LIPOSOME AND POLYCATION-TRANSFERRIN-CONJUGATE MEDIATED DELIVERY OF DNA FOR THE TRANSIENT TRANSFECTION OF HAEMOPOIETIC CELL LINES ~~WITHOUT SIGNIFICANT TOXICITY~~.

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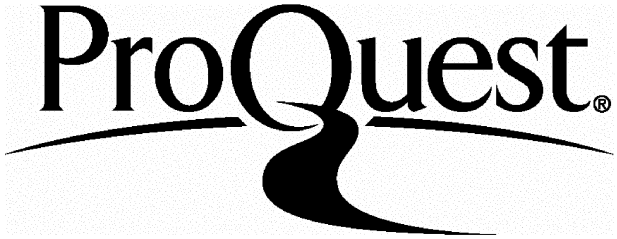

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ABSTRACT.

The aim of this work was to develop a non toxic transfection protocol suitable for the transient transfection of haemopoietic cell lines. There is a need for such a system since current methods of transfection of non-adherent haemopoietic cells are problematic. The standard method of transfection is electroporation but, to be effective, it is necessary to kill over 80 % of the cells treated, so the small surviving population of cells may not be representative of the original population. I initially optimised the electroporation of various haemopoietic cell lines. I then used fluorescent dyes as a model system for macromolecular delivery and investigated various liposome methods for the delivery of DNA, including hybrid vesicles with reconstituted Sendai virus membrane proteins. I showed that fluorescent dyes could be associated with cells but that delivery to the cytosol was inadequate for the efficient transient expression of introduced DNA. I investigated reasons why adequate expression was not seen and adopted a number of strategies to try to correct this. An alternative means of transient transfection in haemopoietic cells with low toxicity is the use of transferrin conjugated to polycation as a DNA delivery system (Transferrinfection). These conjugates bind DNA by a polycation-DNA interaction and undergo receptor mediated endocytosis. In K562 and TF1 cells transferrinfection allows efficient expression of the reporter gene chloramphenicol-acetyl-transferase with virtually no cellular toxicity. Transfection is improved by the pre-incubation of the cells in an iron-chelator and the effect of the 3-Hydroxypyridin-4-one iron chelator Cp 94 and desferrioxamine was compared. An efficient non toxic transfection system would enable the study of the mechanisms involved in the proliferation and differentiation of haemopoietic cells. Polycation-transferrin conjugates are able to mediate transient expression of a reporter gene and therefore may be valuable in future studies.

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COMMONLY USED ABBREVIATIONS

ADV-R	Adenovirus receptor
APAAP	Alkaline phosphatase / anti alkaline phosphatase
βgal	β-galactosidase
BMT	Bone Marrow Transplant
C/M	Chloroform/ Methanol
CAT	Chloramphenicol acetyl transferase
CF	Carboxyfluorescein
CHAPS	3-[3-chloroamidopropyl]dimethylamonio)-1-propane-sulphonate
Chol.	Cholesterol
CMV-CAT	Cytomegalovirus (promoter)- Chloramphenicol acetyl transferase (gene)
Cp 94	Compound 94
cpm	Counts per minute
CQ	Chloroquine
DC	Direct Cochleates
DetLUV	Large unilamellar liposomes made by detergent removal
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DPPC	Dipalmitoylphosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
ELISA	Enzyme linked immunosorbent assay
F PROTEIN	Fusion protein
FAB	French-American-British (Classification)
FCS	Fetal calf serum
Gang.	Ganglioside
+G	+ Ganglioside
GMCSF	Granulocyte-macrophage colony stimulating factor
H4	Histone H4
HA PROTEIN	Haemagglutinin protein
HAU	Haemagglutinating units
HGF	Haematological growth factor
HN	Haemagglutinin-neuraminidase
HSV-CAT	Herpes simplex virus (promoter)- Chloramphenicol acetyl transferase (gene)
HVJ	Human Virus of Japan = Sendai virus

IBE	Iron binding equivalent
IL3	Interleukin 3
IRE	Iron responsive element
LB	Luria-Bertani
LC	Liposome (before virus) cochleate
LUV	Large unilamellar vesicles
MCF	Mean cell fluorescence
MCF/MCFctl	Ratio of Mean cell fluorescence/ Mean cell fluorescence of control cells
MLV	Multilamellar vesicle
MRA	Mycoplasma removal agent
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethylene-glycol
PKC	Protein kinase C
PS	Phosphatidylserine
rbc's	red blood cell's
REV	Reverse phase evaporation
RISH	Reconstituted Influenza-Sendai hybrid
RIVE	Reconstituted Influenza virus hybrid
RSVE	Reconstituted Sendai virus hybrid
SDS	Sodium dodecyl sulphate
SUV	Small unilamellar vesicle
TA	Transfectace
TBE	Tris borate EDTA
TBS	Tris buffered saline
TE	Tris EDTA
TF's	Transcription factor's
TF-R	Transferrin receptor
TFPLCo	Transferrin-Polycation Conjugate
T_c	Transition temperature
+V	+ virus
w/v	weight / volume

CHAPTER 1 INTRODUCTION.

1.1 SUMMARY OF THE AIMS OF THE PROJECT:

The aim was to optimise transfection of human haemopoietic cells, firstly cell lines and then primary cells. The purpose of this was to facilitate studies of haemopoietic differentiation and to develop alternatives to retroviruses for gene therapy. High level transfection proved elusive and therefore the work remained focused on the development of successful transfection strategies.

1.2 HAEMOPOIETIC PROLIFERATION AND DIFFERENTIATION.

1.2.1. The Haemopoietic stem cell.

The concept of the Haemopoietic stem cell from which all cells in the haemopoietic system are derived is fundamental to the understanding of haemopoiesis. The concept has been gradually defined during the last thirty years but was largely proven by Keller et al [1] in 1985 when they reported experiments in which the retroviral mediated gene marking of stem cells was followed by the demonstration of the introduced marker in the cells of multiple lineages of the progeny. Metcalf [2] in a review published in 1989 defined four classes of haemopoietic cells each with successively larger numbers of cells within it, these are; haemopoietic stem cells, committed progenitor cells, morphologically identifiable immature cells and mature cells. This subdivision of cells can be applied to all lineages. Haemopoietic stem cells are defined as cells that are able to undergo self renewal and can give rise to the cells of the different lineages. In practical terms, stem cells are those cells which when given to recipients are capable of long term reconstitution of the haemopoietic system. It is generally believed that, in the steady state, the majority of stem cells are not in cycle and only a relatively few stem cells supply all the haemopoietic

cells whereas differentiation is associated with cells being in cycle undergoing cell doubling [3]. Transfer of true stem cells between HLA compatible individuals during allogeneic bone marrow transplantation has been shown to give rise to long term haemopoiesis of donor origin and this cell has become the natural target for the implementation of gene therapy protocols for the correction of single gene disorders (see later 1.5.).

There has been much debate as to the mechanism of lineage commitment in stem cells and this has recently been reviewed by Ogawa [3] In brief, the possibilities are that the process may be stochastic (defn: "governed by the laws of probability") or deterministic. Possible mechanisms for a stochastic process could be either the random lineage restriction of lineage potential in progenitor cells, or a stem cell could self-renew or be randomly committed to a single lineage with this process governed by the random activation of a group of differentiation genes involved in single lineage expression. Within the haemopoietic system the process of immunoglobulin and T cell receptor gene rearrangement can be cited as an example of a stochastic solution to a similar problem. By contrast, two deterministic models have been proposed where stem cells are influenced either by a "haemopoietic inductive microenvironment " or by "stem cell competition".

Ogawa [3], in his recent review, quotes evidence that colonies derived from 2 daughter cells, which were derived from a single parent cell, showed dissimilar combinations of lineages in many instances and that also the number of lineages expressed in each of the pairs of colonies differed. This data can be seen to support the operation of a stochastic process not only in the choice of lineage but also in the number of lineages expressed by the progeny of these pairs. The need for the haemopoietic system to respond to outside environmental stimuli means however that some element of determinism is probably required to fully explain lineage commitment *in vivo*.

1.2.2. Control of Proliferation and Differentiation in Haemopoietic cells.

The control of proliferation and differentiation, after the earliest stages of stem cell lineage commitment discussed in the previous paragraph, is under the control of a family of haemopoietic growth factors (HGF's). The nature and biology of these HGF's is now increasingly well understood [2-6]. HGF's are, by definition, members of the wider class of substances which mediate intercellular communication that are known as cytokines. HGF's are divided broadly into three categories; firstly those which act predominantly on primitive cells and are concerned with the cycling of dormant progenitors e.g. Interleukin 1 (IL1) (also known as Haemopoietin-1) and Stem cell factor (SCF), secondly non-lineage restricted intermediate factors acting on both intermediate and later progenitor cells e.g. Granulocyte macrophage colony stimulating factor (GM-CSF) and Interleukin 3 (IL-3) (also known as Multi-CSF) and thirdly those lineage restricted factors which act predominantly on late progenitors and mature cells e.g. Macrophage Colony stimulating factor (M-CSF) and Granulocyte Colony Stimulating factor (G-CSF), though G-CSF does have activity on some early cells. These factors act on specific cell surface receptors which for the most part have now been identified and the genes coding for them have been cloned e.g. for GM-CSF [7]. Growth factor receptors are often composed of two or more subunits (e.g. for GM-CSF [8]) both of which are required for the receptor to bind its cytokine ligand at high affinity [8]. In some cases one of the subunits is common to two or more receptors [9] (e.g. GMCSF and IL-3 [10]). Many of the subunits conform to a common basic structure which characterises the members of the so-called cytokine receptor superfamily [11]. The demands placed upon the haemopoietic system are for rapid mobilisation of mature effector cells to carry out the various functions for which the differentiated end cells are adapted and

these end cells are post mitotic and incapable of further cell division in some lineages. The time required for differentiation and proliferation from the level of the stem cell to mature effectors is incompatible with the required rapidity of response. Therefore flexibility of response is achieved by the occurrence of "Apoptosis" or programmed cell death of committed progenitor cells and their progeny if their survival is not triggered by the regulating cytokines. This process has been recently reviewed [3] and it is postulated that the presence of the relevant cytokines prevents the apoptosis of required cells and their progenitors. This model suggests that the cells which are not required undergo apoptosis and therefore the resources invested in them can be readily re-utilised. Flexibility is also given to the system by a range of inhibitory cytokines which are notable for being active at very low concentrations [3]. These include the Interferons, Tumour necrosis factor (TNF), Transforming growth factor β (TGF β) and macrophage inhibitory factor 1 α, (Mip1α) . Of these TGF-β and Mip1α appear to act early in haemopoiesis.

1.2.3. Signal transduction from HGF receptors.

At present, despite considerable increases in our understanding of receptor mediated signalling many of the details of this process remain unclear. One of the earliest events after ligand binding is protein phosphorylation and this is probably triggered by interaction between the intracytoplasmic domains of two of the receptor subunits, as shown by receptor deletion mutant studies [11]. Tyrosine kinase activity occurs even though there is no tyrosine kinase activity intrinsic to members of the Haemopoietin receptor family. However the most recent evidence suggests that a member of the Janus kinase family, Jak2, is rapidly phosphorylated as it is activated when ligand binds to these HGF receptors and then has a critical role in linking the receptor to the other downstream signalling pathways [12-14]. These

events can occur because this protein is thought to be closely associated with the intracytoplasmic portion of the receptor even prior to ligand binding. This single kinase has been implicated in signalling from a wide variety of different growth factor receptors. The central role of Jak2 in the signal transduction from different HGF receptors might be explained by the suggestion that the diversity of growth factors exists to allow target cell specificity rather than diversity of message. However deletion mutant receptor studies [11] also suggest that in some cases a specific deletion may abrogate the signal to differentiation but leave the proliferation signal intact thus showing that a single receptor may signal to different downstream pathways. The further pathways, beyond Jak2, involved in the transmission of the HGF binding signal are complex and under constant review, in particular the role of PK-C has not been fully elucidated. At the time of the commencement of this project the varying isoforms of Protein Kinase C (PK-C) had been implicated in growth factor signal transduction [6, 15-17] and the PK-C isoform profile in normal haemopoietic cells of different lineage and stage of differentiation was under study in the Department of Haematology, University College London. The results of this work have now been published [18] and show that during differentiation towards monocytes and neutrophils there is an upregulation of mRNA for both the β1 and β2 isoforms of PK-C. Terminal neutrophil differentiation, but not monocyte differentiation, is associated with a down regulation of PK-C α mRNA and later PK-C α protein. This work lead to the question of whether these changes in PK-C isoform profile were a consequence of differentiation or the trigger for differentiation to occur. It was reasoned that one approach to defining the role of specific PK-C isoforms in the mediation of growth factor effects would be to modulate the levels of the individual isoforms in myeloid cells and observe the effects of such manipulation. This was one of the questions to resolve which required the development of

a non-toxic means of transfecting haemopoietic cells. In particular, HL 60 cells which by exposure to certain agents can be induced to move either towards a monocytic or a neutrophilic phenotype and growth factor responsive cells such as TF-1 [19] were to be studied.

The current state of knowledge of growth factor signal transduction has been summarised by Miyajima [11] in the aforementioned review. In this review Miyajima suggests that the intermediate pathways involved after the initial tyrosine kinase may include firstly Phospholipase C (PLC) and Phosphatidyl Inositol 3 kinase (PI3K) (both of which may act via PK-C) and secondly other complex signalling pathways involving molecules such as the GTP binding protein, Ras and two serine / threonine kinases Raf and MAP kinase (Mitogen activated protein kinase). The nuclear pathways which have been implicated at the end of these cascade pathways include c-fos, c-jun and c-myc and therefore the nuclear events resulting from transduction of the original receptor signal include modulation of the expression of transcription factors.

1.2.4. Transcription factors in the regulation of Haemopoiesis.

The regulation of gene expression within haemopoietic cells is also controlled by a system of nuclear-regulatory proteins or transcription factors (TF's). Transcription factors are DNA-binding proteins which by combining with specific recognition nucleotide sequences in the promoter and enhancer regions of genes are involved in the regulation of the expression of those genes. The GATA family of TF's were named because of the characteristic 4 nucleotide motif included in the nucleotide sequence {(T/A)GATA(A/G)}. The binding of the GATA TF's to this motif regulates the expression of genes downstream on the sense strand from the GATA motif (that is it is cis-regulatory). GATA TF's have been highly conserved throughout evolution which underlines their importance across many

species. Member's of the GATA family are found in other systems but have been most closely studied within the haemopoietic system where they are differentially expressed in the different lineages so, for example, the binding of GATA -1 to a GATA motif was described first in the locus control region of the α and β globin gene loci and is essential for normal erythroid differentiation [20]. GATA 1 however is also expressed in megakaryocytes and mast cells. TF's have also been implicated in leukaemogenesis by virtue of putative transcription factor genes being present at sites of chromosomal translocations [20]. A recently reported study of the dynamics of TF expression in peripheral blood progenitor cells during erythroid differentiation [21] showed that the expression of GATA factors in erythroid cells is distinct from that of the myeloid or lymphoid lineages with a significant decline in the level of GATA-2 with erythroid differentiation. GATA -3 seems to be confined in human haemopoiesis to the lymphoid (T cell) lineage and is not expressed in erythroid or myeloid development. In addition when this group examined the effect of erythropoietin (Epo.) in this system it was found that those cells deprived of Epo. did not differentiate and maintained high levels of both GATA-1 and GATA-2 whereas in cells with Epo. GATA-1 increased and GATA-2 declined as predicted from the results observed with erythroid differentiation. The authors conclude from these experiments that the levels of different GATA factors may influence the progression to specific haemopoietic pathways. The controlling influence of the GATA factors in haemopoiesis has also now been demonstrated by the manipulation of the expression of the members of this family by gene transfer. In these experiments reported by Visvader et al [22] the levels of GATA-2 and GATA-3 were modulated by electroporation and selection of stably transfected cells which expressed the transfected gene in a myeloid cell line which did not normally differentiate. The experiments resulted in megakaryocytic differentiation, though interestingly

endogenous GATA-1 was greatly increased and was probably the dominant regulator of this megakaryocytic differentiation. The authors postulate that this was because GATA-2 and 3 lie upstream of GATA-1 in a regulatory pathway and that therefore GATA-1 may mediate the phenotypic changes caused by modulation of GATA-2 or 3.

To summarise therefore in terms of haemopoietic development the current view is that GATA-1 is expressed in multipotential progenitor cells and then downregulated in myeloid cells and upregulated in erythroid cells. The control of GATA-1 is not well understood but includes an autoregulatory loop and in addition GATA-1 expression plays a role in the regulation of the expression of the receptor for erythropoietin thereby linking the role of the transcription proteins of the GATA family with the cellular response to growth factor stimulation [20]. Also in his review Orkin [20] discusses the possibility that one target of GATA-1 may be another transcription factor, the stem cell leukaemia (SCL) gene (which has a GATA motif), thus it is possible that different TF's may form an order of regulatory events in haemopoietic development. Much remains to be learnt about the role of these factors in the control of haemopoiesis and it is clear that a non-toxic transfection system would be of great value in manipulating the expression of these factors to observe the phenotypic consequences of such manipulation.

1.3. REVIEW OF METHODS OF TRANSFECTION

1.3.1. Definition of Types of Transfection:

Transfection is the introduction of exogenous DNA into cells leading to its expression by the target cell. Transfection or Gene transfer may be either stable or transient. Transient transfection does not require the introduced DNA to be integrated into the target cell chromatin, may normally be detected within hours of the transfection process and will rarely persist beyond 80 hours. The degree to which the transfected genes are expressed can be monitored by the expression of reporter genes, that is genes which lead to the expression of an easily monitored protein which is normally absent or present only at very low levels in the untransfected target cells. An example of this is the bacterial protein Chloramphenicol acetyl transferase [23].

Stable transfection occurs when the introduced DNA has been stably integrated into the target cell chromatin. Stable integration of introduced DNA even with the most efficient methods will occur at a low frequency so most protocols using stable transfection will also employ a selection step so that transfected cells are selectively encouraged to survive, normally by the ability to grow in an otherwise toxic environment e.g. by the acquisition of an antibiotic resistance gene which confers resistance to an antibiotic such as neomycin.

1.3.2. Conventional Methods of Transfection: Calcium Phosphate Precipitation and the DEAE dextran method.

Many methods of transfecting cells have been described but conventionally the two most widely used methods have been Calcium phosphate precipitation and the DEAE dextran method. These methods are described in detail in a review of mammalian gene transfer by Gorman [24]. Both these methods depend on presenting the DNA to the cells to be transfected

in a physical form that it is suitable to be taken into the cytoplasm during an incubation step either as a calcium phosphate -DNA precipitate in the former method or as a DEAE-dextran-DNA complex in the latter case. The physical nature of the precipitate in the former case is critical to uptake by the cells, for example it is said that the best precipitate is "small grains which cover the cell" whereas if the conditions are unsuitable "large spheres of precipitate will float in the medium" [24]. Careful optimisation of the parameters has been found to be essential in either case however inspite of their widespread application in many gene-transfer protocols both techniques are of limited value in the transfection of haemopoietic cells [25, 26].

1.3.3. Electroporation.

Greater success in the transfection of haemopoietic cells has been reported following the use of electroporation [25-27]. Electroporation involves the passage of high voltage electric pulses between two plates in a specially engineered cuvette. These electric pulses create pores in the target cell membrane through which the target DNA is able to pass into the cytosol of the cell from a surrounding culture medium containing purified DNA. These pores are believed to occur at lipid-protein junctions and /or protein sites in the cell membrane [27]. This "poration" of the membrane however is highly toxic to many of the cells since, if the pore size is too great, rapid restoration of membrane continuity is not achieved. Conversely, if insufficient "poration" occurs then the pores do not allow the effective delivery of the DNA from the surrounding DNA suspension. While toxicity is not of prime importance when cell lines are used, because of the relatively unlimited availability of target cells, it is of much greater importance if primary haemopoietic cells are to be used since the target stem cells to be studied will be in short supply. A further consideration is that if transfection

experiments are to be used in the study of mechanisms of differentiation then sub-lethal toxicity may result in an unrepresentative situation occurring which is of limited value in understanding the normal control of differentiation.

1.3.4. Commercially available Lipids used for DNA delivery.

There are a number of cationic lipids which are marketed for use in transfection studies. These compounds depend on the formation of a tight DNA-lipid complex during an incubation step which, in the same way as with calcium phosphate precipitation, presents the DNA in a suitable form to be taken up by the target cells. The first of these compounds, was described by Felgner et al [28] and its full chemical name is ; N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) it is now commercially available in combination with phosphatidylethanolamine (PE) as Lipofectin (Gibco-BRL, UK). The original paper described Lipofectin mediated transfection in two animal cell lines COS-7 and CV-1 (Simian Kidney). There do not appear to be any reports of successful transfection in human myeloid cell lines. A related compound, Transfectace (TA), has also been marketed by the same company (Gibco BRL) and is reported (in commercial information-[29]) to be effective in transfecting baby hamster kidney and HeLa cells. Transfectace is a combination of a cationic lipid : dimethyldioctadecyl ammonium bromide (DDAB) and a neutral phospholipid dioleoyl-phosphatidylethanolamine (DOPE) and is conformed as liposomes to which the DNA is externally attached but not encapsulated. Another product, Transfectam, is a lipopolyamine molecule with a spermine group fixed in the terminal position by covalent attachment (peptidic attachment). Transfectam has been reported to transfect primary endocrine cells [30].

The use of these compounds should be distinguished from the use of neutral or negatively charged lipids in liposomes, in these true liposomal methods, which are described in the next section, the macromolecule to be delivered is encapsulated in the aqueous interior of the liposomes whereas in these protocols the lipid and DNA exist as a tight complex which is taken up by the cell.

1.3.5. Liposomes and their use in Transfection studies:

1.3.5.1. Introduction to liposomes:

Liposomes are artificial lipid vesicles normally composed of phospholipids. Phospholipids are amphipathic molecules because of their composition of hydrophilic head groups and hydrophobic acyl chains. The nature and charge of the polar head group as well as the nature of the fatty acyl chains will determine a lipid's physical properties. When phospholipids are placed in an aqueous solution they spontaneously form continuous bilayers with all the polar head groups facing either externally to the surrounding aqueous medium or internally to an aqueous internal space and with all the fatty acyl chains arranged internally within the bilayer. These can either convert spontaneously to liposomes or be converted to liposomes by a variety of physical methods. The liposomes thus formed are made up of closed spherical micelles composed of these bilayers with either one lamella or multiple lamellae. Macromolecules can be trapped either within the aqueous internal space or within the hydrophobic space within the bilayer or molecules maybe attached to the exterior of the liposome as discussed above. The analogy between lipid bilayers and cellular membranes is readily apparent and this has provoked many years of interest in their potential therapeutic value as a delivery vehicles.

The major determinants of the nature of liposomes are the particular lipids used in their manufacture (both the polar head group and the fatty acyl

chains), the number of lamellae and the size of the liposome. In addition to phospholipids, cholesterol maybe included in liposomes and this will tend to have the effect of stabilising them and reducing leakage from the internal space [25]. The physical properties of a lipid are also related to it's transition temperature (T_C) between the gel state and the liquid crystalline state, which exists at higher temperatures, for lipids dispersed in a bilayer arrangement in an aqueous solution. This temperature is characteristic for any particular lipid. In the former state the fatty acyl chains are tightly packed but above the (T_C) the fatty acyl chains are more mobile and the thickness of the bilayer is decreased (reviewed in Gray et al [31]). The state of liposomes either side of this transition temperature may differ significantly, for example some phosphatidylserine liposomes are unstable below their transition temperature [32] which lies between 25 and 30°C. The physical properties of the lipid are related to its acyl chains as well and the T_C for a particular head group will tend to increase with increasing fatty acyl chain length and a higher degree of unsaturation [31].

1.3.5.2. Use of Liposomes to deliver molecules to cells.

The ability of a liposome to deliver DNA or any other encapsulated macromolecule is also dependent on the size of the vesicle. The size of the vesicle will determine not only the volume of encapsulation but also the route of any interaction with the target cell. With regard to encapsulation, since the volume of a sphere is represented by $4/3\ \pi\ r^3$, the increase in the volume of encapsulation and hence the amount of DNA encapsulated increases with the cube of the vesicle radius. In simple quantitative terms this means that a small unilamellar vesicle (SUV) with a typical radius of approximately 50 nm will have 1/ 10 000 th the encapsulated volume of a large unilamellar vesicle (LUV) of a typical radius of 1 μm though this will be offset by the larger numbers of the smaller liposomes made from a given

amount of lipid. Thus larger liposomes will demonstrate dramatically increased encapsulation efficiency and this encapsulation efficiency is further increased if the speed of vesicle formation is slow as with the rotary dialysis process developed by Gould-Fogerite and Manino [33]. However, in addition the nature of the cell: vesicle interaction will also be different for differently sized vesicles. SUV tend to be endocytosed [34] but LUV, because of their size, are unlikely be endocytosed and will only be delivered by fusion of the liposome with the cell membrane. This fusion may be influenced by the lipid composition of the liposomes [35] or because of the utilisation of additional targeting strategies [25].

The fate of liposomes was examined by Straubinger et al [36] who reported, in 1990, the use of the fluorescent probe pyranine to follow the fate of liposomes after interaction with target cells. In this paper they suggested that material delivered by the endocytic pathway to liposomes was progressively acidified to approximately pH 5.5. The material then remained within acidic cellular vesicles for 2-3 days with little appearing in the cytoplasm suggesting that fusion of these vesicles with cellular membranes rarely occurs. Two strategies have been adopted by other groups to overcome this apparent failure to deliver liposomal contents to the cytoplasm. Firstly large unilamellar liposomes can be made entirely from phosphatidylserine, a negatively charged lipid which is highly fusogenic, by a process described by Papahadjopoulos et al [37]. This method involves the formation of an intermediate complex of calcium and lipid which is precipitated from an aqueous suspension of the lipid by the addition of calcium. When the correct conditions are used the intermediate complex, or cochleate, formed has the conformation of a rolled up sheet which in cross-section has a cochlear appearance. The calcium can then be removed from this cochleate by chelation with EDTA causing large

unilamellar vesicles to be formed (for detailed method see chapter 2.5.3.) This strategy was adopted by Itani et al with apparently successful transfection achieved in a variety of cell lines [35]. Alternatively, the failure of delivery from the endocytic pathway can be overcome by the inclusion in the liposomes of the pH sensitive lipid phosphoethanolamine, which becomes unstable at acid pH. This strategy has been reported in at least two papers [38, 39] but does require the liposomal contents to pass through the endosomal compartment and, at least transiently, be exposed to their acidic pH and a range of enzymes which may digest the macromolecule to be delivered.

1.3.5.3. Use of Viral Membrane Proteins for enhancing Liposome delivery:
The most promising strategy for overcoming the failure of liposomes to fuse with the target cell membrane is the use of enveloped virus membrane proteins. A significant proportion of the liposomal transfection protocols reported to date have attempted to utilise these proteins to promote fusion of liposomes, loaded with DNA, with the target cell. While there are anxieties, as discussed later, associated with the use of adenoviruses and retroviruses there is no human tumour association with the ortho and paramyxoviruses. These two groups of viruses are enveloped, RNA viruses that have these membrane associated proteins which can be utilised in order to fuse liposomes with target cells. Most attention has focused on the use of the Human Virus of Japan (HVJ) also known as Sendai virus and on the Human Influenza virus. Both viruses have membrane proteins with Haemagglutinin (HA) , Neuraminidase (NA) and Fusion (F) activity but they differ in that in the case of Sendai Virus HA and NA are combined in one molecule (HN) [40].

