or for determination of encapsulation, is normally achieved by lysis of the liposomes in a detergent such as Triton X-100, however if detergents denature the enzyme, or interfere with the subsequent assay, an alternative procedure for release of the liposomal contents must be used. Kirby and Gregoriadis (1984) have investigated this problem when trying to ascertain the biological activity of factor V111 encapsulated within liposomes. These authors found that factor V111 activity was decreased when the enzyme had come into contact with detergent, and hence

tried alternative techniques such as sonication, solvent extraction, freeze-drying, and detergent extraction. None of these techniques for disrupting liposomes when applied to factor V111 alone did not decrease the activity. It would therefore be desirable to have a reagent which caused total lysis of the liposomes, but did not affect enzymatic activity, and since it would be convenient to take the sample of lysed liposomes and assay activity directly without having to separate the enzyme first, the reagent should not intefere with any assay.

Liposomal encapsulation of DNase1.

DNase 1 at 3 mg/ml in 20% glycerol in PBS was encapsulated in liposomes by the reverse phase evaporation procedure of Szoka and Papahadjopopoulos (1978). It was found that the presence of 20% glycerol in the aqueous phase prevented the DNase 1 coming out of solution when the diethyl ether was added, and was necesary to prevent the complete inactivation of the enzyme during sonication in the presence of diethyl ether.

The liposomes were separated from unencasulated enzyme by ficoll gradient desity centrifugation. An aliquot of the purified liposomes was lysed with triton X-100 (final concentration 0.1%), and the various stages of preparation assayed for enzymatic activity by monitoring increased absorbence at 260 nm of a 0.1 mg/ml calf thymus DNA in PBS/Ca/Mg solution. The activities are shown in fig 1.

Comparing the enzymatic activity of free DNase 1 with that of enzyme in liposomes prior to Ficoll separation, it appears that the process of encapsulation causes an increase in the activity of the enzyme. This is probably due to an association of the enzyme with the lipid bilayer because incubating free enzyme with empty liposomes also causes an

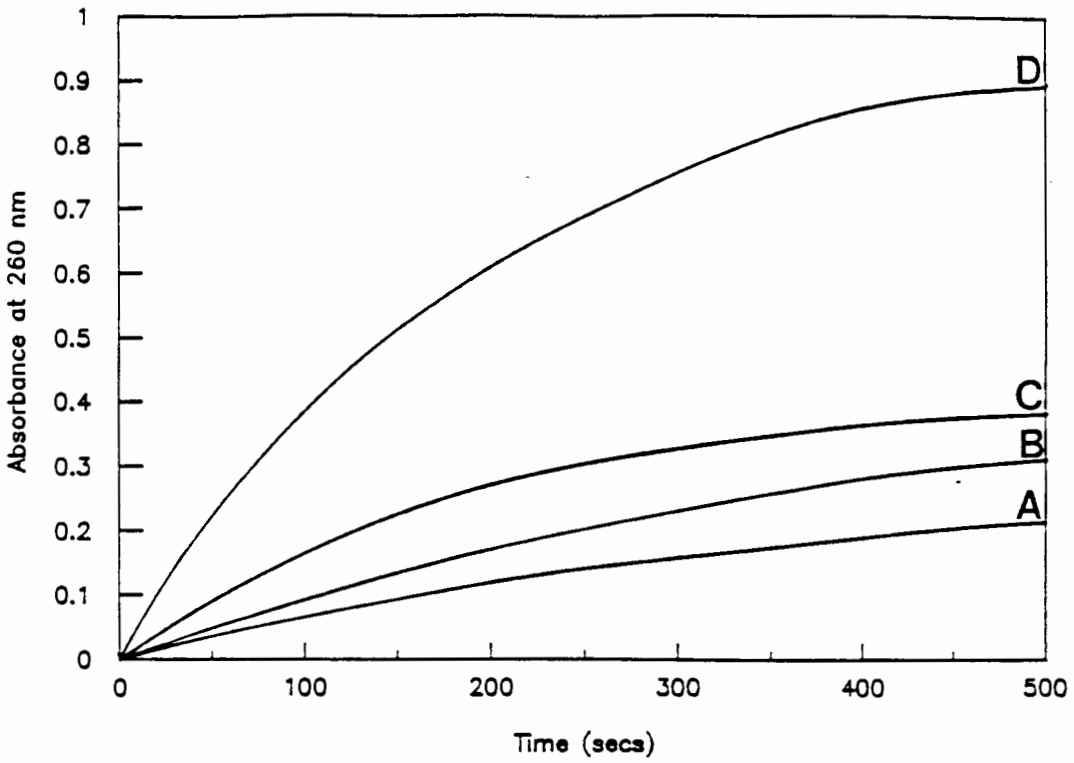

Fig 1. DNase 1 activity of various stages of preparation of liposomally encapsulated DNase1. 10 µg DNase 1 in 20% glycerol in PBS (B), liposome sample (100 nmoles lipid, 10 µg enzyme) prior to separation on Ficoll (D), liposomes subsequent to separation (100 nmoles lipid) (A), 10 µg DNase 1 in the presence of empty liposomes (100 nmoles lipid) (C). The samples were incubated with calf thymus DNA (0.1 mg/ml in PBS/Ca/Mg), and the increase in absorbance at 260 nm with time monitored.