Reconstitution of Viral Envelopes:

Three strategies for the utilisation of these proteins have been tried, firstly disruption with detergent and reconstitution into virus envelopes after detergent dialysis, secondly using ultraviolet light inactivated virus and thirdly the creation of a combination of reconstituted virus protein and liposomes in a hybrid vesicle called a chimerasome. These strategies have been pursued independently by different research groups.

The first strategy of using reconstituted virus envelopes has been pursued by a group in Jerusalem who have investigated both Sendai virus and Influenza virus reconstituted envelopes in great detail. Initially in fluorescence experiments they used the dequenching phenomenon, where an increase in fluorescence can be observed when a very concentrated solution is diluted, to demonstrate an interaction between reconstituted Sendai virus envelopes (RSVE) and red cell ghosts or hepatoma cell lines. They noted that dequenching occurred with fusion of liposomes with cells or, to a lesser extent, endocytosis by cells [41]. It is important to note that the fluorescent label was associated with the vesicle bilayer and not the aqueous interior of the vesicles, as is the case in experiments described in Chapter 4, and it should be noted that the erythrocyte ghosts used are not a typical target since the Haemagglutinin molecule will have a specific interaction with them. In addition the process of viral reconstitution is inefficient and results in small virus like vesicles, which will not encapsulate as much of the selected macromolecule as larger liposomes. A further report [40] from this group using reconstituted influenza virus envelopes (RIVE) showed that RIVE when incubated with phophatidylcholine SUV fused in a non lytic manner (no fluorescence dequenching). The interaction of RIVE with mouse S-49 lymphoma cells at pH 7.4 and pH 5.0 showed dequenching at both pH's however at pH 7.4 though not at pH 5.0 the process could be blocked by lysosomotropic agents. This suggested

that at the higher pH the dequenching was due, at least in part, to endocytosis. On the basis of these and other studies in 1990 they investigated DNA delivery to COS-7 cells. For the reasons outlined above, however, they did not use reconstituted virus envelopes alone but in order to overcome the relative inefficiency of encapsulation they loaded the DNA in liposomes made by reverse phase evaporation (REV) and then incubated these with the RIVE or RSVE prior to addition to the cells. This is based on the technique of Kaneda et al which will be described below [42]. In addition they used the intact viruses in the same manner. They report the use of both RIVE and RSVE and state that vesicles derived from either Sendai or Influenza virus, or in some cases a combination of the two (RISH), are equally effective [43]. In the case of RIVE the mechanism of introduction of the DNA is probably by release from endosomes after receptor mediated endocytosis of the RIVE / liposome pair because the influenza virus fusion protein is only activated at the acid pH found within endosomes. They also report that transfection of cells is blocked by treating the virus or viral envelopes with Dithiothreitol (DTT), which inactivates viral glycoproteins, whereas transfection is not blocked by treatment of the liposomes with DNAses which are unable to digest the DNA when it is encapsulated in the liposomes. It is notable, however, that in this paper that they do not report any transfection using RSVE or RIVE alone, nor give any advantages for the use of reconstituted virus particles over the use of intact virus as discussed below. Neither do they report any results in cells other than COS-7 even though they do report fluorescence data on the interaction of a variety of liposomes with HeLa cells, a human cervical cancer cell line.

Use of Ultraviolet Irradiation to Inactivate Human Sendai virus:

The second group in this field followed a strategy of using UV inactivated Sendai virus and liposomes in combination [42]. This group initially

reported in 1979 [44] that using a detergent dialysis technique liposomes could be reconstituted with viral membrane proteins but that DNA was not successfully encapsulated. They therefore adopted a strategy where DNA was encapsulated by using a REV technique to make liposomes and then these liposomes were allowed to interact with intact Sendai virus which had been U/V inactivated to prevent viral replication. This interaction was initially of low efficiency until gangliosides were included in the liposomes to act as a virus receptor for the haemagglutinin protein to promote the formation of a fusogenic liposome / virus complex . This technique was published [42] in 1987 with successful stable transfection of adherent Mouse L cells, of Ehrlich's Ascites tumour cells in suspension (also murine) and most importantly, but at lower efficiency, of the HeLa human cervical carcinoma cell line. The potential of this system as a macromolecular delivery system was accentuated by the successful delivery of, in addition to DNA, mRNA and proteins such as IgM (with the use of red cell ghosts). The efficiency of the transfections, in parallel experiments, surpassed that of the phosphatidyl serine liposomes (PS LUV) made by calcium chelation which were used in transfection studies by Itani et al [35], with PS LUV no advantage was detected in the presence of the inactivated HVJ probably because the inclusion of gangliosides was not effective in this liposome type. The authors concluded that the process was a significant advance over previous techniques such as Calcium phosphate precipitation. Since 1987 the same group has improved the transfection efficiency achieved with this technique by the introduction of the DNA after it has been associated, by pre-incubation of the DNA with the non-histone nuclear protein, HMG-1 which binds specifically to double stranded DNA. It appears that the nuclear protein in the DNA-nuclear protein complex facilitates migration of the plasmid DNA into the nucleus. These

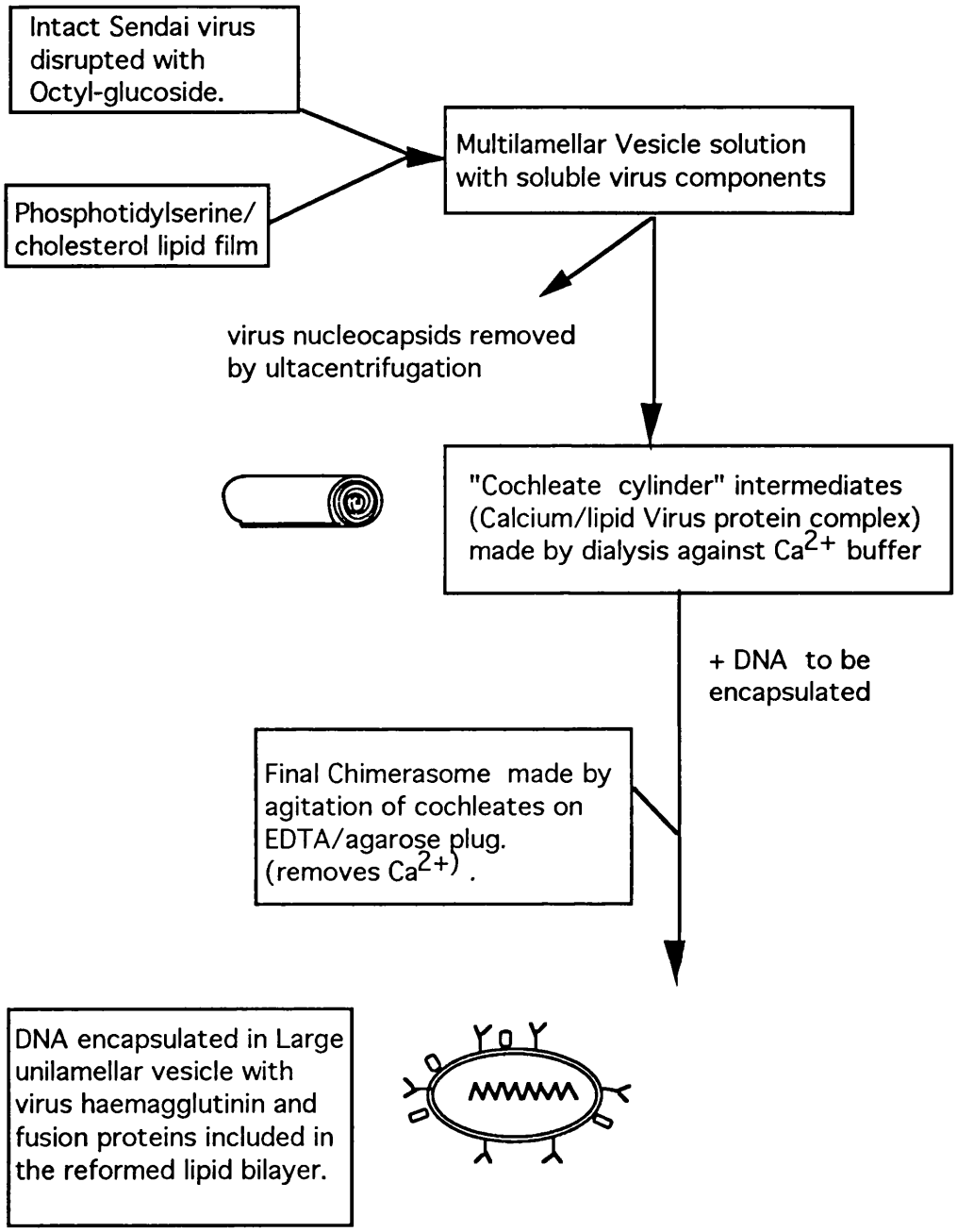
improvements have enabled the technique to be extended to *in vivo* experiments [45] [46].

Synthesis of Chimerasomes:

The third group which has published on a technique for virus protein mediated liposomal transfection is that of Gould-Fogerite and Manino whose protocol combines elements of the strategies discussed above [25, 33]. This protocol was based on the PS liposomes made by calcium chelation originally described by Papahadjopoulos et al [37] and used for transfection by Itani et al [35] but combines the Sendai virus membrane Haemagglutinin / Neuraminidase and Fusion proteins into the cochleate cylinder intermediates. Therefore after calcium chelation a hybrid particle or " chimerasome" is formed which is of sufficient size (up to 0.1-4 μm) to allow highly efficient DNA encapsulation compared with the size of the RSVE formed when virus envelopes alone are reconstituted (see FIGURE 1.1). In addition the virus membrane lipids and proteins are included within the hybrid particle without the need for a separate incubation step between liposomes and virus, as with the Kaneda protocol. This technique also avoids the use of organic solvents during macromolecular encapsulation which increases the ability to encapsulate a range of molecules, especially proteins which would be denatured by the REV process used by the Kaneda group (hence their need to use red cell ghosts). The high encapsulation efficiencies were best achieved by slow removal of the calcium with Rotary dialysis but the protocol has more recently been much simplified (Dr S Gould-Fogerite-Personal Communication) by the use of an agarose / EDTA plug technique to allow slow calcium chelation. The transfection studies reported by this group in 1989 [25] stated that the technique was 100 000 times more efficient than Calcium Phosphate precipitation. This paper included firstly demonstration of delivery of antibody conjugated fluorescence to Rat PTK_1 cells and then the technique

was used, *in vitro* , to transfect a mouse mammary tumour cell line C127 and *in vivo* to cause tumours in mice by the introduction of the early region of the polyoma virus. Both Sendai virus and Influenza virus proteins were studied but only Sendai virus data was reported for the *in vitro* studies. Overall this protocol appeared to be a highly efficient method of transfecting cells *in vitro* and *in vivo* though as with the other two groups no data was available on the use of these techniques for the transfection of either human haemopoietic cell lines or primary haemopoietic cells. In addition to these groups Sechoy et al [47] have followed a related strategy based on Sendai virus membrane proteins incorporated into phospholipid liposomes by detergent lysis, mixing and then detergent dialysis. In addition to targeting by the virus protein they used a specific antibody directed against a plasma membrane antigen of the target T101 cells. They demonstrated the efficacy of this technique by using Hygromycin to decrease cellular tritiated thymidine uptake because the hygromycin, if successfully delivered, had an inhibitory effect on the target cells. A potential limitation of this technique is that it requires in the case of each cell to be targeted a specific antibody to be available for incorporation in the liposomes and there are as yet no reports of whether DNA delivery by this protocol is effective.

FIGURE 1.1. SUMMARY DIAGRAM OF CHIMERASOME SYNTHESIS

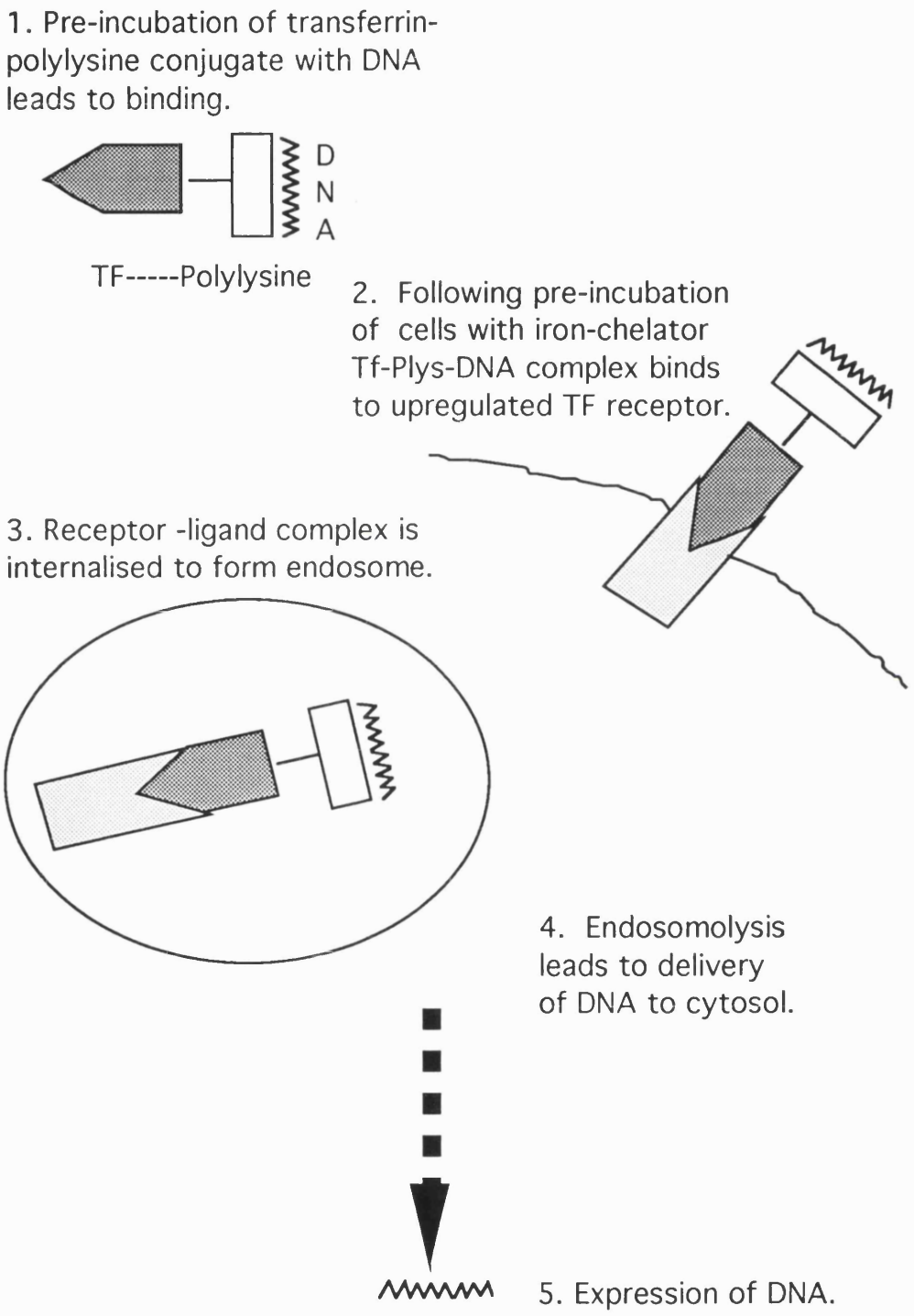


1.3.6. TRANSFERRINFECTION:

1.3.6.1. Introduction to Transferrinfection:

Transferrinfection is a transfection protocol in which DNA is delivered by receptor mediated internalisation of transferrin-Polylysine-DNA complexes. The technique was described by Wagner et al as recently as 1990 [48] and immediately reported to be effective in the transfection of a haemopoietic cell line K562 [49]. The technique has rapidly become established as a means of DNA delivery to cells so that Mulligan [50] in his recent review comments that with regard to non-viral methods of gene transfer ; "interest is centred on methods that rely upon receptor mediated endocytic pathways". Wu and colleagues have also reported success with a modification of the technique in transfecting hepatic cells [51] and it also appears to be suitable for *in vivo* use in animal experiments [52]. In brief, the iron binding protein transferrin is coupled to poly-L- Lysine by means of disulphide bonds after modification with the bifunctional reagent succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to make a transferrin / polylysine complex (TFPLCo) which is able to associate with DNA. These complexes can then be delivered to cells via the transferrin receptor (TF-R) on the cell membrane because the transferrin part of the conjugate associates with this receptor (see FIGURE 1.2.). The level of expression reported without two early modifications to the protocol was very low and was quickly improved by their implementation. Firstly upregulation of TF-R expression was achieved by means of the iron chelating agent Desferrioxamine [53]. This process is dependent on the relationship between TF-R messenger RNA levels and the availability of iron which is mediated by a group of Iron responsive elements (IRE) which are present within the 3' untranslated region (UTR) of the human TF-R mRNA [54]. The problem with the use of desferrioxamine in this situation is that it is known to inhibit haemopoietic cell proliferation [55] which would not be desirable if

FIGURE 1.2. Summary Scheme to describe Transferrinfection.



rapid expression of the introduced gene was required. Secondly since the endocytic pathway leads to the delivery of the conjugates to endosomes some degradation of the DNA can be expected due to the acidic pH and the nucleases present in endosomes. This process can be counteracted, at least in part, by the use of Chloroquine (CQ) which has a range of effects particularly on blocking acidification of the endosomes. The use of CQ leads to a marked increase in the expression of the introduced DNA [53]. The authors of this paper also comment that the transferrin receptor system differs from many other cellular ligands in that the transferrin cycle is thought not to interact with the lysosomal compartment, however they believe, from their own data on the inhibitory effect of cell chilling, that the TFPL complexes probably are processed through the lysosomal compartment.

This group have concentrated from an early stage on a haemopoietic cell line ; K562, a robust cell line which is relatively easy to transfect by other means such as electroporation [56]. K562 [57] is derived from a patient who had an erythroleukaemic blast crisis of Chronic Myelogenous Leukaemia (CML). One of the reasons for further studying this technique in this project was to identify whether this technique could be easily extended to a range of other myeloid cells lines since transferrin receptors are expressed by all neoplastic or fast dividing haemopoietic cells [58]. The need for chloroquine is not a problem in the context of K562 cells because they are able to tolerate the required concentration of 100 μM for the 4 hour duration used in the protocol but the degree of improvement reported raises the possibility that this effect may be related to some particular property of the K562 cell line which might not be found in other haemopoietic cell lines. Indeed the originators of this technique speculate in a review article [59] that a lack of the Na+, K+-ATPase regulation of endosomal acidification, specifically in K562, may lead to rapid endosomal

lysis in the presence of CQ. This raises the question of whether this technique can be applied to use with other target cells.

1.3.6.2. Development of Transferrinfection 1: DNA conformation:

In order to attempt to optimise this technique there has been study of the importance of the DNA conformation and how this is related to DNA delivery to cells [60]. Polylysine has been known to complex with DNA causing the DNA to collapse into very compact particles for many years [61]. The understanding is that the conformation of the TFPL-DNA complex adopted after this condensation process may have importance for transfection. It is suggested that the formation of a "DNA Donut" form, characterised by a condensed DNA/Polylysine core and transferrin molecules forming an outer coat, appears to be optimal. This has lead to the investigation of the use of other proteins in association with TFPLCo. For example additional unconjugated polylysine or Histones (specifically H4) can be added to the TFPL and DNA incubation which has the effect of increasing the efficiency of transfection [60]. Modification of the original protocol using Ethidium homodimer [62] may also be valuable since this enables an alternative TF attachment to the original TFPL complexes. These transferrin-ethidium homodimer conjugates however do not condense the DNA in the way described above unless they are mixed with Polylysine to ensure that, in a ternary complex with DNA, condensation by the polylysine is achieved.

1.3.6.3. Development of Transferrinfection 2: Use of Adenovirus:

The success of the use of chloroquine to enhance the release of the transfected DNA from the endosomes before degradation occurred lead to the investigation of other techniques for promoting endosomolysis. Birnstiel and colleagues have now reported two related techniques involving the use of adenovirus to cause endosomolysis of the introduced DNA. In the first, defective or chemically inactivated adenovirus is used to promote endosomolysis [63]. The adenovirus is added to the target cells at the same time as the TFPL complexes and, though the two are internalised via the adenovirus receptor and the TF-R respectively, they are both internalised to a common endosome which is then lysed by the adenovirus thus causing release of the TFPL Co to the cytosol. The adenovirus has not been genetically modified and is used only as a tool to deliver the conjugates. Safety concerns are reduced because the strain used is defective for transcription and replication and in addition, since the virus endosomolytic activity lies within the protein coat, the virus core can be inactivated by UV irradiation. A further advantage is that the size constraints imposed by the need to include constructs within viral genomes, in the case of retroviral transfection, do not apply. This use of adenovirus enables transfection of cells such as HeLa which are not usefully transfected by the use of TFPLCo and Chloroquine alone. This report, however, does not include data on human haemopoietic cells since they are relatively deficient in adenovirus receptors and the presence of both adenovirus receptors and TF-R are required for the best functioning of this technique. However a further refinement of the use of adenovirus with Transferrinfection is one in which the TFPLCo and the adenovirus are physically linked by means of either an enzymatic transglutaminase link or via a biotin-streptavidin link [64]. This alternative approach ensures that the whole complex will pass to the same endosome so that the adenovirus can

promote endosomolysis to release the introduced DNA to the cytosol. With this modification of physically linking the adenovirus and the TFPL-Co the ability to transfect haemopoietic cells is restored. In K562 cells, for example, in which there are relatively few adenovirus receptors the virus is delivered as a complex with the TFPLCo via the TF-R. Once internalised, the virus is then able to promote endosomolysis after delivery to the endosome.

1.3.6.4. Development of Transferrinfection 3: Use of Synthetic Influenza Virus Polypeptide:

The most recent innovation in the development of transferrinfection is the use of a synthetic polypeptide based on the N-terminal region of the Influenza virus Haemagglutinin HA-2 molecule to replace the role of the adenovirus as described above [65]. This represents a major step forward in potential safety because this is a synthetic rather than virus derived molecule for though it is based on the Influenza protein's structure it is not obtained from virus culture. The technique is effective in HeLa cells as well as K562 cells and involves the use of TFPLCo bound to DNA being mixed with polylysine-conjugated peptides to form one complex. The influenza polypeptide replaces and duplicates the endosomolytic effect of the adenovirus.

1.3.6.5. Duration of Expression of Introduced DNA after Transferrinfection:

K562 cells will express the transfected genes for up to 2-3 weeks after transferrinfection with TFPL conjugates. In this situation between 0.5 -1.0 % of cells may become stable transfectants when the *neo* selectable marker is used [66]. The degree of successful establishment of stable transfection by transferrinfection has recently been reported [67] to be much increased in HeLa cells and a myoblast cell line by the use of a

Chicken Adenovirus (CELO) in combination with transferrinfection, however no data on haemopoietic cell lines was included in this report. CELO virus has the advantage of being replication deficient in mammalian cells.

1.3.7. Retroviral transfection systems.

Retroviral vectors are infectious, replication deficient retroviruses which contain in the place of the normal retrovirus sequences the sequences coding for the gene to be introduced to the target cells. The vector also contains virus long terminal repeats (LTR's) and adjacent short regions (denoted by the notation ψ) that provide replication, packaging, insertion, transcriptional control and RNA processing signals. They are used in combination with packaging cell lines (normally murine fibroblasts) which have an integrated proviral genome which expresses the proteins required for the production of infectious virus particles but lack the ψ regions. Once the packaging cell lines have been transfected with vector they are described as "Producer cell lines". Retroviruses have the advantage over most other transfection techniques of efficient and stable integration, they also have a wide host range and the ability to infect large numbers of cells. On the other hand the size of the DNA sequence to be introduced is limited by the size of the virus and there is difficulty in targeting the virus if a mixed population of cells is to be treated since the virus receptors for most retroviruses are not known [50]. Most important, however, is that for the purposes of application to clinical gene therapy studies they have significant safety disadvantages because of the concern that recombination events might lead to the generation of replication competent virus (for further discussion of safety issues see later 1.5.1.). These recombinations may involve the packaging helper virus or unidentified

retroviruses integrated in host DNA. Two measures have been used to try and prevent such significant recombination events occurring:

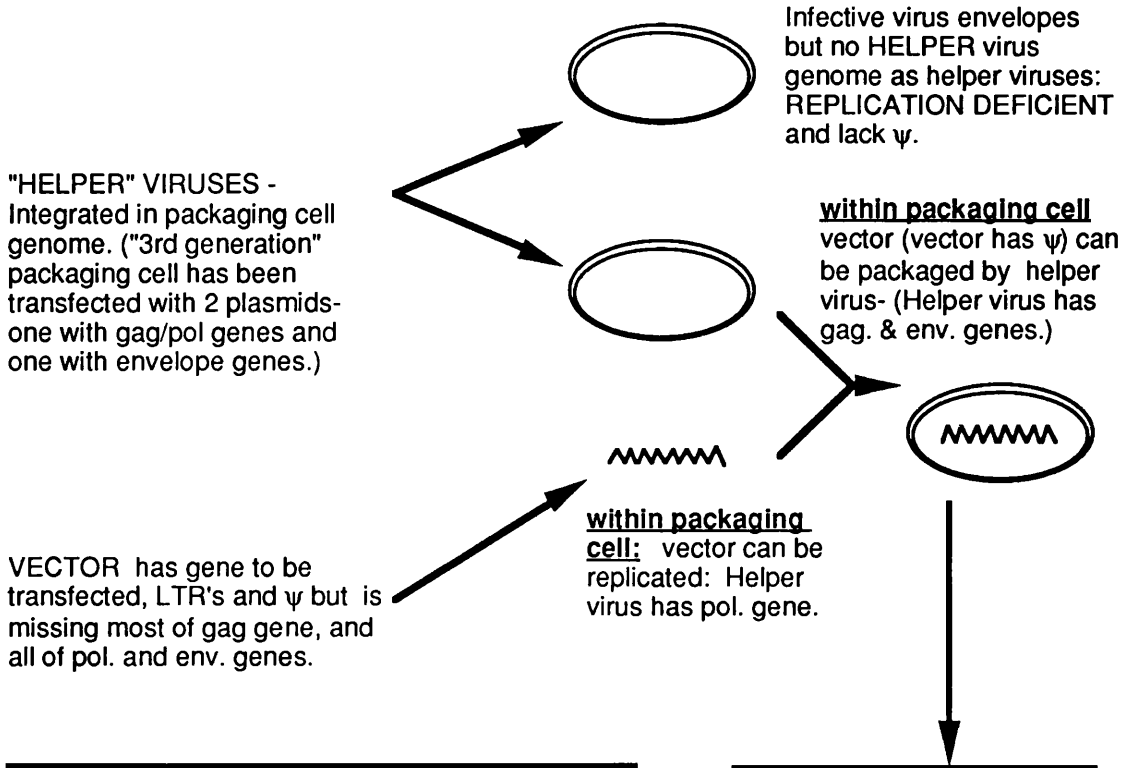
Firstly, as stated above, the retroviral vectors are produced in packaging cell lines. In these cells the vector proviral genome containing the gene to be transfected and the retroviral sequences necessary to achieve this are "packaged" into infectious particles by a second "Helper virus " present as a proviral genome in the packaging cell line, and, due to introduced mutations the packaging cell line is unable to produce replication competent virus.

Secondly in the so called third generation packaging lines additional safety is added by the separation of the genes coding for the ecotropic (ecotropic = capable of growth only in cells of the natural host) gag / pol sequences (which code for internal virus proteins and the viral polymerase respectively) and the amphotropic envelope proteins (amphotropic = capable of growth in cells of the natural host and cells of other species) envelope proteins on different transcriptional units. Also a portion of the gag gene is retained in the vector that is necessary for packaging of the vector RNA and further mutations have been introduced into this gene to reduce the chance of recombination producing an infective amphotropic virus.

These two measures are thought to make recombination events unlikely to lead to the production of replication competent virus.

In addition, in most cases, the retroviral vectors used are based on the Moloney murine leukaemia virus and initial data suggested this virus to be non- pathogenic in primates [68, 69], though this virus does cause a T cell leukaemia / lymphoma in rodents and might be considered to carry the potential to induce similar events in Human patients treated in gene therapy programmes. (see also later 1.5.1.)

FIGURE 1.3. SCHEME TO SUMMARISE PREPARATION OF RETROVIRAL VECTORS IN PACKAGING CELL LINES.



KEY:
- gag.- Gene coding for virus internal proteins which associates virus RNA with envelope.
- pol.- Virus polymerase gene.
- env.- Gene coding for viral envelope proteins.
- LTR's Long terminal repeat's -replication, etc signalling sequences.

1.3.8. Adenovirus Vectors in Gene therapy.

Adenovirus vectors have also been investigated extensively but have not been used in the early *in vivo* gene transfer studies to the extent that retroviruses have been. The reasons for this have been reviewed in Mulligan et al [50] but in brief are principally because viral integration is not an integral part of the life cycle and is relatively inefficient and of course there remains the concern that adenoviruses have known oncogenic potential particularly in relation to the transforming protein E1A. However the use of defective adenovirus may have potential in the delivery of transferrin polycation conjugates, as has been discussed already (1.3.6.3).

1.4. THE USE OF ANTISENSE OLIGONUCLEOTIDES TO DOWNREGULATE GENE EXPRESSION.

The techniques described above for transfection studies could enable the expression of genes of interest to be upregulated to study their effect on cellular events in growth and differentiation. However it is important to be able to downregulate the expression of genes as well as this would facilitate the understanding of the role of their products in these events. This may be achieved in two ways, firstly; antisense DNA constructs, that is DNA sequences which are complimentary to the coding DNA, can be introduced, by the methods described above so that an antisense RNA is transcribed and will interfere with translation of the gene under study. Secondly the antisense oligonucleotide can be introduced directly to interfere with expression of the "sense" messenger RNA.

Antisense oligonucleotides can in some circumstances be added to the culture medium that the cells are grown in but delivery would probably be more reliable and repeatable if they were delivered by a macromolecular delivery system. Examples of delivery of antisense oligonucleotides to

haemopoietic cells using delivery systems have been reported, by Citro et al [70] who used transferrin-polylysine complexes to deliver antisense oligonucleotides by receptor mediated internalisation to HL60 cells and by Loke et al [71] who used large unilamellar Vesicles (LUV) made by calcium chelation to deliver c-myc antisense phosphorothioate oligodeoxynucleotides also to HL60 cells with the additional use of a polyethylene glycol mediated fusion step.

Thus it appears that probably similar mechanisms can be used for the delivery of antisense oligonucleotides to cause downregulation of gene expression as can be used for upregulation of gene expression by transfection.

1.5. GENE THERAPY

1.5.1. Introduction.

The emphasis in gene therapy studies is mainly on stable transfection of cells because long duration of expression is required. In addition it is important that a high efficiency of transfection is achieved in *in vivo* studies since the acquisition of a sufficient number of target cells becomes difficult if a significant proportion of these cells are either not transfected or damaged by the transfection procedure. This has lead to methods of retroviral gene transfer being used in the majority of pre-clinical and early clinical protocols. However, as suggested earlier, the use of retroviruses has safety implications which need to be addressed.

1.5.2. Safety considerations in the use of Retroviruses.

The work of Donohue et al [72] has caused an increase in concern over the safety of retroviral gene therapy protocols in clinical studies. As discussed above (1.3.7.) there are a number of strategies which are used to attempt to prevent the generation of replication competent virus in association with the use of retroviruses in gene therapy and the experiments of Donohue et al. concentrated on the question of the safety of packaging cell lines. This was particularly with reference to the transfection of the human haemopoietic stem cell, which as they state, is acknowledged to be difficult to transfect. The producer cell line they studied, while producing a very high concentration of retroviral vector particles, also produced replication competent virus at low levels. When they used this packaging cell line to transfect haemopoietic stem cells derived from autologous marrow and re-infused the transduced marrow after total body irradiation (TBI) they were able to detect the presence of replication competent virus in five out of eight non-human primates. This was then followed six to seven months later in three animals by the development of a rapidly progressing T cell neoplasm, analogous to the disease described in rodents. In addition, replication competent virus without the inserted retroviral vector genome could be demonstrated in the Lymphoma cells of these animals. It is important to note that the producer cell line used was known to produce replication competent virus at a level which would not be acceptable for use in a clinical gene therapy programme where current packaging lines produce no detectable replication competent virus [73]. However this strategy was used because of the difficulty in transfecting haemopoietic stem cells and the belief that recombinant amphotropic virus was not pathogenic to primates. The authors also note that the recipient animals were significantly more immuno-suppressed than animals in previous studies because of the use of TBI, and the positive stem cell selection of CD-34

positive cells meant that T cells were not included in the re-infused marrow. It must be remembered however that such a protocol may well be required in order to establish long term haemopoiesis from re-infused stem cells *in vivo*, for example in the correction of single gene disorders. In summary therefore the paper clearly establishes, at least in non-human primates, the potential risks of retroviral mediated gene therapy. The difference in the amount of replication competent virus produced by the packaging cell line studied in the paper and those in clinical gene therapy programmes is great but is a quantitative not a qualitative difference. The long term safety considerations are accentuated if the clinical programmes were to include patients with reproductive potential for then the possibility of inadvertent germ line gene transduction is raised. This paper contrasts with the previously held views that the retroviral gene therapy protocols carried little or no risk of adverse events (reviewed in [74]).

1.5.3. Gene therapy Protocols using Non Retroviral Methods.

A number of gene therapy protocols in laboratory animals, which have studied cells which are more susceptible to transfection, have employed non-retroviral systems, either one of the commercially available cationic lipid complex / liposome preparations (e.g. Lipofectin [28], one of the later related compounds or transferrin-polycation complexes [48]). An example of the use of Lipofectin to deliver a β galactosidase gene to endothelial cells and vascular smooth muscle in parallel with retroviral transfection was reported by Nabel et al. [75]. The results of lipid-mediated and retroviral transfection were comparable in this setting though expression persisted for up to 3 months with retrovirus as opposed to 6 weeks after lipofection. The approach used by this group termed "direct gene therapy" is to directly inject *in vivo* the DNA / lipid complexes either into vessels after distal

occlusion to isolate the target segment or directly into cutaneous tumour masses. This circumvents the problems of delivery via the circulatory system, in the case of direct gene transfer, or the need to re-introduce the transfected cells when the material has been transfected *in vitro*. The results of experiments using the former technique to transfect vascular endothelium have been reported and, importantly, this technique did not lead to inadvertent gene transduction in other organs, specifically the gonads [76] as well as not having any significant acute toxicity [77]. The strategy of using direct gene transfer to transfect cutaneous tumour masses is based on the premise that the introduction of a foreign major histocompatibility antigen, in this case HLA B**7**, will lead to an immune response directed against the transfected tumour cells which will not only kill the transfected tumour cells but may also have activity against untransfected tumour cells at distant sites. These experiments were first reported in mice [78] and have, most recently, been reported in patients with advanced malignant melanoma treated in a Phase 1 clinical trial using a similar protocol [79]. The current liposomes being used in this group's most recently reported work [78, 79] are made of dioleoyl phosphatidylethanolamine / 3b-[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol. Once again the safety and toxicity studies have not revealed transfected cells beyond the site of injection using sensitive Polymerase chain reaction (PCR) based techniques to detect the transfected gene and the protocol does not have any significant acute toxicity. With regard to efficacy, the transfected protein was detectable by immunocytochemistry and immune responses both to the transfected MHC antigen and to autologous tumour cells could be detected. In addition objective tumour reduction was seen in one patient both at the site of injection and at untreated distant sites. This protocol appears to demonstrate a potentially clinically valuable non-retroviral transfection method and is fundamentally

different from the other strategies for gene transfer in humans reported to date in that cells are transfected directly *in vivo*.

Much work has also been focused on the potential therapeutic delivery of the cystic fibrosis gene to the respiratory epithelium by lipid mediated methods of DNA delivery e.g. [80]. Receptor mediated internalisation has also been investigated with preliminary results *in vitro* [81] and *in vivo* in laboratory animals [82]. The problem, however, in most of the cases where cells from tissues such as respiratory endothelium, muscle or fibroblasts have been used, is that the duration of expression has been relatively limited and so if these techniques were to be applied to therapeutic applications they would require to be delivered by multiple treatment courses.

The cells transfected in these experiments have either been in tissue culture or at a directly accessible site and this has also been true of other protocols reporting *in vivo* liposome mediated gene therapy in laboratory animals. For example the chimerasomes used by Gould-Fogerite et al in the production of dermal tumours in mice were delivered by subcutaneous injection [25] and the resulting tumours developed at the site of those injections.

Delivery to the liver via the intravenous route of both liposomes and polylysine -conjugates has been reported by injection of liposomes and U/V inactivated HVJ into the portal vein for hepatic delivery [46], by intravenous administration of liposomes [83] or by the delivery of polycation conjugates to the liver [51]. The apparent ease of delivery to the liver may be explained by the rapid removal of liposomes or polycation conjugates from the circulation during the passage of the blood on the first pass through the liver.

Delivery of the liposomes or conjugates to the target cells via the intravenous route beyond the liver remains unproven since, firstly, the rapid

removal of liposomes from the circulation may be expected by the reticulo-endothelial system, secondly the liposomes may be destabilised by plasma and its constituent lipoproteins, and thirdly access to cells outside the circulation requires the passage of the liposomes across the vascular endothelium. (For a table detailing survival times in plasma of different liposome types see the table in Hug et al. [84]) A note of caution to be observed with respect to this issue is that, inspite of the great enthusiasm for liposomes as drug delivery vehicles, liposomes have not become established in routine clinical practice for drug delivery. The only current preparation [85] which has been licensed and used in frequent practice is liposomal amphotericin [86] [87]. The high lipid solubility of amphotericin and the possible desirability of delivery to the R-E system probably make this preparation a special case .

For all the above reasons the use of liposomes in transfection studies is most likely to be in the context of the reinfusion of transfected stem cells using a protocol similar to the protocol that has been followed in the gene marking studies reported by Brenner et al [88, 89] using retrovirally mediated transfected cells. The use of techniques such as CD34 positive stem cell selection [90] to reduce the proportion of cells other than stem cells present in the aliquot of cells treated would probably be necessary depending on the efficiency of transfection achieved. The acquisition of stem cells may be further improved either by *ex vivo* stem cell expansion [91] or by using 5 Fluorouracil treatment to cause stem cell enrichment in subsequent stem cell collections [77].

1.5.4. Current Retroviral Gene therapy protocols.

The first gene therapy protocols to be reported such as the gene marking of tumour infiltrating lymphocytes [92] and the correction of Adenosine deaminase deficiency [93] used lymphocytes as the target cell because of the difficulties in transfecting the totipotential stem cell. However the limited life span of these cells limits the expected duration of gene expression. This limited duration of expression of an introduced gene will probably not apply to transduction of the haemopoietic stem cell if the introduced material can be integrated in a stable manner because of the capacity of these cells to undergo self renewal. This makes haemopoietic stem cells one of the most important targets for the therapeutic application of gene therapy. Circumstantial evidence for this view can be derived from the success of allogeneic bone marrow transplantation (BMT) in the treatment of many single gene disorders [94]. The success of BMT in many disorders can be seen to exemplify the diverse progeny of the haemopoietic stem cell. In biochemical single gene disorders the long term expression of transplanted, biochemically normal, HLA matched sibling bone marrow is a close analogy for the potential application of gene therapy.

The gene marking of cells involved in either leukaemic relapse or normal haemopoietic reconstitution following the transfection of autologous bone marrow cells reported by Brenner et al. [89] [88] is evidence that earlier haemopoietic precursors had been successfully transfected.

An important paper by Hajihosseini et al [95] has recently examined the question of the timing of retroviral integration within the cell cycle and its consequences for gene therapy. They did this by an elegant single cell assay using a β–galactosidase indicator dye (X-gal) to detect transfected daughter cells including the transfected reporter gene. The results using this reporter system had a 100% correlation with the PCR detection of provirus incorporation. The results of these experiments showed that 80 %

of the single cells which gave rise to colonies with transfected cells contained both transfected and untransfected cells. The remaining 20% of transfected colonies which contained only transfected cells could be explained by the death of one of the daughter cells at the two cell stage (this approximately corresponds to the predicted level of toxicity). In the mixed colonies the transfected and untransfected cells were present in approximately equal proportions (0.82 : 1) and because the colonies had arisen from a single plated cell this strongly suggested that proviral integration had only occurred in half the cells representing the progeny from only one of the two daughter cells of the first post transfection cell division. The authors' conclusion from this data is that integration occurs late in the cell cycle after DNA replication so that, if only one provirus was integrated per cell, a maximum of 50 % of the progeny would contain the integrated provirus. In their discussion of this finding the authors make the point that this could be particularly important if a transfected stem cell divides "asymmetrically" to give one daughter stem cell and one committed progenitor cell because in that case the stem cell progeny may not include the integrated provirus. These findings provide another potentially significant problem with the use of retroviruses for the gene therapy of haemopoietic stem cells and will certainly place a limit to the efficiency of transfection at a maximum of 50%. However, since this figure of 50 % applies to only a single round of replication, with the use of multiple rounds of replication it should be possible to achieve the transfection of a significantly greater final proportion of cells.

1.6. SUMMARY: THE NEED FOR NON RETROVIRAL MEANS OF MACROMOLECULAR DELIVERY.

The protocols described above which use retrovirus technology illustrate the great therapeutic potential that any successful gene therapy clinical programme could hope to achieve. The aim of establishing a non-retroviral system for high efficiency transfection would be the avoidance of the potentially serious hazards, previously discussed, which could arise when retroviruses were used in the context of a clinical gene therapy protocol.

The key to the development of a non-toxic delivery system is to deliver the macromolecules across the cell membrane without compromising its integrity. Many non-retroviral delivery systems involve the physical disruption of the membrane for example as in the use of electroporation, however for the transfection of haemopoietic cells electroporation appeared to be the most efficient of the physical methods [26] available.

The review of the literature described above illustrated the very many potential means of investigating the non-retroviral, non toxic delivery of macromolecules to haemopoietic cells. The great variety of choice necessitated the reasoned choice of a small number of the most promising techniques to investigate. The lack of any convincing data demonstrating that any of these techniques could be used in the generality of human, haemopoietic cells justified the study of these techniques with a view to optimising them for application to this context. The study of methods which maintained the integrity of the cell membrane appeared to be the best strategy to pursue in order to minimise toxicity. The potential approaches available were therefore liposomes, delivered either by fusion or phagocytosis, or the use of ligand-polycation-DNA complexes delivered by receptor mediated internalisation.

CHAPTER 2 : GENERAL METHODS

2.1. BUFFERS, CULTURE MEDIA AND REAGENTS.

2.1.1. Buffers

Phosphate Buffered Saline (PBS) was made by dissolving one tablet of PBS-A (Unipath ltd, Basingstoke, Hants.) in deionised water or as Dulbecco's PBS (without Calcium and Magnesium, without Sodium Bicarbonate) from Gibco-BRL, Paisley, Scotland, in cell culture experiments.

For Reconstituted Sendai Virus Envelopes (RSVE), Detergent Removal Large Unilamellar Vesicles (DetLUV) & Small Unilamellar Vesicle preparation (SUV) :

Tris/NaCl Buffer: NaCl 100 mM , Tris [hydroxy methyl] aminomethane hydrochloride.(Tris) 50 mM , pH 7.4.

Solution "A" 160 mM NaCl, 20 mM Tricine, pH 7.4

For Large Unilamellar Vesicles (LUV) from Cochleate Intermediates:

Tris Buffered Saline (TBS) (pH 7.6) 136 mM Na Cl, 5 mM K Cl

25 mM Tris.

+ Calcium TBS + 20 mM Ca Cl_2

+ EDTA TBS + 30 mM Ethylenediaminetetra-acetic acid disodium salt

For Chimerasome Preparation:

TES Buffer 2 mM Tris, 2mM Histidine, 100 mM NaCl

For Calcium Dialysis Buffer : TES + 3 mM Ca Cl_2

For EDTA Dialysis Buffer TES + 10 mM EDTA.

Virus Extraction Buffer: 2M NaCl , 0.02 Na PO_4 pH 7.4.

For Molecular Biology Procedures:

Tris / EDTA (TE) Tris 10 mM, EDTA 2 mM.

Tris / Borate / EDTA (TBE) 0.5 x 0.045M Tris Borate, 1mM EDTA.

Balanced Salt Solutions:

Hanks Balanced Salt Solution (HBSS) (Gibco-BRL, Paisley, Scotland)

HBSS + Hepes HBSS + 20 mM Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid)

2.1.2 Tissue Culture Media:

RPMI 1640 with L-Glutamine was obtained from Gibco-BRL Europe Ltd. Paisley, Scotland. It was used for cell washing, culturing and freezing procedures. Where appropriate the addition of the antibiotics penicillin (at 50 units /ml) and streptomycin (at 50 µg/ml) (GIBCO) was made to media prior to use. The preparation (RPMI + Hepes) with additional 25 mM Hepes was used for electroporation to avoid pH changes during cell manipulation.

Dulbecco's Modified Eagle's Medium (DMEM). Gibco-BRL Europe Ltd, Paisley, Scotland. This was used with added glutamine (Final concentration 584 mg/l) and Non-essential amino acids (1/100 x MEM-Non essential amino acids-Gibco-BRL) for the growth of HeLa cells.

Modified DMEM (Calcium 0.5 mM Calcium and Magnesium free, low phenol red- Gibco-BRL). A custom made modification of Dulbecco's modified Eagle's Medium with a calcium concentration of less than O.5 mM and free of magnesium was used with Phosphatidyl-serine liposomes to avoid the precipitation of lipid that can occur with higher concentrations of calcium.

OPTIMEM ™ Gibco-BRL- Special Formulation of DMEM for use with Lipofectin.

OPTIMEM ™ Special Formulation of DMEM with Calcium concentration limited to O.5 mM and Magnesium free.

2.1.3. Fetal Calf Serum (FCS).

FCS was supplied by Gibco-BRL, in 500ml bottles, it was inactivated by heating at 56°C or 65°C (for DNA experiments) for 30 minutes, aliquotted into 50 ml polypropylene tubes and stored at -70 °C. For use with chimerasome experiments serum obtained from Gibco-BRL, USA was used to reproduce most closely the original methods of Gould -Fogerite et al. [25]

2.1.4. Reagents.

All reagents used were of Analar ™ or 'Tissue culture grade' unless stated otherwise.

2.1.4.1. Constituent Lipids in Liposomes:

L-α-Phosphatidyl choline (PC) - egg derived, stored at -20° C under 95% : 5% v/ v Chloroform / Methanol (C / M) : Sigma (Poole, Dorset)
Nutfield Lipids.(Surrey)

Di-palmitoyl-phosphatidyl -choline (DPPC)- synthetic, stored at -20° C under 95% : 5% v/v C / M. Sigma.

L-α-Phosphatidyl -serine (PS)- Bovine brain derived stored at -80° C under 95% : 5% v/v Chloroform / Methanol, normally used direct from a sealed whole ampoule as received from the supplier. Ampoules of the correct

amount for an individual experiment were obtained to avoid oxidation during storage after opening of the ampoules. From-:

Sigma

Nutfield lipids

Avanti Polar Lipids (Birmingham, USA)

Cholesterol (Chol.) stored at -20° C under 95% : 5% v/v C/M (Sigma.)

Cholesterol -Porcine stored at -20° C under 95% : 5% v/v C/M (Sigma.)

Gangliosides: Bovine Brain Type III - N-acetyl neuraminic acid : stored at -20° C under 95% : 5% v/v C/M Sigma.

2.1.4.2. Flourochromes: Carboxyfluorescein (CF): Carboxyfluorescein was obtained from Molecular probes (Cambridge).

This preparation contained impurities which interfered with encapsulation by lipids. Therefore the CF was purified as follows; 5 g was boiled in 100 ml of ethanol with 5g of activated charcoal. The solution was filtered through Whatman No.1 filter paper to remove the charcoal, cooled on ice and the CF was reprecipitated by slowly adding ice cold distilled water. The precipitate was washed in a Buchner funnel with more cold water and dissolved in 6M sodium hydroxide adding sufficient to give a final pH of 7.4. This solution (the sodium salt) was separated by column chromatography (Sephadex LH-20, Pharmacia.) by eluting with distilled water at room temperature. The first peak was brown and non-fluorescent and was discarded, the main peak (orange in appearance) was the purified CF and was collected, freeze dried and stored in the dark in an air tight vessel. Lipophilic contaminants remained at the top of the column or trailed behind the CF band.

SNARF ™ (Molecular Probes) This pH. sensitive dye was obtained as a pure compound and used unmodified.

Acridine Orange/ Ethidium Bromide Stock Solution:
For this viability stain a stock solution of 4 ml of Ethidium Bromide (4 mg/ml) and 10 ml of Acridine Orange (0.1% w/v solution) was diluted to 1 litre with PBS and stored in a dark glass bottle at 4°C.

2.1.4.3. Detergents (all from Sigma) :
CHAPS:(3-[3-chloroamidopropyl]dimethylamonio)-1-propane-sulphononate.) at 15m**M** CHAPS in Tris / NaCl buffer pH 7.4.

n-Octyl β- D -glucopyranoside (Octyl glucoside) 1% by volume in Solution A pH 7.4 or added as desiccated dry powder to sample to make a final 2 % w/v mixture.

TRITON X-100 . Used as 5-10% w/v solution.

2.2. PLASTICS AND GLASSWARE.
All items used in experimental procedures were either tissue culture grade polystyrene / polypropylene. Laboratory glassware used for liposome work was rinsed in chromic acid (BDH) and then rinsed ten times with deionised water and sterilised by autoclaving. Where indicated glassware or plastics were siliconised with REPELCOTE (BDH) and rinsed ten times with de-ionised water to remove residual acid before autoclaving.

7ml bijou bottles (Bibby-Sterilin Ltd, Feltham, Middlesex)
15 ml Falcon tubes (Becton Dickinson, Falcon)
30 ml Universal containers (Sterilin Ltd.)

50 ml Universal containers (Becton Dickinson, Falcon)

Long, unplugged, glass Pasteur pipettes (John Poulton Ltd, Barking, Essex for Volac Lab. Glass)

1ml graduated glass pipettes (John Poulton Ltd for Volac Lab. Glass)

5 ml graduated pipettes (Bibby-Sterilin Ltd)

10 ml graduated pipettes (Bibby-Sterilin Ltd)

25 ml graduated pipettes (Bibby-Sterilin Ltd)

15 cm Petri dishes (Becton Dickinson, Falcon)

6, 12 & 24 well Costar ™ multiwell plates

(Costar, Cambridge, MA, USA)

Polystyrene U bottomed 96 well plates. (Becton Dickinson, Falcon)

1.8 ml cryotubes (Nunc).

25 cm^2 tissue culture flasks

75 cm^2 tissue culture flasks

175 cm^2 tissue culture flasks

(Becton Dickinson & Co., Falcon Division, Lincoln Park, New Jersey 07035)

Syringes (1 - 50 ml capacity) (Sabre International Products Ltd.)

Pipette tips P20, P200, P1000

(Alpha Labs. Ltd.)

0.22 µm Nalgene cellulose nitrate membrane (Nalge Co., Rochester, NY, USA.)

2.3. CELL CULTURE.

The haemopoietic cell lines used were all obtained from co-workers at the Department of Haematology, University College Medical School. Cells were cultured for a maximum of 6-8 weeks before being discarded. New cells grown from stocks cryopreserved in liquid nitrogen. This was done to avoid progressive changes in the cell populations.

K562, a human myeloid cell line derived from cells from a patient with Chronic Granulocytic Leukaemia with myeloid (FAB type M6-erythroleukaemic) blast crisis.

HL60, a human myeloid cell line derived from cells from a patient with Acute Myeloid Leukaemia (FAB type M3).

Daudi, a human B cell lymphoid cell line.

TF1, a growth factor dependent human myeloid cell line derived from a patient with Acute Myeloid Leukaemia (FAB type M6-erythroleukaemia). [19] Recombinant human GM-CSF (either material produced in *E. coli* or yeast) for use with TF1 cell line was provided by Behringwerke AG, Marburg, Germany.

HeLa, a human cell line derived from a patient with carcinoma of the cervix. (A gift from Dr. PA Lawton, CRC Gray Laboratory, Northwood, Middx.)

All cell **lines** were cultured in RPMI medium supplemented with 10% heat inactivated fetal calf serum, except where stated that DMEM, OPTIMEM or low Calcium, Magnesium free DMEM or OPTIMEM was used. Cells were incubated at 37 ^{o}C in a 5% CO_2 humidified atmosphere.

Antibiotics (streptomycin and penicillin) were not added routinely but were added after electroporation or lipofection. Mycoplasma Removal Agent (MRA-ICN FLOW: High Wycombe) was added to the medium when indicated for a period of 7 days at a final concentration of 0.5 µg/ml. Prior to use in experiments MRA was removed by washing cells three times in RPMI /10% HI FCS and the cells were cultured for at least a further 24 hours.

Cell numbers were determined using a haemocytometer. Either Trypan blue or Acridine Orange /Ethidium Bromide was added to assess viability. Samples of cell suspensions were mixed with an equal volume of this staining solution and were examined in a haemocytometer under an epi-fluorescent microscope (Zeiss). Dead cells appeared stained red and live (viable) cells stained green.

Cell lines were frozen by combining an equal volume of freezing medium (40% RPMI, 40% FCS and 20% Dimethyl Sulphoxide (DMSO)) with an equal volume of cells (5-8 x 10^6 per aliquot) in RPMI / 10% HI FCS. The mixture was then cooled to -70°C with a NALGENE cell freezer and transferred to liquid nitrogen for long term storage.

2.4. HUMAN SENDAI VIRUS: SOURCE, PURIFICATION & TITRATION.

2.4.1. Human Sendai Virus (Human Virus of Japan-HVJ).

HVJ was obtained from The Department of Virology, St Bartholomew's Hospital London, where it had been grown for 72 hours after inoculation of the allantoic fluid of 10 day old fertilised eggs (White Leghorn-Gallus gallus).

2.4.2. Purification by Differential Centrifugation.

HVJ was purified from the crude allantoic fluid to obtain a final suspension in PBS by firstly, clarification of the allantoic fluid at 900 g for 20 minutes at

4 °C. The pellet was discarded and the supernatant was then centrifuged for 2 hours at 5° C at 14750 rpm (30 000 g) in a Beckman J2-21 centrifuge (JA 17 rotor). The pellet was retained and resuspended in 10 ml of PBS before further centrifugation in the same rotor at 5000 rpm (3500 g) for 20 minutes at 5°C. This was done three times. The supernatant was retained on each occasion and the pellet was resuspended with a further 10 ml of PBS. Finally each supernatant was pooled and centrifuged for 2 hours at 5° C at 14750 rpm (30 000 g). The pellet was resuspended in 5 ml of PBS and frozen at -70° C until use.