increase in activity. It is possible that association of the enzyme with a lipid bilayer decreases its mobility and hence increases the chance of the enzyme cutting both strands of DNA within close proximity. This will have the effect of increasing the rate at which the hyperchromic shift in the DNA occurs, and therefore increasing the activity.

The increase in activity of free enzyme in the presence of 100 nmoles of lipid is not as great as when the enzyme was present during liposome formation. This is probably due to greater association of the enzyme with the lipid during bilayer formation than if added subsequent to the formation.

That there is association of the enzyme with the liposome membrane is further shown by the enzymatic activity of liposomes after separation on Ficoll. The activity in this sample is most readily ascribed to the presence of adsorbed enzyme on the outside of the liposomes.

When the purified liposomes containing enzyme are lysed with triton X-100 all of the enzymatic activity is lost (fig 2), indicating an inability of the enzyme to function in the presence of the detergent, and hence the unsuitability of triton X-100 for releasing encapsulated enzyme. On the other hand when melittin is used to lyse the liposomes there is a very significant increase in activity compared to that of intact liposomes.

An exact interpretation of how much enzyme is released on lysis, and hence an estimate of the encapsulation efficiency, is hampered by the effect of the bilayer on the enzymatic activity. A comparison with free enzyme in the presence of added lipid is not really valid since this appears to give less association than when the enzyme is present during liposome formation, hence a comparison was made against liposomes which had not been separated from the

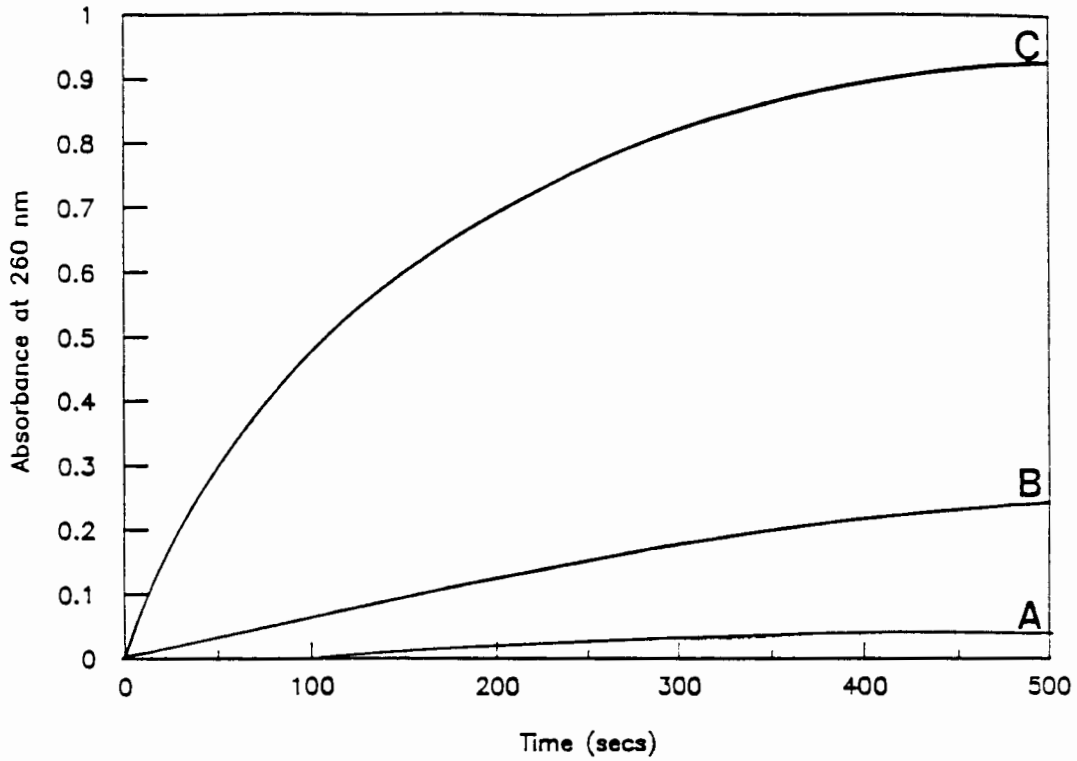

Fig 2: Action of triton X-100 and melittin on liposomaly entrapped DNase 1. Liposomes (100 nmoles of lipid) was incubated with either PBS (B), 0.1 nmoles of melittin (C) or 10 nmoles of triton X-100 (A) for 20 minutes at 4°C. The enzymatic activity was monitored as above.

unencapsulated enzyme, and yet had been lysed to release any encapsulated enzyme with 0.1 nmoles of melittin (Fig 3).