2.4.3. Haemagglutination Assay for Virus activity.

This assay is based on the ability of the HVJ to agglutinate chicken red blood cells (rbc's) caused by the virus membrane haemagglutinin protein. The assays were performed in 96 well U bottomed polystyrene plates (Becton Dickinson, Falcon). Chicken rbc's in alsevers medium (Tissue Culture services Ltd, Buckingham) were washed twice in PBS and suspended at 0.5% by volume (Haematocrit checked in automated cell counter, Coulter, UK). 50 µl of virus suspension was pre-diluted to 1/10 and 1/100 in PBS in duplicate and then, in a microtitre plate, doubling dilutions were made to a maximum of 1/2056 in the 12th well and the final 50 µl was discarded. Fifty µl of the dilute suspension of chicken rbc's was added to each well and the plate was stored at 4°C until agglutination had occurred. The last well in which complete agglutination had taken place was taken to represent the Haemagglutinating unit (HAU) titre of the virus suspension. The HAU / ml titre was obtained by multiplication of the HAU titre by 20 (1ml / 50 µl) .

2.5. LIPOSOME PREPARATION.

2.5.1 Preparation of Multilamellar Vesicles. (MLV)

To make MLV for conversion to Small Unilamellar Vesicles: The MLV suspension was made by vortexing a dry lipid film of either egg phosphatidylcholine and cholesterol of molar ratio 2:1 or for PS/PC/Chol/ ganglioside (G) MLV lipid with a composition, by weight, of 1:4.8:2:0.001 in the presence of an aqueous solution of 200 mM CF or a solution of HSV-CAT or CMV-CAT DNA for DNA containing liposomes. In some cases, as detailed in the text, the synthetic phosphatidyl choline DPPC was substituted for egg derived PC and Gangliosides were added or omitted. Constituent lipids were taken from stocks stored under chloroform at -20° C or -80 °C and dried in a glass tube with a rotary evaporator. The final lipid solution was separated from unencapsulated dye by column chromatography (G50 Sephadex, Pharmacia).

MLV for use in preparation of Large Unilamellar Vesicles via cochleate intermediates or Chimerasomes were made by vortexing the lipid for LUV in 2 ml TBS or for chimerasomes in the virus/ detergent suspension obtained after disruption of the purified virus suspension by Octyl Glucoside in extraction buffer. Unlike the preparation of SUV the material to be encapsulated is not included until a later stage in the protocol. The dry lipid film was prepared with either Phosphatidyl serine alone, Phosphatidyl serine and Cholesterol in a 9: 1 w/w ratio or these lipids with 1% gangliosides.

2.5.2. Preparation Of Small Unilamellar Vesicles (SUV) from MLV.

SUV Synthesis by high pressure extrusion:

These liposomes were prepared from the MLV suspension in an extruder (WHITEY-USA) [96] by 10 passages through a 200 nm filter, then 10 passages through a 100 nm filter under high pressure (1000 lbf / m^2) generated via a high pressure head from an N^2 cylinder. The filters used were polycarbonate to minimise lipid losses. (Nucleopore, Surrey)

SUV Synthesis by Sonication:

Sonication was done in a constant temperature vessel cooled by water, using a 10 mm titanium probe using a MSE Soniprep 150. An amplitude of 6 μm was used with a cycle time of 20s ON / 40s OFF for 60-120 cycles. A clear suspension of SUV was obtained by centrifugation of the suspension to remove lipid aggregates and titanium micro-fragments. Unencapsulated chemical was removed by chromatography through Sephadex G50 (Pharmacia) and eluted with Tris /NaCl buffer. Unencapsulated chemical elutes later than the liposomes and was discarded.

2.5.3. Large Unilamellar Vesicles (LUV) via Cochleate Intermediates from MLV.

These liposomes were prepared from the method of Itani et al [35] with modifications (personal communication Dr Farzaneh, King's College Hospital Medical School). A suspension of MLV was made, as detailed above, and these were then converted to SUV by sonication in a sterile constant temperature sonication vessel in an MSE Sonicator at 27° C (maintained by water circulation from a water bath) at an amplitude of 6 μm for 60 cycles (20s ON / 40s OFF-unless otherwise stated) using a 1 cm diameter titanium probe. The clear solution of SUV, in sterile, siliconised eppendorf tubes was then converted to "Cochleates" [37] by the addition of an equal volume (normally 200 μl) of 20 mM Ca Cl_2 in TBS and incubated at 37° C for one hour. The cochleates were pelleted by centrifugation at 1500 g for 5 minutes and then the solution containing the dye (or DNA) to

be encapsulated was added in < 30 µl, mixed and incubated for 30 minutes. Finally Large Unilamellar vesicles (LUV) were made by the addition of an appropriate volume of an EDTA solution (e.g. 200 µl of 30 mM EDTA) thus chelating the Calcium and causing the formation of the LUV containing the dye or DNA. The liposomes were washed free of unencapsulated CF or DNA by repeated centrifugation and resuspension steps at 10 000 g in a benchtop microcentrifuge (MSE Microcentaur) and in the case of CF liposomes size and encapsulation was checked by viewing in a fluorescence microscope. In the case of Plasmid DNA liposomes the encapsulation was checked by lysis and gel electrophoresis.(see below 2.6.4.).

2.5.4. Large Unilamellar Liposomes incorporating Sendai Virus Membrane proteins. (Chimerasomes).

These were made as per the published method of Gould-Fogerite et al [25] with modifications as suggested by personal communication with the author (now of New Jersey Medical School , Newark, New Jersey, USA). Briefly, lipid films were made as described above with PS/ Cholesterol in a ratio of 9:1. To these was added a solution of solubolised human Sendai virus prepared from a purified stock solution of known HAU activity and protein concentration as follows: Virus was quickly thawed from -80 °C to room temperature in a water bath and ultracentrifuged at 60000g at 4 °C in a Beckman SW-55 rotor in a L8-80M ultracentrifuge for 30 minutes in a polycarbonate tube that had been sterilised by rinsing firstly in methanol and then in autoclaved TES buffer. The virus was sequentially resuspended in small volumes of extraction buffer to a concentration of 2 mg total protein /ml as determined by a modified Lowry assay. β–D-octylglucopyranoside (Sigma, UK) was added as a dry desiccated powder (stored sealed at -20 °C) to a final concentration of 2 % w / v and after capping with parafilm, the tube was vortexed and incubated for 45 minutes

with vortexing at 15 minute intervals. This virus solution was then re-centrifuged at 60000g at 4°C in a Beckman SW-55 rotor in a L8-80M ultracentrifuge for 45 minutes to pellet the virus nucleocapsids (which were discarded) and the supernatant containing the virus membrane lipid / protein solution was added to the lipid film. The amount of total lipid used was calculated so that it was equivalent to 4 times the amount of viral lipid present. The original method states [25] that the weight in mg of viral lipid in the virus / detergent suspension is equivalent to the weight in mg of the viral envelope proteins which, in turn, is equivalent to 1/3 of the total virus protein. Therefore since the total virus protein was determined on the purified virus suspension immediately prior to the addition of the detergent then the total lipid required could be calculated as:

Total Lipid = 4/3 x measured total virus protein.

The lipid film was resuspended in this virus solution by vigorous vortexing for 5 minutes and the final clear solution was sterilised by passing through a 0.22 μm polycarbonate filter (Millipore, UK). This suspension was then made into the cochleate intermediates by dialysis in Spectra / Por 7 dialysis tubing (Medicell, London- 10mm flat width, MW cut-off 10 000) versus TES buffer with 3 mM Ca Cl_2 with 4 changes of buffer at 2-3 hrs, 4-5 hrs, overnight and after a further 2-3 hrs. These cochleates are called DC (Direct Cochleates) as opposed to LC (liposomes before cochleates) for those made by removal of the detergent by dialysis prior to the formation of the cochleates. With either method the hybrid cochleates, which include the viral membrane proteins, were recovered by centrifugation at 60000g at 4 °C in a Beckman SW-55 rotor in a L8-80M ultracentrifuge for 60 minutes. The final step is then the formation of the chimerasomes by mixing these cochleates with a solution of the material to be encapsulated and either agitating over an agarose plug containing 100 mM EDTA in TES buffer in an eppendorf tube or by rotary dialysis when the cochleates are placed in

Spectra/Por7 dialysis tubing and rotated by means of a magnetic stirrer in a dialysis tank containing TES buffer with 10 mM EDTA. The chimerasomes were collected after overnight dialysis by ultracentrifugation at 60000g at 20 °C in a Beckman SW-55 rotor in a L8-80M ultracentrifuge for 60 minutes. The chimerasomes were then resuspended in TES buffer, pelleted again and then resuspended in the same way to remove unencapsulated DNA. Samples used for encapsulation studies were washed and resuspended three times at 10000g in a microfuge (MSE microcentaur). The final chimerasomes were resuspended in TES buffer.

2.6. LIPOSOME CONSTITUENT DETERMINATION.

2.6.1. Phospholipid assay [97].

This assay is based on the formation of a 1:1 complex between ammonium ferrothiocyanate and phospholipids.

An ammonium ferrothiocyanate stock solution (27.03 g Ferric Chloride + 30.4 g Ammonium thiocyanate) was made in 1 litre of distilled water. A stock standard at 0.1mg / ml in chloroform of the test lipid was added to glass tubes in volumes of 0.1ml to 1.0 ml, in duplicate, to give data points on a standard curve representing lipid concentrations between 0.01mg /ml and 0.1 mg / ml. The volume of chloroform in all samples was made to 2 ml with the addition of further chloroform. Test samples using 0.05 or 0.1 ml according to the lipid content of the sample were also made to 2 ml with chloroform. 2 ml of ammonium ferrothiocyanate was added and covered with parafilm and vortexed for 1 minute before centrifugation (750g) for 15 minutes. The organic (lower) phase was removed using a glass Pasteur pipette and the absorbence at 488 nm was measured using a spectrophotometer (Pye Unicam SP8-200 UV / VIS.) Mean absorbence was plotted against phospholipid concentration and the lipid concentration was read from the standard curve.

2.6.2. Protein Assay (Based on Modified LOWRY method [98])

Reagent A : Equal Volumes Of:

1. Copper sulphate tartrate

(1 part 0.4% $CuSO_4$ 5 H_2O., 1 part 0.8% Potassium tartrate, 2 parts 20% Sodium Carbonate)

2. 10 % SDS

3. O.8 M Sodium Hydroxide.

4. Distilled Water

Reagent B: Folin-Ciocalteau phenol reagent (BDH) mixed 1:6 with distilled water.

The sample was brought to 0.5 ml volume with distilled water and 0.5 ml of reagent A was added and stood at room temperature for 10 minutes. 0.25 ml of Reagent B was added and the mixture was further incubated for 30 minutes at room temperature. The absorbence was then read at 750 nm on a spectrophotometer and compared to a standard curve plotted with measured absorbence of samples with known amounts of bovine serum albumin.

2.6.3. Protein Gel Electrophoresis to demonstrate incorporation of Viral Proteins in Chimerasomes.

In order to assess whether the virus membrane proteins were reconstituted into the chimerasomes, samples of 1 µl, 5 µl or 10 µl of chimerasomes (1 mg/ml total protein) or stock, purified virus (1.7 mg/ml) were run on a 10 % polyacrylamide gel using the BioRad MiniProtean gel system. A 10% gel was made with 10 % Pre-weighed BioRad Acrylamide /Bis (37.5: 1 mixture) in 0.375 M Tris/HCl pH 8.8, 0.1 % w/v Sodium dodecyl Sulphate, 0.05% Ammonium persulphate and 5 µl N,N,N, Tetramethylethylenediamine (TEMED) (Sigma). In addition a stacking gel was mixed with 4% Pre-weighed BioRad Acrylamide / Bis (37.5: 1 mixture), 0.125 Tris-HCl pH 6.8, 0.1 % w/v SDS, 0.05% Ammonium persulphate and 10 µl N,N,N, Tetramethylethylenediamine. Samples were mixed 1:1 with sample buffer (8 ml Sodium dodecyl Sulphate (SDS) reducing Buffer-: Distilled water 4.0 ml, 0.5 M Tris-HCl pH 6.8 1 ml , Glycerol 0.8 ml, !0% (w/v) SDS 1.6 ml, 2 β mercapto ethanol 0.4 ml, 0.05% (w/v) Bromophenol blue 0.2 ml.) 15 µl of final sample was loaded to the 10% gel and run for 1 hour at 100V

(Running buffer: Tris base 3 g/l, Glycine 8.6 g/l, SDS 1 g/l). Amersham Rainbow Marker was run in the first lane to be loaded. The gel was then stained for 1 hour with Coomassie blue (0.1% Coomassie Brilliant blue R - Sigma) in 40% methanol and 10% acetic acid), destained with 40% methanol and 10% acetic acid, dried on a gel dryer and then photographed.

2.6.4. Assessment of DNA encapsulation in Liposomes by Agarose Gel Electrophoresis:

Duplicate samples of liposomes to be assessed were washed repeatedly by pelleting (10000g x 5 minutes in Benchtop microcentrifuge) and resuspension in TBS. The final aliquot (10 µl or less) was then either loaded directly onto the gel or lysed by the addition of 2 µl of 10% Triton X. In addition to the lysed or intact liposome samples, 2.5 µl of a 1kb ladder (GIBCO-BRL) and a known amount of the stock plasmid were loaded in parallel lanes, all lanes also included 2 µl of bromophenol blue for normal light visualisation. One percent agarose in TBE was used for the gels with ethidium bromide (5µl of 10mg/ml EtBr / 50 ml TBE) and electrophoresis was at 70 mA for approximately 40 minutes or until the bands could best be discriminated. DNA was detected by exposure to ultraviolet light and photographed with a Polaroid camera.

2.7. PLASMID PREPARATION

Two plasmids were used with the reporter gene Chloramphenicol Acetyl Transferase under the control of either a Herpes simplex promoter (HSV-CAT) or the Cytomegalovirus promoter (CMV-CAT). HSV-CAT was Hs4 immediate early 4/5 promoter-CAT. The plasmid vector for CMV was pBR322. These were available from co-workers in the Dept. of Haematology and plasmid DNA was purified from Maxipreps of bacterial broth inoculated from glycerol stocks stored at -80 ° C. The bacteria used in both cases was E. coli DH 5.1.

Glycerol stock of transfected Eschericia coli was spread onto a bacterial plate and incubated overnight at 37 °C in the presence of ampicillin 25 µg/ml. A single discrete colony was picked and added to 20 ml of LB medium (see below) was incubated until turbid (usually 8-10 hours) and then added to 1-4 litres of LB medium and incubated overnight at 37°C.

Luria- Bertani Medium (LB)

For each litre of broth (corrected to pH 7.5 with 10 Molar Na OH):

Bactotryptone 10g

(Difco, Detroit, Michigan, USA)

Bacto -Yeast Extract 5g

(Betalab, E Molesy, Surrey)

Na Cl 5 g

Autoclaved after constitution and Ampicillin 25 mg/ml added.

For bacterial plates: Bacto Agar (Betalab, E Molesy, Surrey) 15 g / litre of medium

The bacterial broth was chilled and then centrifuged at 3000 rpm (2000 g) for 30 minutes at 4º C in an MSE Mistral 3000i centrifuge. The supernatant was poured off and discarded into Chloros disinfectant for 24 hours to decontaminate. The bacterial pellet was resuspended in 50 ml of Plasmid solution I (50 mM glucose, 25 mM Tris HCl (pH 8), 10 mM EDTA (pH 8)) and incubated for 10 minutes. Then 100 ml of freshly made Plasmid solution II (0.2 M Na OH, 1% w/v SDS) was added, mixed gently and incubated for 10 minutes. Then 75 ml of plasmid solution III (60 ml 5M Potassium acetate, 11.5 ml Glacial acetic acid, 28.5 ml distilled water per 100 ml of solution) was added and incubated on ice for 20 minutes. The solution was then centrifuged again for 20 minutes at 3000 rpm at 20 ºC. The supernatant was then passed through a muslin filter and 0.6 volumes of Propan-2-ol added. This mixture was centrifuged at 6000 rpm in a Beckman J2-21M centrifuge (JA 10 rotor) at 20 ºC. The resultant pellet, which includes the plasmid DNA was then washed with 70% ethanol / 30% 0.1mM Tris/ 2 mM EDTA (pH 8.0), and vacuum dried to remove any remaining ethanol. The pellet was re dissolved in 0.1M Tris HCl , 2 mM EDTA pH 8.0. and to this solution was added 10g of caesium chloride and 0.5 µl of 10 mg/ml EtBr solution. This solution was added to a 12.5 ml quick seal Beckman centrifuge tube and centrifuged at 55 000 rpm, 20 °C for 24 hrs in a Ti 80 rotor in a Beckman L8-80M ultracentrifuge. The lower band (supercoiled plasmid DNA) was extracted with a needle and syringe and recentrifuged under similar conditions with a similar density Caesium gradient. The final plasmid band was then again extracted and separated from the Et/Br by repeated extraction with equal volumes of water-saturated Butan-2-ol. Finally the plasmid DNA was precipitated by storage at -20 °C overnight with 70 % Ethanol in the presence of a final concentration of 0.3M Sodium acetate (pH 7.0). The DNA was recovered by centrifugation at 10000 rpm (13800 g) in a JA-17 rotor in the J2-21M centrifuge. The pellet

was dried in a freeze drier and then redissolved in TE pH 8.0 and aliquotted into 200 μg aliquots at 1-2 μg/μl after determination of the DNA concentration and 260/280 ratio spectrophotometrically at 260 & 280 nm. The 260/280 ratio was in all cases > 1.7:1. The plasmid was stored either as a dry powder at 4 °C or at -80 °C in solution in TE.

2.8. CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY -

2.8.1. Radioactive C^{14} incorporation:

This assay is based on the principle that chloramphenicol becomes radio-labelled when acetylated by CAT using the C^{14} Acetyl Co-A provided in the reaction mixture. The labelled chloramphenicol partitions into the organic phase during separation and is thus separated from the unincorporated C^{14} Acetyl Co-A. Cells to be assayed were lysed in NP40 lysis buffer (0.15 M NaCl, 10mM Tris pH 7.4, 1.5 mM Mg Cl_2, 0.65% v/v NP40) . 100 µl reaction volume was comprised of 20 µl of 8 mM chloramphenicol in distilled water, 30 µl of cell lysate, 20 µl of Acetyl Co-A (0.5 mM non-radioactive Acetyl Co-A + 0.1 µCi C^{14} Acetyl Co-A (Amersham International) and 30 µl 250 mM Tris pH 7.8. This was incubated at 37 °C for 1 hour. 100 µl ethyl acetate was then added and mixed in a vortimixer and then spun at 10000g for 5 minutes in a benchtop microcentrifuge. 80 µl of the organic phase was then extracted, a further 100 µl of ethyl acetate added and the process repeated. The 160 µl of pooled organic phase was then mixed with an equal volume of 75 mM Tris HCl pH 7.8, spun and the organic layer was added to a scintillant tube with scintillant (Optiphase Hisafe, WALLAC, Leics.). 10 µl of the original aqueous layer was counted to act as the denominator to calculate C^{14} uptake to the organic layer. The samples were counted twice for each specimen in a β counter (LKB , Upsala, Sweden).

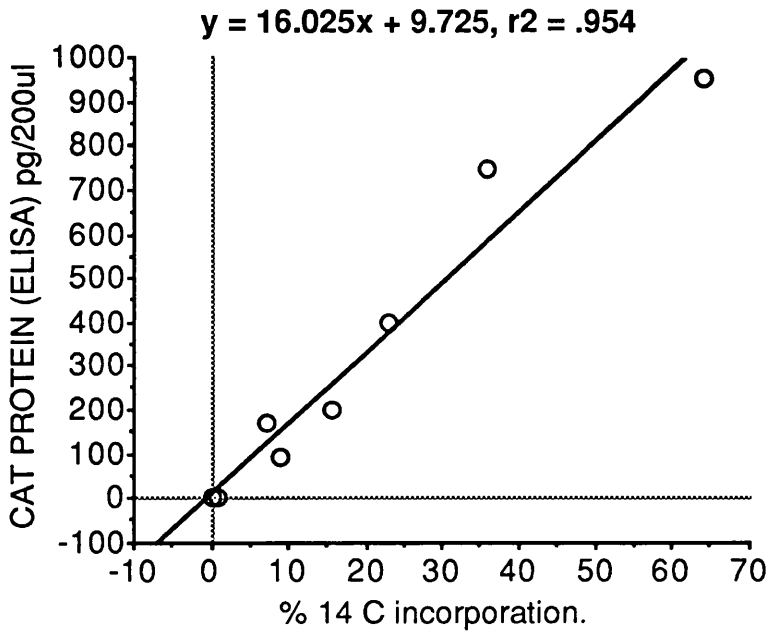
2.8.2. ELISA CAT assay (Boehinger Mannheim Biochemica)

A new commercial ELISA CAT assay (Boehinger Mannheim Biochemica) became available during the period of study which has the safety advantage of not involving the use of radio isotopes. The method was as follows:

Cells were lysed with 500 µl of lysis buffer (provided in the kit) and incubated at room temperature for 30 minutes. 200 µl of cell extract was added to pre-hydrated ELISA multiwell plates and incubated at 37 ° C for 1 hour, the plates were then washed 4 times with 300 µl wash buffer (provided in kit) and then 200µl of a solution of an anti-Chloramphenicol Acetyl Transferase antibody conjugated to Digoxigenin was added in sample buffer (provided in the kit) at the recommended dilution of 0.2 mg/ml. This was incubated for another hour at 37 °C before washing with 4 x 300 µl wash buffer as before. Next 200 µl of an anti-digoxigenin antibody conjugated with peroxidase was added in sample buffer at the recommended dilution of 25 units /ml. This was incubated for another hour at 37 °C before washing with 4 x 300 µl Wash buffer. Finally 200 µl of ABTS Peroxidase substrate was added and the optical density was read at 410 nm in a Dynatech Microplate reader (MR 700). A reagent blank was measured by reading a well in which sample buffer only was added for the first incubation step and this value subtracted from all other readings. A standard curve for 10-200 pg/ 200 µl of sample was established on each occasion and the total amount of CAT protein detected was calculated from the regression line of these results.

When the standard positive (5 µl x 1/100 i.u. /ml) from the radioactive assay, used up to this point was assayed by the ELISA method it was found to correspond to 150 pg /200 µl . Other test samples were assayed by both methods and the results are shown in FIGURE 2.1

FIGURE 2.1. Comparison of Radioactive and ELISA methods of measuring Chloramphenicol Acetyl Transferase activity.



2.9. STATISTICAL TESTS.

STATISTICAL tests were performed on an APPLE MACINTOSH using the StatView SE + Graphics ™ program ,Version 1.02, supplied by Brainpower Inc., Calabasas, California, USA.

CHAPTER 3: ELECTRICAL METHODS.

3.1 INTRODUCTION.

Various physical methods of breaching the cell membrane have been used in order to deliver plasmid DNA to target cells. These methods include Calcium Phosphate Precipitation [24], DEAE Dextran delivery [24] and Electroporation [99]. Of these Electroporation, in which a high charge is passed through a suspension of cells lying in a cuvette causing a transient "poration" of the cell membranes, appears to be the most suitable for the transfection of "difficult to transfect" cell lines such as those of haemopoietic origin [26, 27]. This method was investigated in a variety of cell lines to demonstrate efficacy and to compare against the results of the other means of DNA delivery which are reported later. In addition a variation on conventional electroporation, the BAEKON electroporator, was investigated for this, unlike conventional high current / low voltage methods, uses high voltage with low current. It was possible that this could have been less damaging to the target cells.

3.2 SPECIAL METHODS.

3.2.1. Electroporation.:

DNA was used of high purity with a 260/280 ratio of 1.6-1.8, it was either freshly ethanol precipitated or used from a -80^{o}C stock frozen immediately after resuspension in TE following ethanol precipitation. Cells were pelleted by centrifugation at 1000 rpm (MSE centrifuge), in most cases the growth medium was retained for use after electroporation. The cells were resuspended in 0.2 ml of RPMI/ Hepes / 10% FCS and 6-20 μg of the DNA to be transfected was added. BioRad electroporation cuvettes were used and discarded after a single use. The cells and DNA were incubated for 10-30 minutes prior to electroporation either at room temperature or on ice. The cells were then electroporated in the BioRad electroporator at 960 μF, 0.20-0.25 kV (unless otherwise stated) and the time constant recorded after treatment. The cells were than left for a further period of 15-30 minutes on ice or at room temperature before with extreme care transferring them to a culture flask using a sterile glass Pasteur pipette. The cells were then cultured at a density corresponding to 0.1-1.0 x 10 ^{6} of starting cells / ml, normally in the presence of penicillin and streptomycin (see general methods). After the specified incubation period the cells were harvested, lysed and analysed for expression of the transfected gene. In the case of stable transfectants the cells were grown as above for 24 hours after electroporation and at that time 1mg/ml (for K562 and HeLa cells) or 500μg / ml (for HL60) of Geneticin- G-418 Sulphate (Gibco-BRL) was added. Control cells were also treated with G418 at the same concentration, at 24 hours after electroporation. These concentrations were selected after untransfected cells were grown in the presence of G 418 at a range of concentrations and their growth and survival monitored for up to 2 weeks. When transfected cells were being selected a control, untransfected

population of cells was always used to confirm that the concentration of G418 used was toxic to untransfected cells after approximately 7-10 days of culture. The test cells were transfected with a plasmid (from Dr. S. Devereux, Dept of Haematology, UCL) containing the bacterial Neo DNA sequence which, when expressed, confers resistance to the toxic effects of G-418. [100]

3.2.2. Baekoniser.

This method was similar to that for electroporation except that the cells were resuspended in 0.3M sucrose for treatment and the differing parameters on the Baekoniser were set as recorded in the text. The electroporation was then performed using the BAEKON electroporator. (BAEKON Corp., CA, USA)

3.3 RESULTS.

3.3.1. Electroporation in various cell lines with the HSV promoter:

The efficiency of transfection in various haematological cell lines was assessed with a plasmid containing the Herpes simplex promoter and the chloramphenicol acetyl transferase reporter (CAT) gene. The parameters used were those previously optimised in the laboratory for the electroporation of this plasmid into Daudi cells. The voltage used was 250V and the impedance was 960 μF. 10 μg of HSV CAT DNA, prepared by caesium gradient differential centrifugation, was electroporated with 8 x 10^6 Daudi cells, in duplicate, in 200 μl of RPMI with Hepes and 10% FCS. The final cell pellet was lysed in 50 μl of lysis buffer and the %^{14}C uptake to the organic phase of 10 μl of lysate was assayed. The %^{14}C uptake was 1.5% (1.1%,1.8%) with HSV CAT compared to 1.6% for 5μl of a standard of known CAT protein concentration of 0.01 units / ml and 0.25% for the same cells electroporated with 10 μg of a β Galactosidase plasmid as a negative control.

The same conditions were then used for electroporating Daudi cells and 3 other haematological cells lines; K562, HL60 and TF 1. The results of this experiment are shown in TABLE 3.3.1. Ten μg of HSV CAT DNA was again used with 8 x 10^6 cells, the time constants were in the range 29 - 44 ms and the results are expressed as the uptake of labelled ^{14}C to the organic phase obtained with each aliquot assayed. The activity of each aliquot was counted four times and the mean counts per minute (cpm) calculated. The proportion of ^{14}C uptake to the organic phase is shown for each 20 μl aliquot of lysate assayed. (Total cell lysate 50μl). A second experiment, the results of which are shown in TABLE 3.3.2, showed that large reductions of the voltage lead to the complete loss of transfection

activity in both K562 and HL60, but as shown in TABLE 3.3.3. in K562 cells transfection activity was maintained between 0.2 and 0.25 kV.

TABLE 3.3.1. Electroporation of HSV-CAT into 4 Different Haematological Cell Lines at 2 voltages.

Values are for the % ^{14}C Incorporation to the organic phase for 20µl of 50µl total cell lysate.

Cell Type	DNA (µg)	% ^{14}C uptake.
Non transfected cells (Daudi)		0.12
K562	10	5.25
HL60	10	0.1
TF1	10	0.5
Daudi	10	3.25

TABLE 3.3.2. Effect of Voltage (large range) on Transfection efficiency in K562 and HL60 cells.

Values are for the % ^{14}C Incorporation to the organic phase for 20µl of 50µl total cell lysate. (starting aliquot 5 x 10^6 cells)

Cell Type	Voltage (kV)	DNA (µg)	% ^{14}C Uptake
Standard 5 µl 1/100th i. u. /ml			17.8%
HL60	0.25	0	0.08
	0.12	45	0.07
	0.25	45	34
K562	0.25	0	0.05
	0.12	30	0.04
	0.25	30	6.5

TABLE 3.3.3. Effect of Voltage (Small range) on Transfection efficiency in K562.

Cells	Voltage	DNA /μg	Cell No. Aliquot at harvesting x 10^6 (2.5 at t =0)	CAT protein (pg) / 200μl of lysate. (ELISA assay)
EXPT 1				
K562 cells	0.2	10	4.3	5200
	0.22	10	1.7	>8000
	0.25	10	1.05	>8000
EXPT 2				
K562 cells	0.2	4	0.7	1340
	0.22	4	0.45	1325

3.3.2. Selection of promoters.

The activity of constructs including various promoters had been determined in Daudi cells and HSV had been shown to be superior to two other virus promoters RSV (from Rous Sarcoma Virus) and pSV2 (from SV40 virus). (personal communication Dr A Bybee, Dept. of Haematology, University College, London). As a result of this the initial experiments were done using HSVCAT until the CMVCAT promoter became available which when compared to HSVCAT showed markedly greater activity as shown in TABLE 3.3.4. All further experiments utilised CMVCAT.

TABLE 3.3.4. Comparison of HSV & CMV promoters in K562 cells.

Expt 1

(Starting cell number 4 x10 ^{6}, Time constant 37-40 ms, cells lysed in 100 µl of lysis buffer))

Promoter (20µg of DNA)	kV	Vol. of Lysate used	Cell Count at harvesting	CAT Protein (^{14}C uptake to organic phase).
HSV CAT	0.25	10 µl	0.24 x 10^{6}	23.5%
CMV CAT	0.25	10 µl	1.1 x 10^{6}	off scale
		1 µl		>65%

Expt 2

(Starting cell number 2.5 x 10 ^{6}, Time constant 53-58 ms, cells lysed in 500 µl lysis buffer)

Promoter (10µg of DNA)	kV	Vol. of Lysate used	Cell Count at harvesting	CAT Protein pg / 200µl (ELISA)
HSV CAT	0.2	200 µl	0.45	19
	0.22	200 µl	0.3	83
CMV CAT	0.2	200 µl	0.7	>1000
	0.22	200 µl	0.45	>1000

3.3.3. Electroporation vs Baekonisation.

Electroporation therefore can cause effective transfection in at least 2 haematological cell lines but with high toxicity. The BAEKON electroporator, unlike conventional high current low voltage methods, works on high voltage and low current is speculated to potentially have less toxicity to the treated cells. In these experiments the activity of the CAT gene with both the HSV and the CMV promoter was assessed using electroporation as above and the Baekon electroporator in HL60, Daudi and K562 cells.

The initial criteria used for the Baekoniser were;

Baekon Criteria 1: Amplitude 0.5, Cycles 5, Electrode: Cells suspension gap 3mm, no of pulses 2 ^{8} , burst time 3.2s, pulsetime 180 µs.

The criteria used for electroporation were as before namely a voltage of 250V and an impedance of 960µF. 10µg of CMVCAT DNA was used in all cases. The results in Daudi cells showed that there was less toxicity with

the Baekoniser with the cell viability at 18 hours being 77 ± 5 % compared to 27 ± 7.5 % for electroporation. However there was no CAT activity detected after Baekonisation with these criteria compared with the 12.6 % of ^{14}C observed for the electroporated cells (5 µl of 1 i.u./ µl chloramphenicol acetyl transferase used as a positive control = 12.3%). Therefore the amplitude, burst time and number of cycles was increased on the Baekoniser in an attempt to improve transfection. The 2 sets of Baekon criteria were therefore now :

Baekon setting 2. Amp 7.5, no of pulses 2^8, burst time 3.2s, no of cycles 10, pulsetime 180 µs, electrode distance 1mm.

Baekon setting 3. Amp 7.5, no of pulses 2^8, burst time 12.8s, no of cycles 15, pulsetime 180 µs, electrode distance 1mm.

20µg of either HSV-CAT or CMV-CAT DNA was used in 4 x 10^6 K562 cells and the cells incubated for 48 hr after transfection. The time constants for electroporation were 37-40ms. The results are shown in TABLE 3.3.5. and show the uptake to the organic phase using a 1 or 10µl aliquot of cell lysate. (out of total 50µl) This demonstrates that the Baekon method was able to transfect these cells but at a much reduced level compared to conventional electroporation.

TABLE 3.3.5. Comparison of Electroporation versus Baekonisation with CMV CAT in K562 cells.

10µl of final cell lysate used.

	% ^{14}C uptake to Organic Phase
Electroporation	>84
Baekon-Criteria 1.	8
Baekon Criteria 2.	13.7

This experiment was then repeated in Daudi cells and HL60 cells using the same criteria. In this case 5 x10^6 cells were used with either 20 or 40 µg of plasmid DNA and the cell counts at harvesting recorded. The time constants for the electroporated samples were 55-57 ms. The results are shown in TABLE 3.3.6.-experiments 1 and 2. These show consistently markedly less activity with the Baekoniser though with some reduction in toxicity.

In the second experiment the protocol was modified so that cells to be transfected were treated with mycoplasma removal agent (Flow-MRA) during growth prior to transfection and in addition the cells were incubated on ice before and after electroporation for 30 minutes.

TABLE 3.3.6. Comparison of Electroporation (E/P) and Baekonisation in HL60 and Daudi cells with CMVCAT plasmid.

	DNA (μg)	Cell count at harvesting (x 10^6)	CAT protein (%^{14}C uptake)
EXP.1. (Starting cell Number: 5 x 10^6, cells lysed in 60 μl of lysis buffer and 10 μl used for each data point)			
a)BAEKON			
DAUDI	20	2.0	0.8
	40	5.6	0.09
HL60	20	3.4	0.05
b) E/P			
DAUDI	20	2.2	64
HL60	20	1.9	0
EXP.2. (Starting cell Number: 1.7 x 10^6 for Daudi & 2 x 10^6 for HL60, cells lysed in 60 μl of lysis buffer and 30 μl used for each data point)			
BAEKON			
DAUDI	40	2.8	15.4
HL60	20	7	0.1
E/P			
HL60	20	1	22.7

The method for CAT protein estimation was changed to the new commercial ELISA assay at this time and all CAT assays were measured with this method once the sensitivity and reliability of the assay had been established (see Chapter 2 General methods 2.8.2.) When the positive control used up to this point was compared (5 μl x 1/100 μl /ml) it was found to give an value of 150 pg /200 μl by the ELISA technique.

The settings on the Baekoniser were then maximised with either a very long or short burst time to maximise the efficacy of the procedure.

The settings used were:

Baekon setting 4: amp 10, no of pulses 2^8, burst time 0.05s, no of cycles 30, pulsetime 180μs, distance 1mm.

Baekon setting 5: amp 10, no of pulses 2^8 , burst time 12.8, no of cycles 30, pulsetime 180μs, distance 1mm.

The electroporation settings were as before and the time constants noted were 38 for the Daudi cells and 40 for the HL60 cells. The results showed no transfection in Daudi cells with the Baekoniser compared with high activity with conventional electroporation (> 200 pg/ 200μl of cell lysate) , however for HL60 cells there was no activity with either method. These results lead to the abandoning of further experiments with the Baekoniser.

3.3.4. HL60, K562 and HeLa Transient transfection.

Further experiments with K562, HL60 and with the adherent cervical carcinoma line HeLa were done with the conventional electroporation protocol. In the case of HeLa cells it was found that it was important to maintain the cells in suspension for 24 hours after treatment with Trypsin/EDTA when the cells were mobilised from adherent cell culture if transfection activity was to be seen. HeLa cells were repeatedly shown to express large amounts of CAT protein when assayed at 48 hours. CAT protein expression at 48 hours after electroporation of 20μg of CMV CAT into 6 x 10^6 cells was detected in duplicate samples at more than 200 pg / 200 μl of cell lysate by ELISA CAT assay.

The results for HL60 cells described above showed intermittent expression of CAT protein on some occasions but also failure to express any CAT protein on other occasions. This implied, since the protocol used was similar on these different occasions, that these differences were caused by undefined variation in the target cells used. Factors that maybe involved in this variation include:

1) The frequency of treatment of cells with Mycoplasma removal agent (to treat inapparent MP infection) -see below.

2) Cell passage number.

3) Starting cell density.

Under optimal conditions after electroporation of 20 μg of CMV CAT into 6 x 10^6 HL60 cells CAT protein expression was detected in duplicate samples at greater than 100pg / 200μl lysate (of 500μl total cell lysate).

3.3.5. Stable Transfection.

Coincident with these experiments the efficacy of electroporation in achieving stable transfection was investigated in K562, HL60 and HeLa cells by selection for neomycin resistance after transfection with the neo gene. This was achieved for K562 and HeLa with the cells proliferating rapidly in 1mg/ml of G418. At the time of harvesting and freezing the K562 cells 33 days after transfection the cells were requiring splitting to maintain optimal cell density two times per week in the presence of 1mg/ml of G418. No growth was observed in control untransfected cells beyond 10 days at this concentration of G418. However no HL60 cells survived after the selection procedure.

3.3.6. Transfection in TF1 cells.

Transient transfection was initially achieved only at low efficiency in TF1 cells, however with the CMV-CAT plasmid and optimisation of cell culture as discussed above transfection efficiency was markedly improved. The most important criteria was treatment with Mycoplasma removal agent (ICN -FLOW). This was examined prospectively when the cells were not transfecting efficiently. One aliquot was treated for with MRA while a second aliquot was retained in normal medium as a control. An equal number of cells from both aliquots were transfected with 10μg of CMV CAT DNA, under standard conditions with similar time constants, the cells were

cultured for 20 hours, lysed and assayed. The MRA treated cells expressed 3685 ± 79 pg CAT protein / 200µl of lysate whereas the untreated cells only expressed 97 ±38 pg CAT protein / 200µl of lysate. The use of stored conditioned medium was also noted to be required for high levels of transfection to be seen. Time course experiments demonstrated that CAT protein in transfected cells could be detected as early as 3-5 hours after electroporation. The results are summarised in TABLE 3.3.7.

TABLE 3.3.7. Time course of CAT expression after Electroporation of TF1 cells under different culture conditions.

	CAT ACTIVITY pg/200µl of lysate		
Time Constant 60-80 ms			
TF1 Cells 2.5-3 x10^6 / aliquot			
Incubation Medium	3hrs	5 hrs	overnight
1. CM + IL3-10ng/ml			49
2. RPMI / FCS		12.5	6
RPMI / FCS + IL3 (10 ng/ml)		0	14
3. CM (Starved)	30		>200
CM+ GM CSF 2 ng/m	42.5		>200
4. RPMI / FCS	0	0	0
RPMI / FCS + 2 µg/ml GMCSF	0	23	
RPMI / FCS + 5 µg/ml IL3	0	0	13
5. CM / +2 µg GMCSF		88	>200

CM : Conditioned medium.

3.4. DISCUSSION.

These experiments demonstrated that it is possible to achieve transient expression of CAT protein in a variety of haematological cell lines. The results and findings in terms of the selection of parameters and outcome achieved correspond closely with work published, after this work had been completed, by Pahl et al [26] on transient transfection of HL60 cells using the luciferase reporter gene system. However there is marked toxicity and there is variation within the population of treated cells in the expression of the transfected gene (see Chapter 7)[26]. There was a marked difference between the cell line K562 in which efficiency of transfection was robust as compared with the HL60 and TF-1 cell lines in which expression of the reporter gene varied from zero to high levels according to undefined parameters which may include factors such as passage number, cell density and Mycoplasma (MP) infection. Unfortunately the relative expense and difficulty of mycoplasma detection compared to empirical treatment with MRA means that the role of MP infection remains poorly defined.

These two cell lines; the HL60 cell line, which can be induced to undergo differentiation in response to appropriate stimulation, and the growth factor dependent cell line TF1 are both potentially useful in the study of cell growth and differentiation. Therefore, in order to study a homogenous population of cells either stable transfection must be combined with a selection system such as neomycin resistance or the proportion of cells transiently transfected must be increased. With regard to stable transfection this was only achieved in the non-haemopoietic cell line HeLa and in the robust haematological cell line K562. This therefore left the need to develop a transient transfection system capable of transfecting a high proportion of a variety of myeloid cell lines with low toxicity. Liposome and virosomal transfection offered the possibility of achieving these aims.

CHAPTER 4: CARBOXYFLUORESCEIN DELIVERY BY LIPOSOMES.

4.1. INTRODUCTION.

The delivery of the fluorescent dye carboxy-fluorescein (CF) was chosen as a surrogate for DNA in the initial process of optimising liposomal delivery. Fluorescent liposomes also have the advantage of enabling the assessment and uptake at the single cell level. Delivery of macromolecules by liposomes is dependent, firstly on the nature of the lipid used, especially with regard to charge and the proportions in which different lipids are mixed. Secondly on the method by which they are made which is in turn integrally related to the size of the liposomes synthesised and thirdly it is related to whether or not they are targeted by their association with, for example, viral proteins.

The lipids used in liposomes which had been proposed to be of value in DNA delivery were firstly neutral lipids such as phosphatidyl-choline (PC) (whether egg or synthetically derived) and cholesterol. Liposomes made of only these two lipids were used by the Jerusalem group [43] in association with influenza viral proteins. By contrast the Japanese group led by Kaneda used liposomes including PC, the negatively charged lipid Phosphatidylserine (PS), cholesterol and ganglioside in association with UV inactivated Sendai virus [42]. Alternatively it has been proposed that macromolecules could be delivered in reconstituted viral particles composed only of viral phospholipids after detergent dialysis [101].

With regard to the size of the liposomes this may determine the fate of the liposomes after interaction with cells as it is proposed that the larger liposomes are more likely to fuse with the plasma membrane as opposed to being phagocytosed into endosomes. Small vesicles can be made to be reliably unilamellar which may also assist in delivery; these may be made by high pressure extrusion through filters [96] or by reverse phase

evaporation [42]. Conversely large unilamellar liposomes are best made by detergent dialysis and pure PS liposomes have been reported to be effective even without targeting [35]. Most reports of targeting with liposomes reported in the literature have concentrated on the use of the haemagglutinin and fusion proteins of the RNA enveloped viruses Sendai and Influenza. The most complex system reported [25] used "chimerasomes" which are large unilamellar vesicles incorporating, by reconstitution, Sendai virus phospholipids and viral membrane proteins. At the commencement of this work many of these authors had reported liposome mediated transfection in non human or fibroblast cell lines and only one [35] reported transfection of human haemopoietic cells and even this was at low levels. Therefore all of these methods were examined in this model system to optimise parameters such as encapsulation and fusion prior to commencing DNA work.

4.2 SPECIAL METHODS

4.2.1. LUV Synthesis by detergent removal.

These were made by detergent dialysis against SM2 biobeads (BioRad) after solubolisation of a lipid film of egg phosphatidylcholine and cholesterol (molar ratio 2:1) in a 1% solution of octyl glucoside in buffer A. (Detergent : Beads 1:7 w/w) The concentration of the carboxyfluorescein was 20 or 80 mM. The use of the Biobeads which remove the detergent from the virus solution avoids a long dialysis step and enables the encapsulation of small molecules.

4.2.2. RSVE Synthesis.

Purified Sendai virus (HVJ) was solubolised with the detergent 3-[3-chloroamidopropyl]dimethylamonio)-1-propane-sulphononate (CHAPS). This solution was then centrifuged to remove the insoluble virus cores and then the detergent was removed by either conventional dialysis or by the addition of SM2 Biobeads thus allowing the virus envelopes to reform spontaneously. The low molecular weight of the fluorochrome used (carboxyfluorescein Sodium salt MW 493) prevents encapsulation when conventional dialysis is used due to rapid equilibration with the dialysis buffer. Virus was then recovered by ultracentrifugation at 60000g at 4 °C (Beckmann J2, SW 41 rotor).

4.2.3. Measurement of Intracellular pH.

Liposomes were loaded with the pH sensitive dye SNARF ™ (Molecular Probes) which emits at a different intensity at different wavelengths according to the pH of the surrounding environment. The emission at 604nm (the isobestic point) is uniform at all pH's and therefore can be used to give an assessment of the amount of dye delivered to the cells. The emission at acid pH's is maximal at 588 nm and at basic pH's the maximum

emission is at 634 nm. Therefore an assessment of the pH of an acid environment can be made by calculating a ratio of the emission at 588 nm and the emission at 604 nm. Measurements were done in quartz cuvettes in a Fluorimeter (Locarte, London).

4.2.4 Measurement of fluorescence Dequenching.

The quenched nature of the 200 mM CF liposomes was confirmed by measurement of the fluorescence of a known aliquot of liposomes which had been washed by pelleting and resuspension in buffer. The liposomes were then lysed by the addition of 10 µl of a 10% solution of Triton-X and the measurement repeated with appropriate dilution to achieve a reading within the scale. The dequenching % was then calculated as :

$$\text{dequenching \%} = \frac{\text{Fluorescence of Lysed Liposomes}}{\text{Fluorescence of intact liposomes}} \times 100.$$

The fluorescence of the lysed liposomes was measured in the presence of the Triton -X but the fluorescence of the detergent itself was insignificant (<0.1%) of the fluorescence of the released CF. An effect of the detergent on the fluorescence of the dye was not excluded, however, since the degree of dequenching was used comparatively between liposome types it is unlikely that any such effect, if present, would have caused any significant differences to the results obtained.

4.2.5. Preparation of Peripheral Blood Lymphocytes (PBL) Density gradient separation.

Mononuclear cell (MNC) preparations were obtained by layering blood (diluted to 50% with RPMI) onto an equal volume of Lymphoprep (Nycomed, Oslo, Norway.) followed by centrifugation at 400g for twenty minutes at room temperature. Low density cells at the interface were collected and washed twice in RPMI1640 and finally resuspended in RPMI1640/10% HIFCS and the cell count and viability determined before experimental use.

4.3 RESULTS

4.3.1 Large unilamellar liposomes made by detergent dialysis

Large unilamellar liposomes were made by detergent dialysis (detLUV) in the presence of carboxyfluorescein which therefore becomes encapsulated within the internal aqueous space. The liposomes were then incubated with 2 x 10^5 K562 cells at 37° C for 30 minutes followed by incubation at 37° C for one hour. Following the incubation the cells were then washed and analysed on an EPICS (COULTER) flow cytometer to determine the mean cell fluorescence of the population of cells within a bit map selected on the forward angle light scatter and log 90° scatter of control cells. Unbound liposomes are mainly lost during the washing steps but in addition are excluded from the cell fluorescence estimation by use of the bitmap since they are of a smaller size.

The fluorescence is expressed as a ratio of the Mean Cell Fluorescence (MCF) of the cells incubated with liposomes divided by the MCF of control cells (MCF:MCF ctl). In the case of det-LUV this ratio was 16.5.

4.3.2. Reconstituted Sendai Virus Envelopes (RSVE)

RSVE were investigated as a means of delivering CF either alone or in combination with CF loaded liposomes. The liposomes and virus were pre-incubated to allow them to interact together before addition to the cells to be treated. Three batches of RSVE were made as described above and the virus protein measured. The use of the detergent Triton-X to initially disrupt the virus before reconstitution by detergent removal by dialysis did not result in the reconstitution of functional virus envelopes but with the detergent CHAPS functional RSVE were obtained which were able to agglutinate chicken red blood cells (Tissue Culture Service, Bucks, UK). In terms of detectable protein in the final RSVE solution in the three batches the yield was 30 μg (from 25 mls virus stock solution), 180 μg from 72 mls of

virus stock solution and 45µg from 15 mls stock solution respectively. The HAU titre of the stock solution prior to purification of the virus from the harvested allantoic fluid was 128 000 HAU /ml. The yield in terms of HAU reconstituted in the final RSVE solution compared to the starting virus stock solution was 2.8 % for batch 2 and 1.7 % for batch 3. Approximately 50 % of these losses were accounted for by the differential centrifugation and purification step and the remainder from losses during dissolution, dialysis and ultracentrifugation of the RSVE. For batch 3 the recovery of 45 µg of virus protein as RSVE represents 10 % of the total virus protein assayed in the purified virus aliquot. The viral membrane associated proteins represent approximately one third of the total virus protein [25] therefore the implication is that 50% (1.7 / 10 x 0.33) of the viral membrane proteins are functional when reconstituted in the RSVE. These RSVE did not encapsulate significant amounts of carboxy-fluorescein when it was included in the reconstitution buffer and therefore the use of RSVE in combination with LUV was investigated. The RSVE were combined with CF containing LUV at 37° C for 30 minutes followed by incubation for one hour at 37° C with 2 x 10^5 K562 cells. The cells were analysed as above and the mean cell fluorescence of the cells after incubation with RSVE + CF liposomes was 17.4 times that of the cells alone (MCF:MCF ctl ratio) however this was no greater than the ratio of 16.5 obtained for liposomes without RSVE added to the cells. In a further experiment RSVE and liposomes (200 µl of 0.7 mg/ml total lipid) were incubated at 4° C for 30 minutes , then added to 2 x 10^5 K562 cells at 4° C for 30 minutes and then further incubated at 37° C for 2 hours. Again there was no increase in the MCF ratio compared with the CF liposomes alone over a range of virus HAU titre between 100 and 1000.

4.3.3. Sendai Virus and Large unilamellar liposomes.

The activity of whole virus added with CF loaded liposomes to the cells was then examined. 200 µl (2000 HAU) was added to 200 µl (0.7 mg/ml) of liposomes and incubated at 4° C for 15 minutes to allow interaction between the liposomes and virus and then the mixture was added to 1 x 10^6 K562 cells at 4° C for 15 minutes before a final incubation at 37° C for 4 hours. The ratio of the MCF of these cells to control cells was 2.2 times that detected if the virus was omitted. This was repeated with 5 HAU titres between 400 and 2000 and the MCF/ MCF:ctl ratios were all between 1.7 and 2 times that seen with the liposomes alone though this ratio did not increase with the dose of virus used.

4.3.4. Sendai Virus and Small unilamellar vesicles.

The encapsulation of CF by LUV made by detergent dialysis is relatively inefficient so the use of small unilamellar vesicles (SUV) made by high pressure extrusion through filters was investigated. These liposomes were compared with the LUV's made by detergent dialysis (detLUV) either with or without virus.

15 µM of lipid : egg phosphatidylcholine and cholesterol (molar ratio 2:1) was used for the synthesis of both liposome types and the final lipid concentration of the suspensions was measured by lipid assay. With the detLUV's 0.5 mls of a suspension of 0.8 mg/ml (i.e. 0.4 mg of final liposome lipid) was obtained whereas with the SUV's 1 ml of an 8 mg/ ml suspension (i.e. 8 mg) was obtained.

When equivalent aliquots of these two liposome types were incubated with virus (HAU titre 0,1000,2000,3000,4000) at 4° C for 20 minutes, added to 2 x 10^5 K562 cells at 4 °C for 20 minutes and 37° C for 2 hours the MCF/MCF:ctl ratio was greater for the SUV's compared to the detLUV's at each dose of virus used;

(0 HAU 1:1.4, 1000 HAU 1: 1:1.3, 2000 HAU 1:1.3, 3000 HAU 1:1.2, 4000 HAU 1:1.3).

Following reference to the method published by Kaneda et al [42] three modifications were made: the virus dose was increased, a 37° C incubation of virus and liposomes was added and calcium chloride was added to the incubations at a final concentration of 1 or 2 mM.

The effect of washing the cells after the cold incubation (when liposome attachment should have occurred) was examined. 200mM CF SUV (2mg total lipid) in 400 µl were added to HVJ 8000,12000 or 16000 HAU in a total volume of 400 µl . The virus was incubated at 4° C for 20 minutes and 37° C for 40 minutes with the liposomes then added to 2 x 10^5 K562 cells at 4° C for 10 minutes and then further incubated at 37° C for 2 hours. In this experiment no increment over liposomes alone was seen with any virus dose and the early washing step reduced the final MCF/MCF:ctl ratio by approximately 40 %, therefore the use of a washing step at this stage was abandoned.

K562 cells and peripheral blood lymphocytes in parallel were then incubated at either 4°C or 37°C to establish whether internalisation was occurring. The MCF/MCF:ctl ratios for K562 were 1.8 at 4° C and 6.0 at 37° C and for Peripheral blood lymphocytes the MCF/MCF:ctl ratios were 2.0 at 4° C and 9.0 at 37° C. The dequenching of these liposomes with detergent lysis was 1000 % since a 1/300 dilution of the liposome stock registered 135 units by fluorimetry at 520nm (excitation at 488nm) this aliquot was then recorded as off scale after Triton-X lysis of the liposomes. The lysed liposomes registered 642 units following a 50/50 dilution so there was an approximately a ten-fold dequenching after lysis.

The role of the temperature of the incubations was further examined. 100 µl of liposomes at 12mg/ml loaded at 200mM CF were incubated with 20000 HAU of HVJ for 30 minutes at 37° C then added to 2 x 10^5 K562 cells and incubated, washed and analysed and the results are shown in TABLE 4.3.1.

TABLE 4.3.1 Incubation of K562 cells with carboxyfluorescein containing liposomes with or without Human Sendai virus (HVJ): Ratio of Mean cell fluorescence of cells incubated with liposomes to cells alone (MCF + liposomes / MCF cells only)

	MCF +liposomes / MCF cells only	
Incubation Conditions.	Liposomes	Liposomes + HVJ
Liposomes + Cells 4° C 30mins & 37° C 2 hours.	14	44
Liposomes + Cells 4° C 30mins, WASHED* & 4° C for 2 hours.	3	7
Liposomes + Cells 4° C 30mins, WASHED* 37° C for 2 hours.	3	3
Liposomes + Cells mixed but no incubation WASHED* & 4° C for 2 hours.	3	3

WASHED* = unbound liposomes removed by centrifugation of cells after initial incubation.