Fig 3 shows that 100 nmoles of melittin-lysed liposomes previously separated from unencapsulated enzyme has approximately the same rate of digestion of DNA as 25 nmoles of melittin-lysed liposomes which have not been separated from unencapsulated enzyme. If the total activity present is composed of three components:

1) Intraliposomal enzyme
2) Membrane associated enzyme
3) extraliposomal enzyme,

the activity of the melittin lysed liposomes previously separated from unencapsulated enzyme can be ascribed to factors 1 and 2. The activity of the melittin lysed liposomes not separated from unencapsulated material is due to factors 1, 2 and 3. Thus subtraction of activity of the former from the latter yields activity not associated with the liposomes, which is 75 to 80 % of the total activity. From this it can be concluded that approximately 20 to 25% of the added enzyme becomes entrapped or associated with the liposomes during liposome formation.

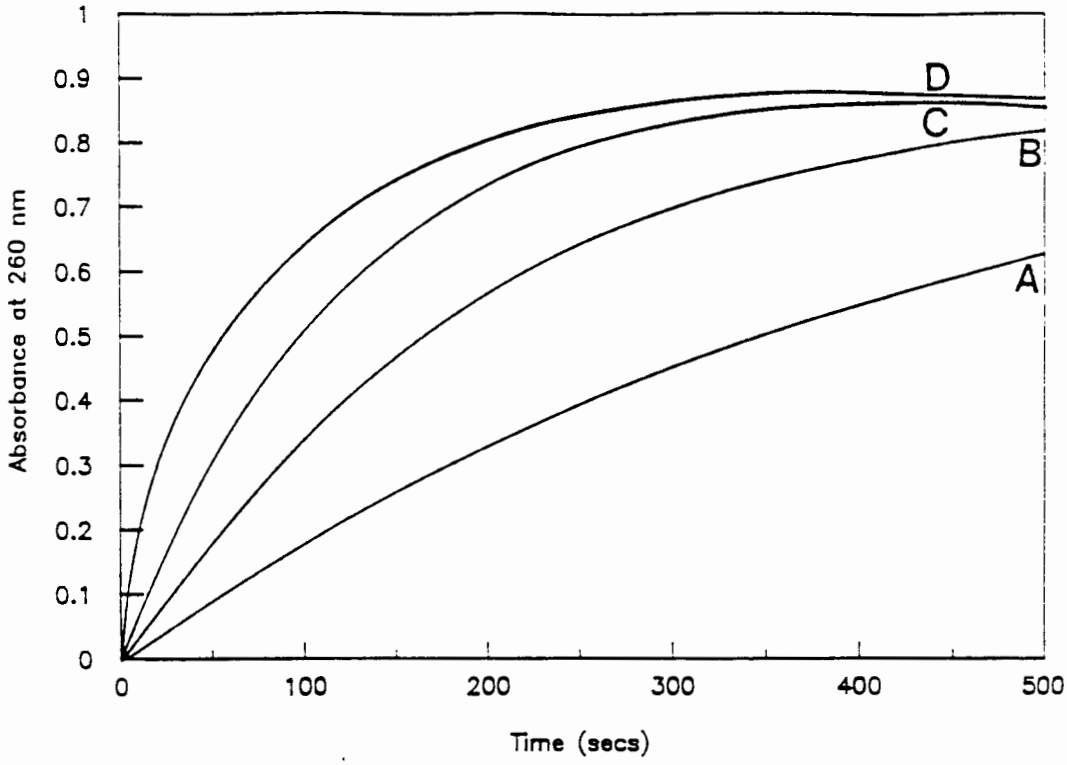

Fig 3: Enzymatic activity of increasing quantities of liposomes prepared in the presence of DNase 1 as described above and lysed with melittin. 0.1 nmole Mellitin was added to unseparated liposomes 5 nmol lipid (**A**), 15 nmol lipid (**B**), 25 nmol lipid (**D**), or to 100 nmol lipid of liposomes separated from unencapsulated enzyme (**C**).