The PC/Chol liposomes were then compared with those with a lipid composition as used by Kaneda et al [42] that is PS / PC / Cholesterol / ganglioside with a composition of 1: 4.8: 2 of PC/PS/Chol plus 1/1000 of ganglioside as a potential virus target. Five similar experiments with Liposomes and 12800 HAU of HVJ and 2 x 10^5 K562 Cells were performed. In each case parallel samples contained an equivalent amount of lipid as determined by lipid assay. The incubations were 1/2 hr at 37° C, 1/2 hr at 4° C and then 2 hr at 37° C and the results are shown in TABLE 4.3.2. and show that the PC/Chol SUV give high values for the MCF/MCF:ctl ratio but with no increase in the presence of virus whereas the PC/PS/Chol/Gang liposomes give a lower value for MCF/ MCF:ctl but that it is consistently increased after a preincubation in the presence of virus.

TABLE 4.3.2. Incubation of K562 cells with carboxyfluorescein containing liposomes of different lipid composition with or without Human Sendai virus (HVJ): Ratio of Mean cell fluorescence of cells incubated with liposomes to cells alone

	MCF +Liposomes / MCF control					Mean
Expt No.:	1	2	3	4	5	
PC/Cholesterol						
Liposomes	18	51	37	38	313	
Liposomes+ virus	16	67	49	52	438	
Ratio +/- virus	0.9	1.3	1.3	1.4	1.4	**1.3± 0.1**
PS/PC/Chol/Gang.						
Liposomes	4	3	3	3*	16	
Liposomes+ virus	8	11	14	10*	43	
Ratio +/- virus	2	4.3	4.7	3.3	2.7	**3.4± 0.5**

* 4° C step omitted.

UV inactivation of Virus:

In order for the technique to be valuable for transfections the virus must be inactivated by ultra violet irradiation [42]. Three minutes of irradiation at 100 erg mm^{-3} s^{-1} was used and it was determined that this had no effect on the HAU titre of the virus.

4.3.5. Use of the pH sensitive dye SNARF for determination of intracellular pH.

2 x 10^{5} K562 cells were incubated with virus and 0.2 mg of liposomes loaded with 1.1 mM SNARF. The cells were then washed and the emission analysed at 588 and 604nm. The un-encapsulated SNARF diacetate used as a positive control is able to freely diffuse into cells whereas SNARF, being a polar molecule, cannot cross the cell membrane.

The results shown in TABLE 4.3.3 suggest that the PS/PC/Chol/G liposomes are being delivered to a more acidic environment when incubated without virus than with virus. By contrast the PC/Chol liposomes seem to be delivered to a neutral pH in both circumstances.

TABLE 4.3.3. Incubation of K562 cells with SNARF containing liposomes of different lipid composition with or without Human Sendai virus (HVJ): Ratio of Total fluorescence of cells at 588 nm to Total fluorescence of cells at 604 nm.

	588/604 Ratio.	
	1	2
PC/Chol. Liposomes	1.0, 1.3	
Liposomes + HVJ (4000 HAU)	1.0, 1.0	
PS/PC/Chol./Gang. Liposomes	2.4, 2.3	2.1
Liposomes	1.2, 1.5	
+ HVJ (6000HAU)		1.3
+ HVJ (12000HAU)		1.5
+ HVJ (24000HAU)		1.2
cells+ Free SNARF diacetate.	1.4, 1.4	1.6

4.3.6. Investigation of different types of liposome with three different cell lines:

Three cells types K562, Daudi and TF1 were incubated with liposomes (total lipid 0.25mg) of two different lipid compositions with or without 12000 HAU of HVJ. Dequenching was assessed by fluorimetry before and after lysis with Triton X. The results are shown in TABLE 4.3.4. This experiment was then repeated for K562 cells with other liposome types to further examine the role of gangliosides and the relative value of egg derived PC versus synthetic DPPC. Total CF incorporated in the liposomes and the

degree of quenching was assessed by triton lysis and fluorimetry of a known aliquot of liposomes and a correction was made for the loading of CF in the liposomes. 2 x 10^5 K562 cells, 0.5 mg of lipid and 6000 HAU of virus was used. The virus was incubated at 4° C for 30 minutes and 37° C for 30 minutes with the liposomes then added to 2 x 10^5 K562 cells at 4° C for 30 minutes and further incubated at 37° C for 2 hours.

The results are shown in TABLE 4.3.5.

TABLE 4.3.4 Incubation of Three cell lines with PC/Cholesterol SUV or PS/PC/Chol/Gang SUV with or without HVJ.

		MCF/ MCF control			
Lipid used.	% Dequenching*	K562	Daudi	TF1	Mean
egg PC/Chol	2100	39	12	22	
egg PC/Chol +virus		20	9	8	
ratio +/- virus		0.5	0.75	0.4	**0.55±0.1**
PS/PC/Chol/Gang	2100	5	5	5	
PS/PC/Chol/Gang +virus		9	8	9	
ratio +/- virus		1.8	1.6	1.8	**1.7±0.1**

* % increase in fluorescence detected after detergent induced lysis of a known aliquot of liposomes.

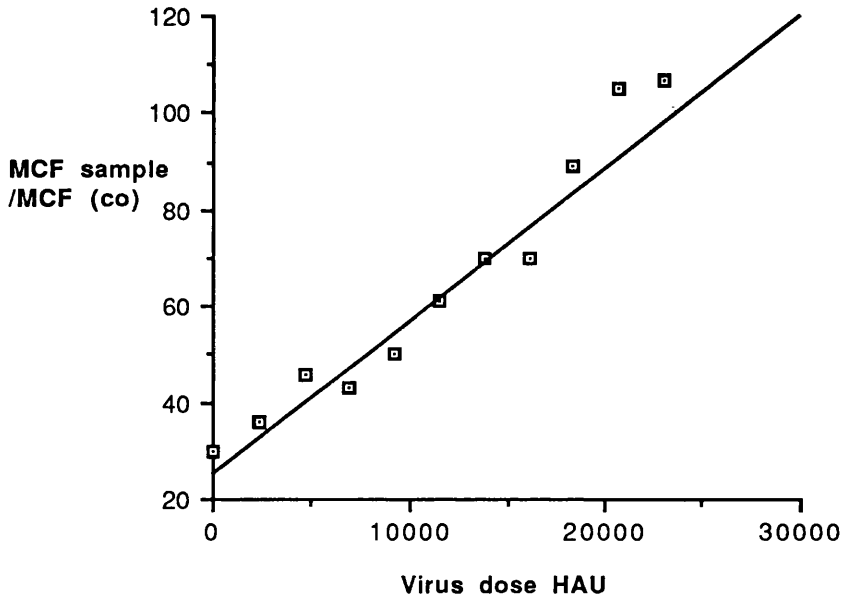
TABLE 4.3.5. K562 cells incubated with PC/Cholesterol SUV, DPPC/ Cholesterol SUV or PS/PC/Chol/Gang SUV with or without HVJ. (MCF treated cells/ MCF control)

	Total CF released from 20 µg of lipid (PC/Chol = 1.0)	% de-quenching	MCF/ MCF control	MCF/ MCF control (corrected for CF loading)
PC/Chol	1.0	300	60	60
+ virus			29	29
ratio +/- virus				**0.5**
PC/Chol/Gang	3.8	500	188	50
+ virus			74	19
ratio +/- virus				**0.4**
DPPC/Chol	2.1	2200	8	4
+ virus			6	3
ratio +/- virus				**0.75**
Chol/DPPC / Gang	0.82	600	6	7
+ virus			5	6
ratio +/- virus				**0.9**
PS/PC/Chol /Gang	2.75	2600	12	4
+ virus			35	13
ratio +/- virus				**3.3**

Each value is the mean of 2 samples.

The conclusion from all of these experiments was that the presence of virus produced an increment during the incubation only with the PS/PC/Chol/Gang liposomes. A dose response curve for virus dose was then produced by repeating this experiment with varying virus doses and this is shown in FIGURE 4.3.1.

FIGURE 4.3.1. Dose response curve for the dose of HVJ used with CF loaded PS/PC/Chol/Gang liposomes: Dose of virus versus MCF of treated cells/ MCF control



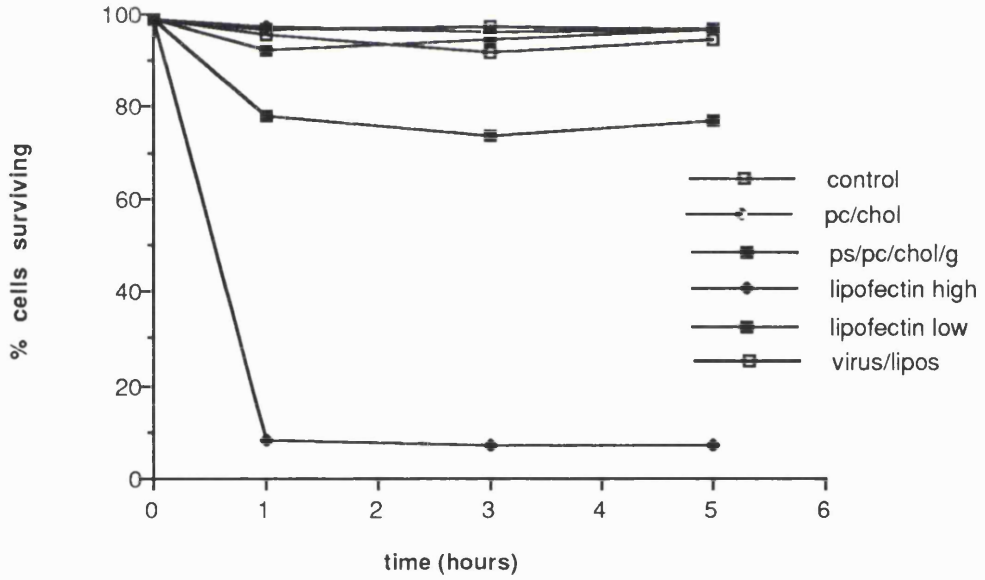
Each data point is the mean of two samples.

The importance of the gangliosides in these liposomes was demonstrated by making liposomes with and without gangliosides. After correction for CF loading the ratio of the MCF/MCF:ctl ratio with and without virus was 2.4 with ganglioside and 1.5 without ganglioside.

4.3.7. Liposome and virus Toxicity

The toxicity of these liposomes with and without virus was tested and also compared to that of the commercially available DNA binding cationic lipid Lipofectin™. The results are shown in FIGURE 4.3.2. Three lipid concentrations for each liposome type were examined 0.01,0.1 and 0.5mg but only the highest dose is shown. Lipofectin™ was used at 10µg/ml (low dose) and 100µg/ml (high dose). The Liposome and virus data is for 1mg/ml of PS/PC/Chol/G liposomes with 2300 HAU of virus. The only significant toxicity observed was with Lipofectin.

FIGURE 4.3.2. Toxicity of PC/Chol and PC/PS/Chol/Gang Liposomes compared to Lipofectin.



Each data point is the mean of two samples.

4.3.8. Large Unilamellar Vesicles (LUV) made from cochleate intermediates with or without Sendai virus.

The use of a large vesicle theoretically enables the delivery of encapsulated molecules from a much larger internal space however detergent dialysis did not achieve this, as discussed above, and resulted in poor encapsulation. The synthesis of large Unilamellar Vesicles (LUV) from cochleate intermediates using negatively charged lipids such as phosphatidylserine was first described by Papahadjopoulos [37] and modified for use in gene transfer by Itani et al [35]. This method was investigated again initially using CF delivery to target cells as a measure of efficacy.

Incubation Temperature : Initially the same lipid combination (PS,PC,Chol, Gang) was used as for SUV but this was inefficient when using the LUV via Cochleate intermediate method due to the low proportion of negatively charged lipids available to take part in the calcium-lipid complex that is formed in the intermediate step. Therefore liposomes composed of only PS were used. CF delivery to K562 cells by these liposomes was compared with that of PC/Chol SUV and the effect of the temperature of incubation was examined with particular reference to the use of 27° C incubations. The use of this temperature for the incubation was suggested by the instability of PS liposomes at higher temperatures because of the physical properties of the lipid. When the two liposome types were compared either an equal amount of lipid or an equivalent amount of encapsulated CF was used. The amount of CF encapsulated was estimated by lysis of the liposomes induced by the detergent Triton -X. This indicated that the cells became markedly more fluorescent after incubation with the LUV compared to the most effective SUV type as determined by the experiments discussed above. The results are shown in TABLE 4.3.6. The temperature of the

incubations was further investigated with regard to the 3 incubations steps in the method. These are:

1. Phosphatidylserine with calcium buffer in the formation of cochleates.
2. Cochleates With EDTA buffer: formation of liposomes.
3. Interaction of liposomes with cells in PBS.

The results of these experiments suggested that the liposome + cell incubation was best done at 27° C, these results are shown in TABLE 4.3.7. The Figures in the second column are corrected for the carboxyfluorescein content of the liposomes as determined by detergent lysis and fluorimetry. The temperature of the first two incubations had little effect on the final cell fluorescence observed.

TABLE 4.3.6 Comparison of PC/Chol and PS/PC/Chol/Gang SUV addition to K562 cells at 2 different temperatures with measured encapsulated CF. MCF of treated cells/ MCF control.

		MCF of treated cells/ MCF control.			
Expt No.	Encaps. CF ratio: PC/Chol. PS/PC/Chol/Gang	PC/Chol. SUV		PS LUV	
		27° C	37° C	27° C	37° C
1	6 / 1		4.4		21.9
2	7 / 1	5.2	5.3	10.2	7.2
3	2 / 1	2.7	1.8	47	24

for Expt . 1 & 2 each value is the mean of two samples.

TABLE 4.3.7. PS LUV addition to K562 cells : Effect of 2 different temperatures during synthesis of liposomes and 2 different temperatures of incubation: MCF of treated cells/ MCF control.

	MCF/ MCF control	MCF / MCF control Corrected for CF loading (27° /27° = 1)
27° /27° at 27°	28	28
27° /37° at 27°	41	26
37° /37° at 27°	28	23
37° /27° at 27°	24	20
27° /27° at 37°	15	15
27° /37° at 37°	26	16
37° /27° at 37°	13	11
37° /37° at 37°	10	17

4.3.9. Addition of Virus with Large Unilamellar Vesicles (LUV) made from cochleate intermediates.

At this point UV inactivated Sendai Virus (9000 HAU) was introduced by pre-incubating these PS liposomes with UV inactivated Sendai virus and comparing the cell fluorescence observed with that induced by the PC/Chol SUV. The pre-incubation at 27° C allows liposome and virus interaction to occur. The cell line HL60 was used for these experiments. The cell and liposome incubations were done in Tris buffered saline for 2 hours at 27° C or 37° C. At 27° C pre-incubation of the PS LUV with virus lead to an increase in the mean cell fluorescence observed (MCF/MCF:ctl was 25 with virus (9000HAU) but 12.9 without virus). However, in contrast, at 37° C a pre-incubation with virus (9000HAU) lead to a decrease in the mean cell fluorescence observed with PS LUV (MCF/MCF:ctl 9.6 with virus vs 19.8 without virus) though both were still greater than the value for MCF/MCF ctl for PC/Chol SUV (1.5).

The role of lipid composition was then examined with reference to the addition of cholesterol, as a membrane stabiliser, and ganglioside as a potential virus target. Experimental conditions used were similar to the previous experiments. The total lipid used per aliquot was the same in each case. These experiments suggested that these lipid modifications had little effect on the final cell fluorescence observed and that a temperature of 27° C for the liposome with cell incubation after preincubation with virus lead to the greatest observed cell fluorescence. This data is shown in TABLE 4.3.8.

TABLE 4.3.8. Comparison of the addition to HL60 cells of CF loaded PS LUV versus PS Cholesterol LUV with or without UV inactivated HVJ at 2 different temperatures: MCF of treated cells/ MCF control.

	MCF of treated cells/ MCF control	
	27° C	37° C
PS	1.5	1.4
PS + virus (3200 HAU)	3.8	1.5
PS Chol.	1.3	1.2
PS Chol + virus (3200 HAU)	3.6	1.7
PS Chol. Gang	1.6	1.4
PS Chol. Gang +Virus (3200 HAU)	3.9	1.9

The importance of the length of the liposome / virus and virosome / cell incubations was tested. If either or both of the incubations was reduced to 1 hour the final observed fluorescence seen fell from 9.3 times control cells to 3.3, 3.3 and 3.4 for 1hour/1hour ,1 hour/2 hour or 2 hour /1 hour incubations respectively. This suggested that a two hour incubation at each stage was necessary to achieve the greatest observed fluorescence. A formal virus dose response relationship was tested by incubating the

liposomes with a range of virus doses and analysed after a two hour incubation at 27° C with cells. The viability of the cells after treatment with liposomes was also monitored. The results shown in TABLE 4.3.9 demonstrate that virus doses of up to 2500 HAU increase the resulting cell fluorescence but that higher doses are associated with decreasing viability and less fluorescence. The dose response curves obtained for doses up to 3000 HAU is shown in FIGURE 4.3.3. and that with lower doses of virus in FIGURE 4.3.4.

TABLE 4.3.9 Addition to HL60 cells of CF loaded PS LUV with increasing doses of UV inactivated HVJ : MCF of treated cells/ MCF control.

Expt.1.	Viability (%)	MCF/ MCF control
Liposomes	97	2.5
+ Virus 2500 HAU	94	45.8
+ Virus 5000 HAU	92	47.6
+ Virus 7500 HAU	80	50.5
Expt 2.	Viability (%)	MCF/ MCF control
Liposomes	82	3.8
+Virus 1000 HAU	34	36.6
+ Virus 5000 HAU	10	27.8
+ Virus 25000 HAU	14	13.9

FIGURE 4.3.3. Effect of virus dose on Fluorescence; % increase in fluorescence observed after incubation of HL60 cells with PS LUV in the presence of increasing virus titre compared with PS LUV alone (100%)

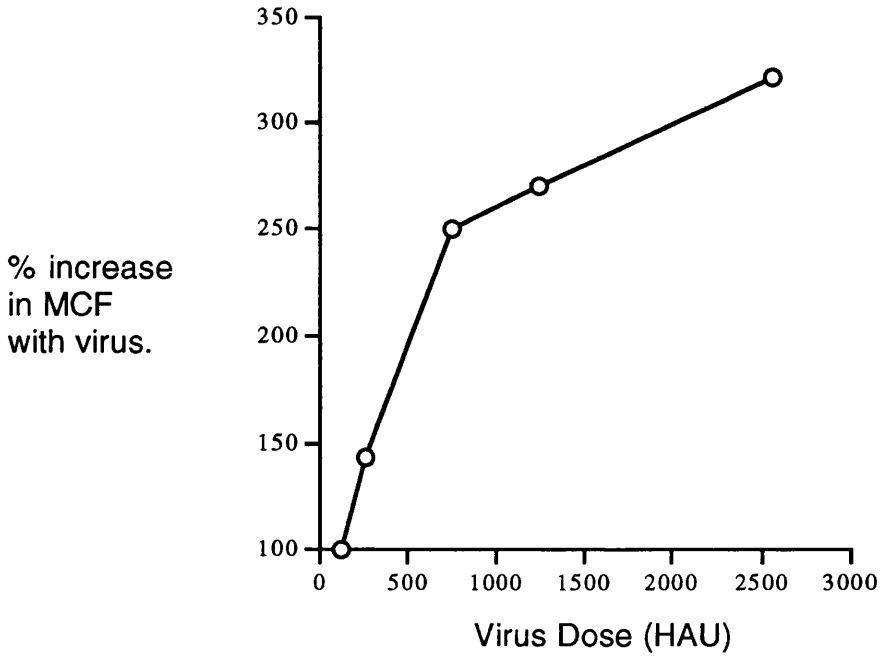
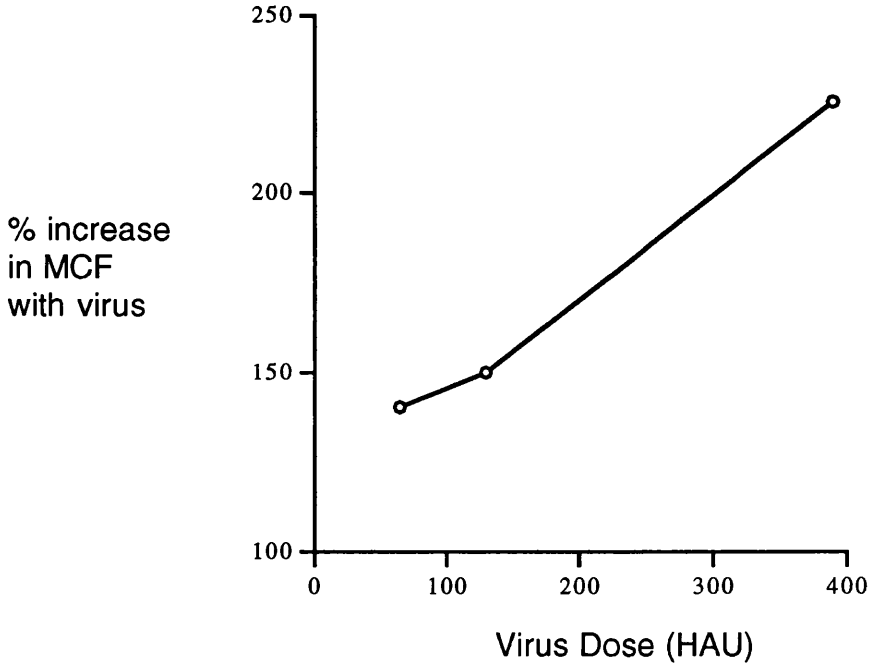


FIGURE 4.3.4. Effect of low doses of virus dose on Fluorescence; % increase in fluorescence observed after incubation of HL60 cells with PS LUV in the presence of increasing virus titre compared with PS LUV alone (100%)



The dye propidium iodide (3 μg/ml x 30 mins) was used to demonstrate that the cells measured within the bitmap excluded the dye and were therefore viable. The effect of virus total dose versus virus concentration for the virus / liposome interaction was examined by adding virus to the liposome aliquot either as neat concentrated virus (5-15 μl) or after dilution to 200 μl with PBS. This suggested that the total virus dose is more important than the concentration of virus during the incubation with liposomes. This data is shown in TABLE 4.3.10.

TABLE 4.3.10. Addition to HL60 cells of CF loaded PS LUV with increasing doses of UV inactivated HVJ, effect of dilute versus concentrated incubation: MCF of treated cells/ MCF control.

	MCF of treated cells/ MCF control	
	Concentrated Virus	Dilute (200μl) Virus
Virus 500 HAU	6.8	6.1
Virus 1000HAU	6.2	5.9
Virus1500 HAU	4.4	4.3

4.3.10. Addition of Chloroquine during incubation of target cells with Large Unilamellar Vesicles (LUV).

The drug chloroquine (CQ) has been shown to increase transfection in some delivery systems probably by blocking acidification of endosomes [53]. Therefore the effect of incubation in 100 μM chloroquine on the final cell fluorescence was determined. This concentration was chosen after determination that this dose was not toxic to the Daudi cells used when they were exposed for short periods of 4 hours or less. The MCF / MCF: ctl ratio was 13 without CQ and 19.5 with CQ for Daudi cells incubated with CF

loaded PS LUV without virus. The MCF/ MCF: ctl ratio was 41.3 without CQ and 36.6 with CQ for Daudi cells incubated with virus and PS LUV. Thus no significant reduction in the cell fluorescence ratio was observed in the presence of chloroquine.

4.3.11. Liposome: cell Incubation: Media and Duration.

The buffering medium used for the liposome /cell incubations up to this point was either tris or phosphate buffered saline. Since it was reasoned that cell viability may be improved by the use of a cell culture medium, as long as liposome integrity was maintained, the effect of various media was examined with or without virus (1280 HAU) and compared to incubations in PBS. Fluorescence microscopy of these cells showed an increase in background, clumping and destruction of liposomes with RPMI, RPMI Hepes and OPTIMEM and so the observed results were unreliable. Therefore a new formulation of the reduced serum medium -OPTIMEM- was obtained and used, this was free of magnesium and with the calcium concentration reduced to 0.5mM, incubation of this with the liposomes did not effect the liposomes and there was no difference from the survival of the liposomes seen with PBS.

A series of 4 experiments comparing incubation in PBS at 27° C with incubation in this modified OPTIMEM at 27° C or 37° C with or without a pre-incubation step with virus was done in Daudi cells. The second experiment included samples incubated in the presence of chloroquine which once again had no significant effect. A fifth experiment was done using HL 60 cells. Monitoring of the liposomes after the liposome/cell incubation step showed that the liposomes remained intact after incubation in this medium. The results, shown in TABLE 4.3.11., show some variation but with the exception of the first experiment the greatest cell fluorescence observed was with either the Modified OPTIMEM or PBS at 27° C when the

TABLE 4.3.11. Increase in Fluorescence (MCF of treated cells/ MCF control) of target cells after incubation of CF loaded PS LUV with different doses of UV inactivated HVJ in different incubation media.

	MCF of treated cells/ MCF control				
Incubation time	2 hrs	3 hrs	3 hrs	2 hrs	2 hrs
Cell type	Daudi	Daudi	Daudi	Daudi	HL60
Liposomes PBS 27°	25.3	6.6	5.8	20.9	
Liposomes PBS 27°					
+ virus 1000HAU	44.4	8.7	13.6	26.7	6.6
+ virus 3250HAU				23.2	
Liposomes PBS 37°	4.8				
+ virus 1000HAU					
Liposomes OPT 27°			7	15	
Liposomes OPT 27°					
+ virus 1000HAU			11	17.4	4.6
+ virus 3250HAU				19.1	
Liposomes OPT 37°	42.8	2.7		11.5	
Liposomes OPT37°					
+virus 1000HAU	51.3	2.5		14.5	3.2
+ virus 3250HAU				14.0	

2 samples/data point- except expt 4.

OPT : Modified (Low Calcium, Magnesium free) OPTIMEM medium.

liposomes were preincubated with virus before adding to the cells. However pre-incubation with a virus dose of greater than 1000 HAU lead to less observed fluorescence than that seen with doses of virus of 1000 HAU, suggesting that there was virus toxicity above 1000HAU. Again there was no effect on the MCF/MCF:ctl ratio of including CQ in the incubation (MCF/MCF:ctl with CQ ±0.5 of MCF/MCF:ctl without CQ for PBS 27° C, PBS +1000HAU Virus 27° C, OPTIMEM 37° C and OPTIMEM 37° C + 1000HAU virus). The effect of longer incubation of the cells with the liposomes was examined to allow a longer time for internalisation of attached liposomes. Daudi cells were incubated overnight in modified OPTIMEM with 10% FCS added. Similar overnight incubation in PBS or modified OPTIMEM without serum resulted in very low cell survival. The cell density was limited at 0.5 x 10^{6}/ml to prevent the medium becoming acidic as this results in the destruction of the liposomes. An initial 30 minute incubation period at high density was used to allow attachment of the liposomes to the cells. In addition to the EPICS analysis the cells were examined on a fluorescence microscope and the fluorescence seen scored on an arbitrary scale. Liposome integrity after the overnight incubation was again confirmed by fluorescence microscopy. The results are shown in TABLE 4.3.12. and suggest that incubation of the liposomes with the cells in OPTIMEM with serum at 37° C gave the greatest observed cell fluorescence.

TABLE 4.3.12. Increase in Fluorescence (MCF of treated cells/ MCF control) of Daudi cells after incubation of CF loaded PS/Chol LUV with or without UV inactivated HVJ at two different incubation temperatures.

	MCF of treated cells / MCF control	%Viable Cells	Fluorescence (Arbitrary score- Fluorescence Microscope.)
27 °C			
Liposomes	5.6	83	+
Liposomes + 1000HAU of virus	10	88	+
37°C			
Liposomes 37°	15.2	53	++
Liposomes + 1000 HAU of virus	27.6	42	+++

2 samples/data point.

A further experiment with K562 and Daudi cells with a long overnight incubation step was done to establish with these conditions whether there was any advantage to the inclusion of gangliosides as a virus target. Again liposome integrity at the end of the incubation was confirmed by fluorescent microscopy. This showed that the inclusion of gangliosides in LUV made from cochleate intermediates is not associated with increased cell fluorescence as shown in TABLE 4.3.13.

TABLE 4.3.13. Increase in Fluorescence (MCF of treated cells/ MCF control) of Daudi cells after incubation of CF loaded LUV : effect of temperature and presence of gangliosides (+G) at two different incubation temperatures in two different cell lines with (+V) or without virus.**(-V)**

18hr Incubation-No added FCS

	1.			2.	
Daudi Cells.					
Medium /Temp.	-G+V	+G-**V**	+G +V	-G+V	+G+V
PBS 27°	6.4	3.3	1.25	10.2	
OPT 27°	4.2	2.0			
OPT 37°	6.2	2.4	1.7	12.1	11.3
K562 Cells.					
Medium /Temp.	-G+V	+G-**V**	+G +V	-G+V	+G+V
PBS 27°	4.1	1.6	2.9	8.9	8.4
OPT 27°	10.1				
OPT 37°	8.3	7.0	8.3	14.5	18.3

2 samples/data point

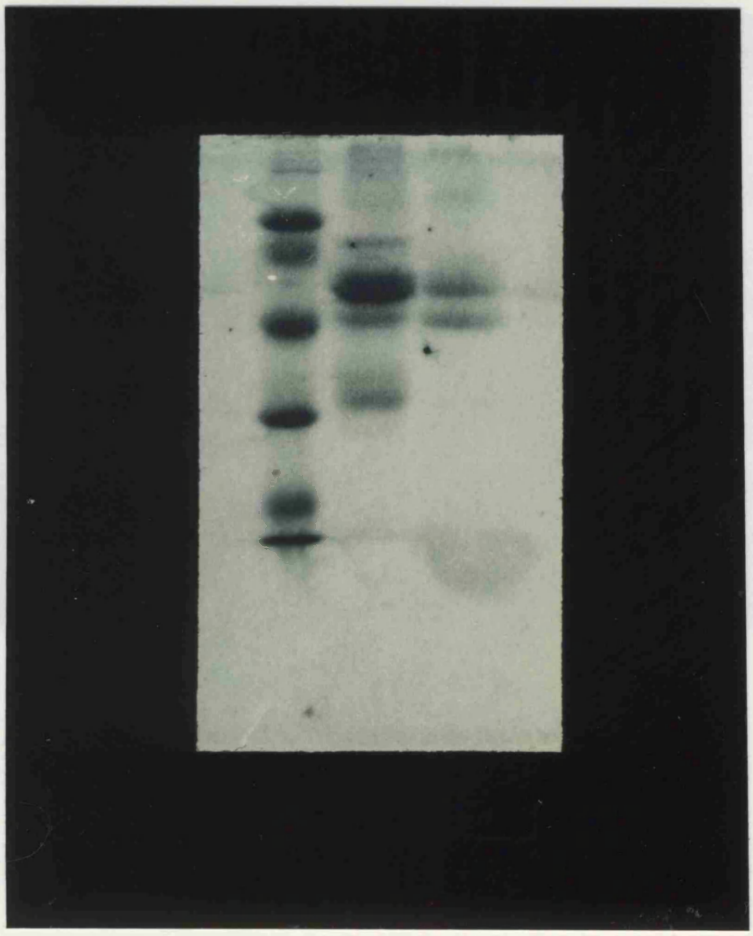
OPT : Modified OPTIMEM (Low Calcium/Magnesium free.)

4.3.12. Chimerasome Synthesis.

The value of the preincubation step in augmenting CF delivery was apparently limited by toxicity above 1000HAU, however if the purified virus is solubolised with detergent and mixed with additional lipid including negatively charged lipids then virus proteins can be incorporated into the cochleate intermediates by dialysis against calcium containing buffer. The LUV can then be made as previously. This method combines the detergent dialysis method originally investigated with the LUV via cochleate intermediate method used later. FIGURE 4.3.5. shows a Poly-acrylamide gel electrophoresis (PAGE) to demonstrate the inclusion of the Sendai virus membrane proteins but not the virus core proteins in the chimerasomes. In this figure when the lane with the chimerasomes is compared to the stock virus it can be seen that the chimerasomes include the two membrane proteins (Haemagglutinin and Fusion Protein) but do not include the three other prominent protein bands which are associated with the virus core seen in the whole virus lane.

Firstly these chimerasomes were used to deliver CF in a similar manner to before and the MCF compared to the MCF of control cells alone. FIGURE 4.3.6. shows K562 cells immediately after mixing with chimerasomes containing carboxyfluorescein. The results of these experiments are shown in TABLE 4.3.14. and indicate that the amount of fluorescence associated with the target cells is five to ten times greater than that seen with the PS LUV pre-incubated with Sendai virus both at 2 hours and after 18 hours incubation.

FIGURE 4.3.5. PAGE Gel to demonstrate the inclusion of Sendai virus Membrane proteins but not Core proteins in Chimerasomes.



Lane 1: Marker.

Lane 2: Purified Sendai virus.

Lane 3: Chimerasomes incorporating Viral Membrane Proteins HA: 64 kD & F 51kD.

FIGURE 4.3.6. Fluorescence Microscopy of K562 cells immediately after mixing with Chimerasomes containing carboxyfluorescein. (x 900).

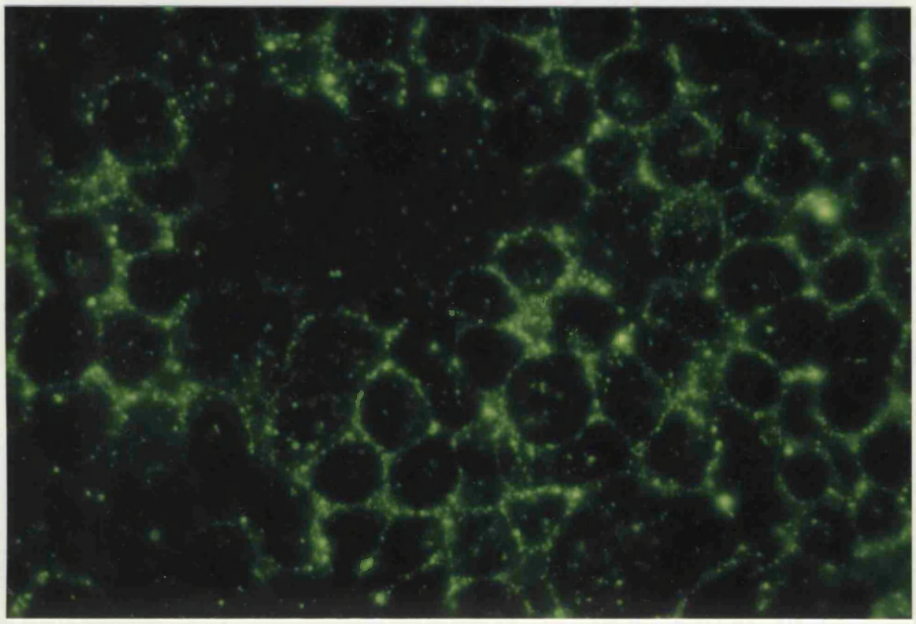


TABLE 4.3.14 Increase in Fluorescence (MCF of treated cells/ MCF control) in two cell lines after incubation of CF loaded PS/Cholesterol/ Virus chimerasomes in PBS or DMEM.

			MCF / MCF:control cells		
	Lipid Dose. (starting PS equiv.)	Incub. Medium	2 HRS	FM Blush +ve	18 HRS
1. K562	0.5 mg	DMEM		-	140
2. `K562	0.1mg	PBS	284	++	
DAUDI.	0.1 mg	PBS	245	+	
3. K562	0.25	PBS	550	+++	
DAUDI	0.25	PBS	828	++	

4.4. DISCUSSION.

The aim of using vesicles to deliver macromolecules to the intracellular space requires them, firstly, to be efficiently encapsulated in the vesicle and secondly for this vesicle to deliver its contents to the cytosol. The reconstituted Sendai virus envelope apparently combined these two properties but in these experiments significant amounts of small molecules could not be encapsulated because of the small size of the envelopes and because of the losses during the long dialysis step, even when SM-2 biobeads were used to extract the detergent as opposed to removal by conventional dialysis. Therefore LUV's were synthesised and both RSVE and virus incubated with them before addition to the cells, because it was believed that they would fuse during this pre-incubation to form a "virosome" which would then be able to fuse with the cell membrane to deliver the liposomal contents. The carboxyfluorescein was encapsulated at a quenched concentration so that a greater increment in cell fluorescence would occur if internalisation occurred compared to the increment in fluorescence due to bound but uninternalised liposomes. When liposomes made by detergent dialysis and HPEF (High Pressure Extrusion through Filters) were compared SUV's made by HPEF gave a greater liposome yield from a given amount of starting lipid and were more efficient at delivering CF to cells. With PC/Chol liposomes, while CF was efficiently delivered, there was no consistent increment when liposomes were preincubated with either RSVE or virus. However when PC/PS/Chol/ Gang liposomes were used though the total amount of delivery was less there was a consistent increment in the presence of virus compared with liposomes alone and this increment increased in a linear manner with increasing virus dose. In addition the use of the pH sensitive dye SNARF suggested that for PC/PS/Chol/ Gang liposomes with virus the SNARF was delivered to a neutral compartment whereas with liposomes alone the

increased emission at 588nm suggested that the SNARF was in an acid compartment. This suggests that the virosome mediated delivery maybe to the cytosol while the liposomes alone were being delivered to acidified endosomes. The inclusion of Phosphatidylserine may increase the fusogenicity of these liposomes and the gangliosides are believed to act as viral targets to promote fusion of virus and liposomes.

These experiments suggested two options for DNA delivery namely PC/Chol liposomes alone and PC/PS/Chol/Gang liposomes in combination with U/V inactivated Sendai virus. The toxicity to the cells of these two options was shown to be minimal in contrast to the commercially available lipid Lipofectin which had significant toxicity at the doses recommended for transfection.

The volume of encapsulation of a liposome increases with the cube of the radius therefore the volume encapsulated by SUV characteristically with a diameter of approximately 100nm or less there is very limited. The volume encapsulated by large unilamellar vesicles, made from cochleate intermediates, with a diameter of up to 1 μm is considerably greater. In addition the method of making LUV by calcium chelation from a lipid/calcium complex allows the liposomes to be loaded at a late stage in the protocol and at higher encapsulations efficiencies. This is an advantage firstly if the substance to be encapsulated is only available in small amounts as is true for most DNA and RNA samples, and secondly the physical methods of preparing vesicles which are unilamellar such as sonication or extrusion are done prior to the point when the vesicles are loaded. Therefore in view of the good cell associated fluorescence achieved with these LUV they represented another liposome type suitable to use for DNA delivery, once the choice of incubation temperature, minor lipid composition and incubation medium type had been optimised.

A 27° C incubation was investigated as a compromise between the need for the lipid to be warm enough to be out of the gel phase while avoiding the increased risk of oxidation with higher temperatures, this temperature appeared to cause the greatest cell fluorescence except in longer incubation experiments when the need for a more physiological temperature for the target cells appeared to override these considerations.

Gangliosides have been suggested to be potential virus targets [42] and so their inclusion was investigated however their inclusion did not increase the cell fluorescence observed with LUV as had been seen with the PS/PC/Chol/Gang SUV, one explanation for this may have been that virus liposome interaction with PS/Chol LUV may result in vesicle lysis rather than fusion [102]. The addition of cholesterol did not increase delivery but because it is likely to stabilise the vesicles [25] it was retained in the protocol, likewise the addition of Chloroquine to the incubation medium had no adverse effect on the cell fluorescence achieved and was therefore an option to be used when DNA delivery was attempted. This is because, by blocking endosomal acidification, it might promote the passage of the DNA to the cytosol un-degraded [53].

The clear positive dose response relationship between pre-incubation of the liposomes with a given dose of virus and the cell fluorescence observed suggests that the virus haemagglutinin and fusion membrane proteins are improving delivery though it is interesting that this advantage is limited to doses of virus up to 1000 HAU above which either the effect is saturated or it is countered by toxicity associated with the virus proteins. The data showing that the virus dose as opposed to virus concentration is important implies that an active targeting process is involved for the dose of virus is the limiting factor and not the volume of incubation.

The effect of the different incubation media is explained by two considerations. Firstly the medium must contain a low (< 0.5 mM)

concentration of di-valent cations otherwise the phosphatidyl serine reverts to the calcium lipid aggregates (cochleates) which are used as an intermediate during their manufacture. This process leads to the release of liposome contents. Likewise the presence of fetal calf serum in most cases leads to liposome destruction, however some batches of FCS do not lead to liposome destruction, in particular heat inactivation at higher temperature (65°C vs 56°C) increased the likelihood of avoiding this effect of serum. This is important as the second consideration is the effect of the incubation temperature and medium on the target cells. This is important not only for increased fluorescence in these experiments but also in later experiments when liposome delivery of plasmid DNA is attempted. This has most effect when the cell/ liposome incubation time is lengthened to overnight when the negative effects on the cells of unfavourable incubation conditions become more apparent.

The largest increase in cell associated fluorescence achieved with liposomes was achieved with "chimerasomes" which are hybrid vesicles made by a combination of detergent removal by dialysis and the calcium cochleate method. The resulting vesicle is principally composed of phosphatidylserine and cholesterol but also includes the Haemagglutinin and Fusion virus membrane proteins, and this was demonstrated on a polyacrylamide gel (FIGURE 4.3.5) on which it was shown that the viral core proteins present in the intact virus are excluded from the reassembled chimerasomes. The ratio of the MCF for targeted cells over negative cells achieved for Carboxyfluorescein with these liposomes was over 800 when incubated at high dose in PBS and this is over ten times the maximum value achieved with other liposome types. These vesicles therefore probably represent the best choice for DNA delivery to cells for transfection.

Therefore, in summary, the results of these experiments identified five different potential sets of conditions suitable for use in liposome mediated DNA delivery experiments. These were firstly, SUV made by extrusion, secondly, SUV with gangliosides in combination with U/V inactivated Sendai virus. Thirdly and fourthly LUV made by removal of Calcium from cochleate intermediates with or without U/V inactivated Sendai virus and lastly the use of Chimerasomes incorporating, in lipid vesicles, reconstituted HVJ membrane proteins.

CHAPTER 5 EXPERIMENTS WITH LIPOSOMES WITH ENCAPSULATED DNA.

5.1 INTRODUCTION

The results detailed in Chapter 3 suggested that the human myeloid cell lines K562, HL60 and TF1 could all be transiently transfected by electroporation with the plasmids available, but that this procedure was toxic to the cells and therefore also probably not valuable to the study of differentiation in populations of these cells. The data from chapter 4 showed that a number of liposome types could associate liposome encapsulated carboxyfluorescein with target cells. This data in association with a number of reports in the literature suggested that liposome mediated transfection with these liposomes could represent an effective means of transiently transfecting these cells. The principal reports of successful liposome transfection came from 4 groups, as has already been discussed, and these were; firstly Kaneda and co-workers [42, 46] with small unilamellar liposomes and Sendai virus showed transfection in Mouse L cells, CHO-K1 cells and HeLa cells. Secondly, Itani and co-workers [35] using Large unilamellar liposomes made from cochleate intermediates reported stable transfection of various cell lines and this technique was modified and adapted at King's College, London (Farzaneh: Personal communication). However Itani et al, though they reported a stable transfection efficiency of 2% in a mouse mammary carcinoma cell line, reported only 0.01% efficiency in HL60 and U937 cells, which were the two human myeloid cell lines that they studied. Thirdly Loyter and Co-workers [43] using a technique of liposome and Influenza envelope proteins also reported DNA delivery to cells in culture, in this case monkey COS-7 cells. Lastly Gould-Fogerite et al [25] using Chimerasome encapsulated DNA showed high efficiency DNA delivery in mouse mammary tumour cell lines.

Thus the principal question to be asked was whether these promising liposome based techniques reported to be successful in a range of animal tumour and human solid tumour cell lines would be suitable for the transfection of human haemopoietic cell lines.

5.2 SPECIAL METHODS.

All methods already described in Chapter 2.

5.3 RESULTS

5.3.1 Small unilamellar vesicles.

The carboxyfluorescein experiments showed that CF could be associated with target cells after incubation with SUV's with (PS/PC/CHOL/Gang) or without (PC/CHOL) a preincubation with virus. SUV's were made from PC/CHOL and PS/PC/CHOL /Gang. The HSV CAT plasmid which had been determined to be active in target cells after delivery by electroporation was encapsulated into MLV's in aqueous solution and then SUV's were made by high pressure extrusion through polycarbonate filters.

DNA was shown to be encapsulated firstly by demonstration that it could be pelleted with the liposomes and secondly by agarose gel electrophoresis in the case of the PS/PC/CHOL/ Gang liposomes. These liposomes remain in the loading well and the encapsulated DNA does not pass into the gel unless the liposomes are disrupted by a detergent such as Triton-X. If any free DNA remaining is removed by repeated washing steps prior to the electrophoresis then, by running liposome aliquots in parallel with and without Triton-X, an estimate of the amount of DNA encapsulated by a given liposome aliquot can be made. In addition gel electrophoresis of the released DNA allows confirmation that the DNA remains intact and in the supercoiled form which is optimal for transient transfection. In the case of the PC/CHOL. liposomes this was not possible as these liposomes, even though they are neutral lipids, move in the gel under the influence of the encapsulated DNA. Therefore it was only possible to demonstrate with these liposomes that the DNA was liposome associated though not necessarily encapsulated. Seven transfection experiments with the two types of SUV were performed using K562 cells and with Daudi cells in addition on two occasions. In each case PC/CHOL liposomes were used alone and the PS/PC/CHOL/Gang liposomes were used with and without a preincubation with UV inactivated Sendai virus. CAT assays on cell lysates

after incubation and harvesting of the cells in these experiments did not demonstrate any expression of the CAT protein from the transfected reporter gene.

5.3.2 Large Unilamellar Vesicles.

Large unilamellar vesicles were identified as an efficient means of encapsulating and associating CF with target cells with or without preincubation with UV inactivated Virus. A series of experiments was undertaken using either HSV-CAT or CMV CAT encapsulated in these liposomes. The total amount of encapsulated DNA and the proportion of the starting DNA encapsulated were estimated from agarose gel electrophoresis with and without detergent and the results from 3 representative experiments are included in TABLE 5.3.1. The optimum number of cycles of sonication required and the optimum concentration of EDTA used to chelate Calcium from the calcium lipid cochleate intermediates were established by comparison of the amount of encapsulated DNA and the results are also shown in TABLE 5.3.1.

TABLE 5.3.1. Encapsulation of DNA by PS LUV in Representative experiments and variation according to Sonication Protocol and EDTA concentration.

Liposome	Starting DNA (μg)	Encapsulated DNA (μg)	%
PS Cochleates	30	0.15	0.5
	30	0.3	1
	30	1	3
PS Cochleates			
30 son. cycles	30	0.2	0.7
60 son cycles	30	0.3	1
90 son. cycles	30	0.25	0.8
PS Cochleates			
EDTA 30mmol	30	<0.1	<0.1
EDTA 150 mmol	30	0.2	0.7
EDTA 300 mmol	30	0.6	2

Liposomes made from PS from ten different batches from three different sources, including that used by Itani et al in their original description of this method, were investigated for efficacy. Most experiments were done using all PS liposomes (26 experiments) but PS/CHOL (9 experiments) and PS/CHOL/Gang liposomes (2 experiments) were also tested. Initially the encapsulated DNA was HSV-CAT but later CMV-CAT was used once it became clear from electroporation experiments that CMV-CAT was more active in the cell lines under study. The mean percentage encapsulation was 1% ± 0.3% and target cells included K562 (18 experiments), HL60 (17 experiments) and Daudi cells (4 experiments). The role of incubation time, temperature and buffering media type was tested in different experiments

with 1,2,3,5 hour and overnight incubations at 27°C or 37°C. The incubation buffer used was PBS (12 experiments), TBS (26 experiments), HBSS (5 experiments), OPTIMEM (low Ca/Mg)(8 experiments) RPMI or RPMI mixed with HBSS (3 experiments).

CAT assays did not reveal that any significant transfection had occurred though on two occasions equivocal results were obtained as detailed in TABLE 5.3.2.

TABLE 5.3.2 PS LUV transfections: Results from experiments where CAT assay revealed activity greater than background CAT

Liposome	Cells	Conditions	CAT assay :OD
	Known Standard:	20pg/200ul	0.059
	K562	Cells only	0.004
PS LUV	Daudi	OPT /2 hr	0.017
		RPMI /2hr	0.022
Standard		20pg/200ul	0.128
	K562	cells only	0.029 ± 0.002
PS LUV	K562	PBS 3hr	0.061 ± 0.012

5.3.3 Chimerasomes : Large Unilamellar Vesicles incorporating Functional Viral Membrane Proteins.

The carboxyfluorescein model suggested that greater cell associated fluorescence was achieved with these liposomes than with any of the other methods investigated. The mean encapsulation efficiency was 5.1% ± 1.3 % of added DNA (see TABLE 5.3.3). This was significantly greater than that of PS LUV made by the previous method of direct addition of the EDTA ($p < 0.025$). This quantitation was done by agarose gel electrophoresis and Triton mediated liposome lysis. (see FIGURE 5.3.1.)

All liposomes were determined to have qualitative haemagglutinating activity using chicken erythrocytes. Target cells and incubation conditions are described in TABLE 5.3.3. No significant levels of CAT protein were detected after incubation of chimerasomes with target cells with the one exception which is detailed in TABLE 5.3.3. when marginal CAT activity was detected.

TABLE 5.3.3. Experimental conditions, encapsulation and outcome of combination of CMV-CAT loaded Chimerasomes with target cells.

Liposome	LC/DC	DNA Encapsulation µg/µg (%)	Cells	Medium	CAT assay
PS/CHOL/Virus	LC	6/300(2)	K562	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	5/300 (2)	K562	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	12.5/300(4)	K562	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	12/300 (4)	K562	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	5/300 (2)	K562	DMEM +/-FCS	See below*
PS/CHOL/Virus	LC	15/300 (5)	K562	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	15/300 (5)	K562 HeLa	DMEM +FCS	None detected
PS/CHOL/Virus	LC	12.5/480 (3)	K562 HeLa	DMEM +/-FCS	None detected
PS/CHOL/Virus	LC	4/770 (0.5)	K562 HeLa HL60	DMEM +FCS	None detected
PS/CHOL/Virus	LC DC	20/500 (2)	K562 HeLa HL60	DMEM +FCS	None detected
PS/CHOL/Virus	LC DC	20/500 (2)	K562 HeLa HL60	DMEM +/-FCS	None detected
PS/CHOL/Virus	DC	80/400 (20)	K562 / FCS HeLa HL60	DMEM RPMI /FCS	None detected
PS/CHOL/Virus (0.1,0.5,1.0 mg/ml of liposome lipid)	DC	108/2160 (5)	K562 HL60	DMEM /FCS	None detected
PS/CHOL/Virus	DC	80/650 (12) 20/176 (11)	K562 HL60	DMEM /FCS	None detected
PS/CHOL/Virus (0.1, 0.5 mg/ml of liposome lipid)	DC	1.4 (135)	K562	RPMI /FCS	None Detected
					Optical Density
*CAT assay result	Standard		20pg/200ul		0.155
	K562		cells only		0.006 ± 0.004
			+chimerasomes		0.046 ± 0.010

FIGURE 5.3.1. Agarose gel demonstrating quantitation of encapsulated DNA by Chimerasomes.

1: 1 kb ladder
2: Unencapsulated CMV-CAT (0.2 ug)
3: Intact CMV-CAT loaded Liposomes
4: Lysed CMV-CAT loaded Liposomes

5.4. DISCUSSION.

The use of liposomes for transfection of mammalian cells has been reported by the four groups discussed in the introduction to this chapter [25, 35, 42, 103] using respectively PC/CHOL SUV, PS/PC/CHOL/Gang SUV, PS LUV and PS/CHOL/Virus chimerasomes. These experiments, in human haematological cell lines, failed to demonstrate that any of these methods could cause expression of the introduced genetic material in the cell lines studied. This is inspite of the demonstration of sufficient quantities of plasmid DNA, still in supercoiled form, within the liposomes. The best encapsulation both in terms of the total amount of DNA included and in terms of the percentage of the starting DNA encapsulated was achieved using the Gould-Fogerite chimerasome protocol. The presence of the viral HA and F proteins in the chimerasomes was demonstrated by PAGE gel electrophoresis (see Chapter 4.3.12. and FIGURE 4.5.) and preservation of their function was suggested by the rapid agglutination of chicken erythrocytes and the clumping of target cells after incubation with the liposomes. These observations confirmed the targeting of the loaded liposomes to the cells inferred from the previous CF experiments.

The failure of these methods to cause effective expression of the CAT reporter gene therefore implies that either there is a failure of fusion of the liposomes with the target cells or that the introduced DNA is being degraded after delivery to an acidified lysosomal compartment and these possibilities were therefore investigated.

CHAPTER 6: EXPERIMENTS TO DETERMINE THE POTENTIAL REASONS FOR FAILURE OF TRANSFECTION AND FURTHER EXPERIMENTS WITH APPROPRIATE MODIFICATIONS TO EXPERIMENTAL METHOD.

6.1 INTRODUCTION

The failure to demonstrate any transfection in the experiments reported in Chapter 5 meant that it had to be questioned whether the CF delivery shown in earlier experiments was being delivered to an appropriate environment for translation of the plasmid DNA to occur. The possibilities were that either the liposome contents were remaining in the liposomes attached to the cell membrane and not delivering their contents to the cytosol or that delivery of the plasmid DNA was occurring but to a compartment where it was being broken down prior to expression, for example in endosomes. This raised the question as to whether additional procedures could be undertaken in order to allow the loaded liposomes to deliver their contents effectively for transfection. The first possibility to be addressed was that the DNA was being broken down after delivery to endosomes, a possibility to overcome this was to modify endosomal acidification with the use of chloroquine, this has been reported to be valuable [53] with receptor mediated internalisation of DNA and Transferrin /Polylysine conjugates as will be discussed in Chapter 7.

A second approach was to use the property of certain proteins to complex with DNA to form compact structures [60] [61] since this might be potentially valuable for liposomal loading of plasmid DNA and also possibly in aiding expression after internalisation. Two proteins: histone H4 and lysosyme have been previously shown to interact with DNA in this way and in addition Kaneda et al [46] reported increased expression after co-introduction of a non-histone protein. Therefore these proteins, in

combination with the chimerasome method, were used to try and transfect human haemopoietic cell lines.

Another alternative explanation for the lack of efficacy of transfection was that the liposomes were failing to fuse with the target cells. To answer whether this was the case the fluorescent techniques used earlier were repeated but this time analysed not by flow cytometry but by confocal microscopy. In addition to carboxyfluorescein, liposomes were loaded with DNA labelled with ethidium bromide, which is also fluorescent. Secondly chimerasomes were loaded with the anti-metabolite Hygromycin and the effect of these chimerasomes compared to an equivalent amount of free hygromycin in terms of tritiated thymidine uptake of the treated cells. Equivalence of the free drug was achieved by high energy sonication of an equal amount of the same chimerasomes to release their contents. This method was based on that described by Sechoy et all [47] who used it to demonstrate delivery of antibody-targeted liposomes containing hygromycin to lymphoblasts.
If failure of fusion was the explanation for the lack of transfection then this might be overcome either using Polyethylene Glycol (PEG) treatment to promote fusion between cell and liposomes [71] or cells and liposomes could be incubated and, once the liposomes were bound to the cell membrane, electroporated to try and cause fusion.

The last approach to be examined in this chapter was to review the use of the commercially available cationic lipids to determine if they had any efficacy in haemopoietic cell lines. Three cationic lipids for use with DNA were commercially available at the time of these experiments and so the effectiveness of each of these was tested. The background to the use of these compounds has been discussed in greater detail in Chapter 1.3.4.

Felgner et al described the use of the first of these related compounds ; N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) for transfection of 2 animal cell lines COS-7 and CV-1 (Simian Kidney). There do not appear to be any reports of successful transfection in human myeloid cell lines. A related compound, Transfectace (TA) is reported (in commercial information-[29]) to be effective in transfecting baby hampster kidney and HeLa cells. Transfectace is a combination of a cationic lipid : dimethyldioctadecyl ammonium bromide (DDAB) and a neutral phospholipid dioleoyl-phosphatidylethanolamine (DOPE). Transfectam is a lipopolyamine molecule with a spermine group fixed in the terminal position by covalent attachment (peptidic attachment). Transfectam has been reported to transfect primary endocrine cells [30]. Lipofectin and Transfectam bind DNA tightly in a complex and are internalised and the DNA is expressed by some cells [28]. Transfectace contains DDAB/DOPE liposomes but the DNA is attached to these during incubation after vesicle formation and is therefore not encapsulated.

The ability of these three compounds to transfect human haemopoietic cells was investigated.

6.2 SPECIAL METHODS

6.2.1. Confocal Microscopy

Cells were combined with chimerasomes as before. Following incubation the cells were washed in buffer and mounted on glass microscope slides and viewed by a confocal microscope (Zeiss epifluorescence microscope [London] with BioRad confocal imaging system [Hemel Hempstead]).

6.2.2. Tritiated Thymidine Uptake:

An aliquot of 7.5 x 10^4 cells was removed from the Chimerasome /cell incubation at the time points specified. Tritiated thymidine (0.5 μCi / well) was added to the cells and they were incubated for 4 hours, washed and then deposited onto a nitro-cellulose filter mat (Whatman) using a cell-washer (Automash 2000 Dynatech, UK). Scintillant (Hisafe) was added in order to measure the ^3H uptake and each sample was counted, in duplicate, in a β counter (LKB. Upsala, Sweden.)

6.2.3. Polyethylene glycol (PEG) fusion protocol .

(modified from Boehringer Mannheim Instruction sheet)

Target cells were pelleted by centrifugation at 300g. The pellet was broken by gentle tapping and kept warm at 37 °C. 1 ml of sterile, tissue culture grade PEG in 50 % PBS solution (PEG-4000, Boehringer Mannheim, Lewes, Sussex.) was added to the pelleted cells over a period of 1 minute with shaking, 5 ml warmed RPMI was added drop by drop over 5 minutes then 15 mls was added over a further 1 minute, the cells were pelleted again and then resuspended in growth medium.

6.2.4. Lipofectin / Transfectace protocol.

3 x 10^6 cells were washed once in OPTIMEM (Gibco-BRL) and resuspended in 0.8 ml OPTIMEM medium. 30 μg of plasmid DNA in sterile water was made to 100 μl with OPTIMEM, 30 μl of Transfectace (or Lipofectin) (both from Gibco-BRL) were also made to 100 μl with OPTIMEM. The lipid and DNA were mixed and incubated at room temperature for 10 minutes prior to further dilution to 1 ml of OPTIMEM and split into two aliquots for addition to the cells. They were incubated for 5 hours after which time a further 4 mls of RPMI/ 20% FCS were added. The cells were incubated further and normally harvested and assayed 72 hours later.

6.2.5. Transfectam protocol.

20 μl of the Transfectam reagent stock solution (Northumberland Biologicals Ltd, Cramlington, Northumberland) was diluted with 250μl of sterile deionised water. 30 μg of plasmid DNA was dissolved in 250 μl of 0.3M saline. The two solutions were mixed and split into 2 aliquots for addition to the cells. The Transfectam / DNA mixture was added to the cells and incubated for 6 hours in a 37°C, 5 % CO_2 incubator, after which they were further diluted, incubated for 72 hours, harvested and assayed.

6.3 RESULTS

6.3.1. The Use of Chloroquine to prevent endosomal degradation:

This strategy was employed in conjunction with the use of CMV CAT loaded PS LUV and PS/CHOL/VIRUS chimerasomes with the liposome / cell incubations in the presence of 100 μM chloroquine for 4 hours. Toxicity was observed to be minimal if the duration of the chloroquine exposure was limited to this 4 hour duration at this 100 μM concentration of the drug. In the case of the CMV-CAT loaded PS LUV the target cells studied were HL60 (5 experiments), K562 (5 experiments) and Daudi cells (1 experiment). In the case of the PS/CHOL/ VIRUS chimerasomes the target cells were K562 (7 experiments), HL60 (5 experiments) and HeLa cells (3 experiments). In no case was any significant amount of the CAT reporter protein detected.

6.3.2. The use of proteins to complex with the DNA to form compact structures.

Histone protein H4 was mixed with plasmid DNA and encapsulated in chimerasomes, as before, in parallel with chimerasomes encapsulating DNA alone. DNA / H4 complex was encapsulated to a similar efficiency as DNA alone by chimerasomes but there was no expression of the CAT gene after incubation of either DNA/H4 or DNA chimerasomes with the K562 cells.

In addition synthetic cationic lipids (Lipofectin and Transfectace) and polylysine which all associate with DNA were premixed with the DNA before loading into chimerasomes but were also ineffective. In the case of

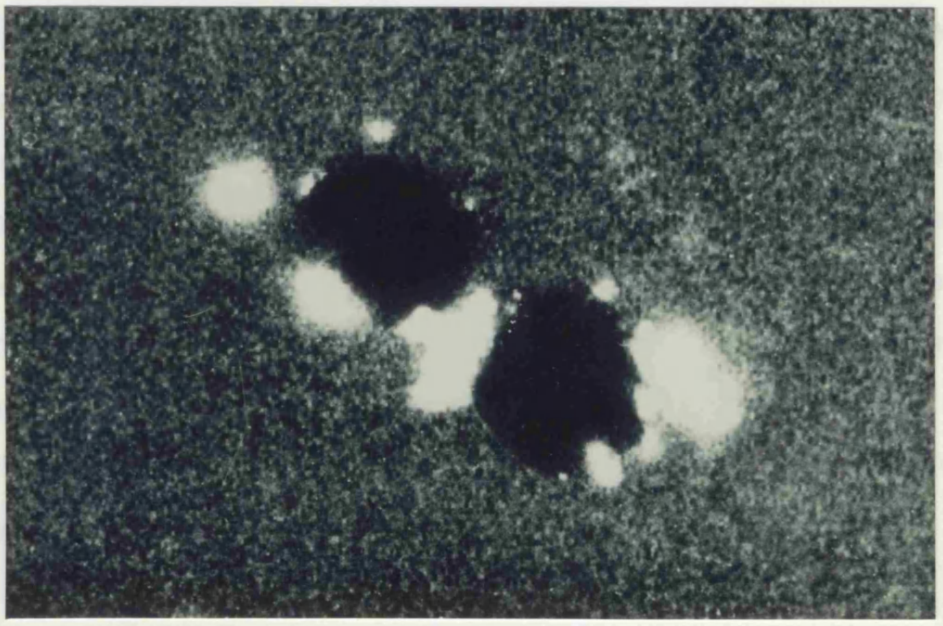
lysozyme DNA encapsulation was markedly decreased when assessed by liposome lysis and agarose gel electrophoresis.

6.3.3 Analysis of Cells incubated with Liposomes by Confocal Microscopy

In order to best understand the reasons for transfection failure PS / Sendai virus chimerasomes were loaded simultaneously with carboxyfluorescein and Ethidium Bromide labelled DNA. To exclude the effect of bound but uninternalised liposomes the cells were examined after a 2 hour incubation at either 4°C or 37 ° C by confocal fluorescence microscopy using settings to visualise the red and green fluorescence emitted by the Carboxyfluorescein and ethidium bromide/DNA respectively. The results showed that while the ethidium bromide DNA appeared to have entered the cells where it was seen as a hazy fluorescence over the cytosol, the carboxyfluorescein could be seen to remain within unfused, bound liposomes with no delivery of CF to the cell cytosol. A representative example of Daudi cells with membrane associated, unfused chimerasomes is shown in FIGURE 6.3.1. The ethidium Bromide labelled DNA (CMV CAT) encapsulated in liposomes was used to attempt to transfect K562 cells under conditions as described in Chapter 5, however no CAT activity could be detected after harvesting, lysis and assay of the cell suspension.

A similar experiment using chimerasomes encapsulating an immunoglobulin- FITC conjugate (Dako, High Wycombe, Bucks) and HL60 cells when examined by fluorescence microscopy did not show any significant cytoplasmic "blush" to the cells as described in the report by Gould-Fogerite et al [25].

FIGURE 6.3.1. Confocal microscopy image to show Daudi cells after 2 hour incubation with Chimerasomes loaded with Carboxyfluorescein. (x 1800)



6.3.4. Effect of Hygromycin containing Liposomes on Cell Tritiated Thymidine uptake.

Chimerasomes were made as previously, loaded with 1 M Hygromycin (Boehringer-Mannheim) and then extensively washed after synthesis so that no free drug remained in the liposome suspension. One aliquot of the liposomes was then sonicated at high power to cause liposome destruction, filtered and added, in identical volumes to the intact liposomes, to the target cells. The results of the ^{3}H uptake was measured over a 4 hour period at 20 and 44 hours and are shown in Figures 6.3.2-5. These show that no inhibitory effect was seen with intact liposomes whereas after the drug is released from the loaded liposomes by sonication there is a marked inhibitory effect on the cellular ^{3}H uptake in a dose dependent manner according to the volume of the aliquot added.

FIGURE 6.3.2. Hygromycin liposomes: Effect on ^{3}H uptake by K562 cells of incubation with Intact or Sonicated liposomes. 27° C -20 hours

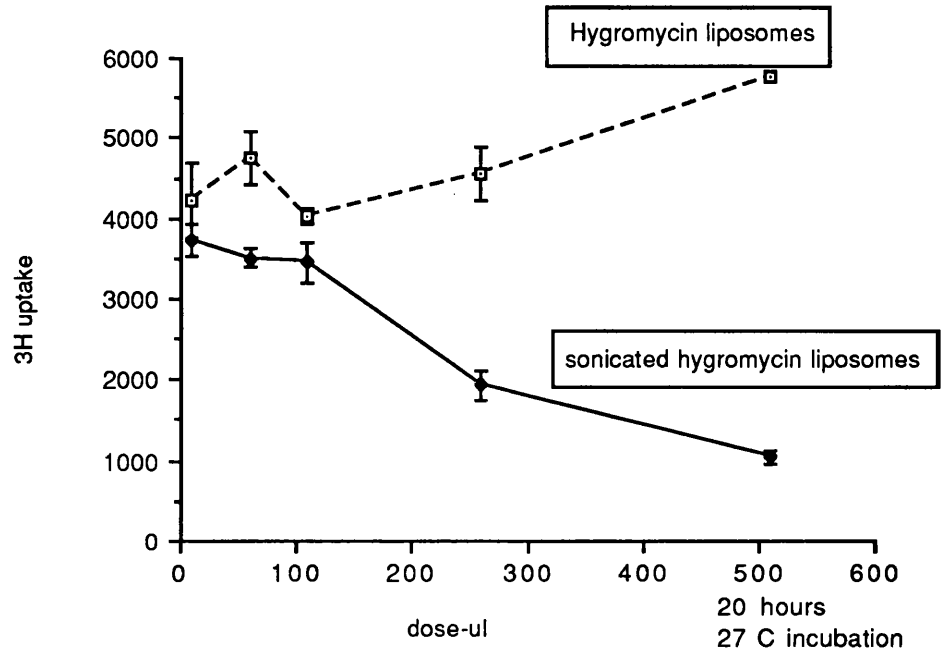


FIGURE 6.3.3. Hygromycin liposomes: Effect on ^{3}H uptake by K562 cells of incubation with Intact or Sonicated liposomes. 27° C -44 hours

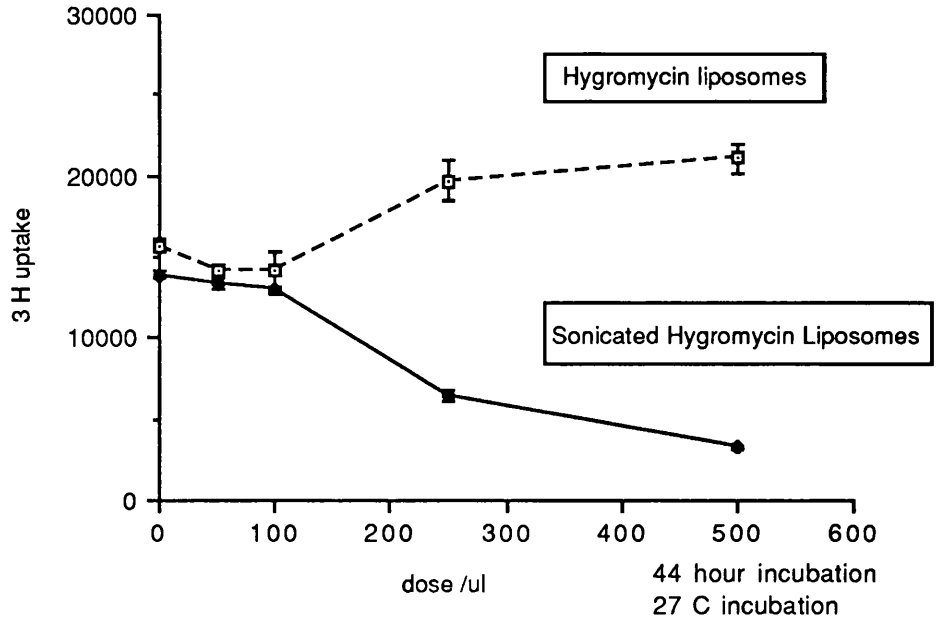


FIGURE 6.3.4. Hygromycin liposomes: Effect on ^{3}H uptake by K562 cells of incubation with Intact or Sonicated liposomes. 37° C -20 hours

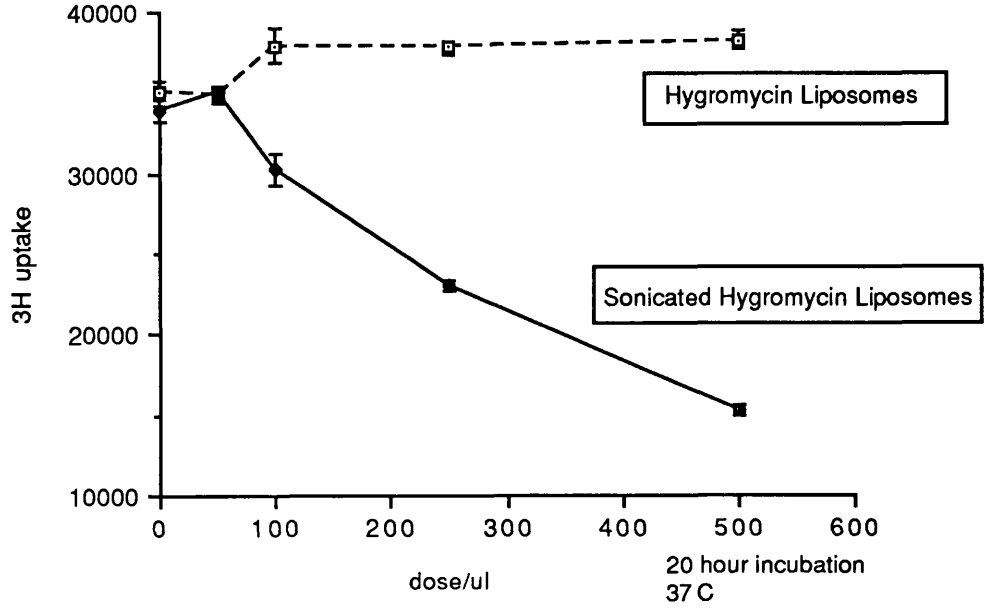
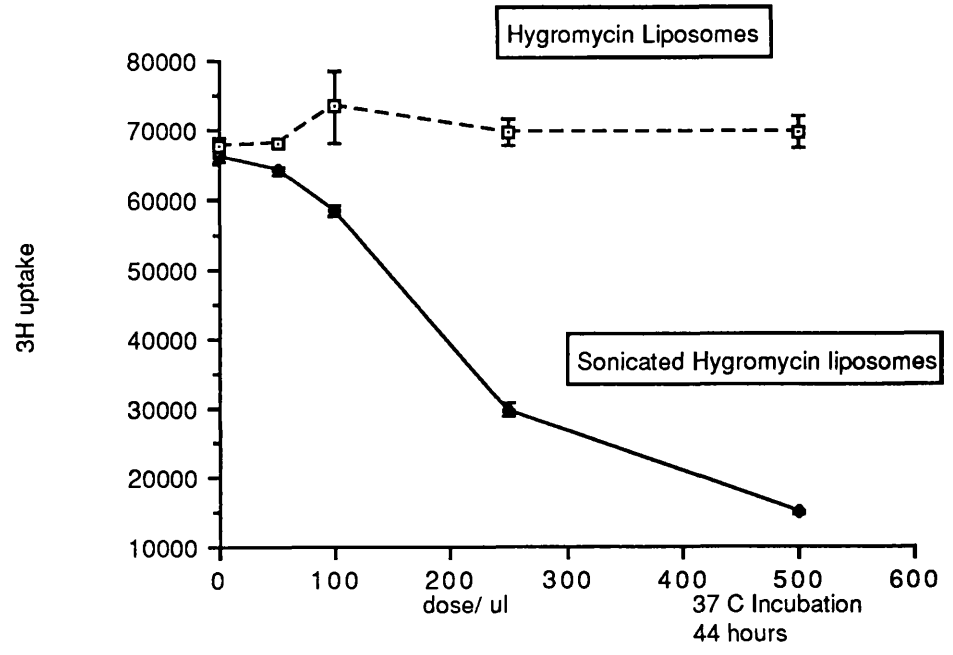


FIGURE 6.3.5. Hygromycin liposomes: Effect on ^{3}H uptake by K562 cells of incubation with Intact or Sonicated liposomes. 37° C -44 hours



6.3.5 Cell liposome Fusion induction using Polyethylene Glycol.

Chimerasomes encapsulating CMV CAT were incubated with target cells as previously and then treated with PEG as described in special methods (6.2.1.) The target cells used were K562 and HL60 cells. Two dose levels were used (0.1mg and 1mg of starting lipid per million cells) and duplicates were incubated in RPMI or DMEM after fusion. CAT assays performed at 72 hours did not detect any CAT protein in the cell lysates. Two further experiments with HL60 and K562 cells as the target cells were performed but again no CAT protein was detected in lysates after incubation.

6.3.6. Electrofusion of DNA containing Chimerasomes.

The strategy of electroporation after incubation of the cells with the chimerasomes was tried. Chimerasomes were prepared as before and then incubated at room temperature for 1 hour prior to transfection in parallel with electroporation of cells as previously described in Chapter 3. Three voltages were used at 0.2 kV, 0.22kV and 0.25 kV and time constants (ms) of 44, 39 and 37 were recorded (49,46,55 for cells with DNA not encapsulated by chimerasomes). The cell toxicity in the samples containing chimerasomes was considerable and there was no significant expression of CAT protein after harvesting, lysis and analysis of the cells, whereas there was good expression of CAT in the cells conventionally electroporated with free DNA. The results are shown in TABLE 6.3.1.

TABLE 6.3.1. Electroporation of Cell / Chimerasome complexes (Electrofusion).

1. Electroporation of Cells after incubation with free CMV CAT DNA:

Voltage (kV)	Final cell count (x 10 6)	CAT activity (pg /200µl of lysate)
0.2	4.3	> 1000
0.22	1.7	> 1000
0.25	1.05	> 1000

2. Electroporation of Cells after incubation with chimerasomes

Voltage (kV)	Final cell count (x 10 6)	CAT activity (pg /200µl of lysate)
0.2	0.25	0
0.22	0.24	29
0.25	0.08	0

6.3.7. Use of Cationic Lipid / DNA complexes.

Three preparations were assessed Lipofectin, Transfectace (both from GIBCO-BRL) and Transfectam (Northumberland Biologicals). These 3 lipids were assessed in Daudi and HL60 cells using CMV CAT (260:280 ratio 1.74) following the recommended methods. There was no significant toxicity observed in these experiments compared to control cells in the presence of DNA contrasting with earlier observations in the absence of DNA when Lipofectin did have a toxicity as demonstrated in Chapter 4 (4.3.6.). Cell lysates were assayed for CAT protein after incubation but in no case was any CAT protein detected.

Further experiments were done with Lipofectin and Transfectace using a longer incubation of the cationic lipid with the DNA and an overnight

incubation of the Lipid /DNA complex with the target cells, using K562 and Daudi cells. The results of these experiments are shown in TABLE 6.3.2. and show detectable levels of CAT protein in cell lysates after the combination of Lipofectin /DNA complex with K562 cells at high DNA doses. These levels of transfection activity however are very low compared to the levels detected using electroporation in experiments done simultaneously in the same cells with lower doses of DNA.

TABLE 6.3.2. Use of Cationic Lipid / DNA complexes to transfect CMV CAT into DAUDI or K562 cells.

Lipid	cell type	DNA /aliquot	Cells (x 10^6)	CAT protein pg/ 200µl
Control cells	DAUDI	0	6.8	2
LIPOFECTIN	DAUDI	25	5.8	1
Control cells	K562	0	4.8	8
LIPOFECTIN	K562	25	7.1	22
Control cells	K562	0	4.6	20
LIPOFECTIN	K562	75	4.6	239
Electroporation (0.25kV)	K562	10	0.4	>2000

6.4. DISCUSSION

These strategies were aimed to identify the reasons for the failure of transfection and then try and overcome the failure of delivery of the DNA to the cytosol. Firstly it was reasoned that it was possible that liposome delivery was occurring but that liposome contents were retained within endosomes and so either destroyed or prevented from translation. In these experiments, however, modulating this by the use of chloroquine had no effect on the transfection efficiency.

Secondly Kaneda et al [46] using a liposome based system showed improved results when nuclear proteins were co-introduced with the DNA possibly due to an effect of the DNA / protein complex on stability or expression of the introduced DNA. This was tested, without success with the Histone protein H4 (Boehinger Mannheim) encapsulated with the DNA and, in addition, with lyzosyme which may cause increased DNA condensation [61] .

The alternative explanation for transfection failure was that inspite of effective targeting of the liposomes to the cell surface no effective delivery of the liposome contents was occurring. Two experiments were performed to test this possibility, in the first experiment, using confocal microscopy, Ethidium Bromide labelled DNA, which probably partitioned to the hydrophobic portion of the lipid bilayer, was apparently delivered to the cells but in contrast the polar CF molecules encapsulated in the aqueous interior of the liposomes were not delivered. However the plasmid DNA bound to the ethidium bromide was not expressed in the treated cells.

Review of the literature to examine the failure of the CF data to predict for macromolecular delivery shows that three approaches have been used. Firstly, the inclusion of the fluorochrome within the lipid bilayer as used by Chejanovsky et al [41] was effective at delineating the nature of liposome

/cell fusion events however the inclusion of the fluorochrome in the lipid bilayer is not an appropriate model for the fate of molecules encapsulated within the aqueous interior of the liposomes. These experiments were done with N-4-nitrobenzo-2-oxa-1,3-diazole-phosphatidyl ethanolamine and liss amine rhodamine-B-sulfonyl phosphatidyl ethanolamine at the appropriate concentrations for quenching /dequenching studies to follow fusion events.

A second system for monitoring the fate of liposomes was published by Straubinger et al [36] from Papahadjopoulos's group, in 1990, they used Pyranine which is a pH dependent fluorescent dye whose behaviour is similar to that of SNARF whose use was described in Chapter 4. In this case the liposomes, which were made by reverse-phase evaporation and rapid extrusion through filters, were rapidly internalised within 1 hour at 37 °C, but to an acid environment and further monitoring showed minimal appearance in the cytoplasm suggesting that liposomes rarely fused with the cell membrane and that the dye when delivered by the endocytic pathway ended up in a variety of intracellular organelles. This is similar to the SNARF data for PC/ Cholesterol SUV made by extrusion described in Chapter 4 which appeared to deliver to an acid environment but which were ineffective at transfection. This was in contrast to the PS/PC/CHOL/Gang SUV which remained at a neutral pH, though this could have been explained by failure of fusion of the liposomes with the target cells resulting in their emission reflecting the pH of the original pH of the encapsulated dye.

A third strategy was employed by Gould-Fogerite et al [25] who followed, with conventional fluorescence microscopy, chimerasomes loaded with fluorescein conjugated immunoglobulin and demonstrated with a Rat cell line (PTK_1) that the pattern of fluorescence went from discrete cell associated liposomes to a more general "blush" over the whole cell. This

could not be shown, in my experience, with HL60 cells using a fluorescein conjugated antibody encapsulated in chimerasomes.

An alternative way of addressing whether internalisation had occurred was the experiment following the ^{3}H thymidine uptake of cells treated with either liposome encapsulated or free hygromycin, a toxic drug which reduces ^{3}H thymidine uptake by affected cells. This experiment represents strong evidence to suggest that the chimerasomes were not effective in macromolecular delivery to these cells. This contrasts with the experience of Sechoy et al [47] using fusogenic liposomes (incorporating Sendai virus membrane proteins in Phosphatidyl choline and Phosphatidyl-ethanolamine liposomes) in a human T lymphoid cell line (CEM) who reported that hygromycin in fusogenic, targeted liposomes could decrease ^{3}H thymidine uptake of the target cells by comparison with appropriate controls. However this protocol included, in the liposomes, a specific covalently coupled antibody (T101) directed against CEM cell plasma membrane antigens as an additional means of targeting delivery.

In order to attempt to overcome this presumed failure of delivery across the cell membrane it was reasoned that it might still be possible to take advantage of the localisation to the cell surface of DNA loaded chimerasomes by using additional techniques to fuse these bound liposomes. In particular two physical methods were tested but unfortunately neither was successful. The failure of the use of PEG was disappointing since Loke et al [71] had shown effective delivery of liposome encapsulated antisense oligonucleotides. In this experiment c-myc protein expression in HL-60 cells was modulated by delivery of the antisense oligonucleotides encapsulated in phosphatidylserine LUV fused with the cells by incubation and PEG treatment. However it seems that this

technique is not effective for the introduction of DNA for transfection of these cells as opposed to the introduction of antisense oligonucleotides. Electrofusion was highly toxic to the target cells as well as being ineffective. Therefore, in conclusion, it appeared that no additional techniques could be combined with incubation of the cells with liposomes to deliver the contents of those liposomes to the cytosol in such a way as to achieve the expression of introduced DNA.

Reports of transfection with the cationic lipids Lipofectin, Transfectace and Transfectam, as discussed in the introduction to this chapter, have come from a number of investigators. However when tested here in human haemopoietic cell lines they did not appear to be of significant value for transfection of plasmid DNA. In the case of Lipofectin, at high doses and with a long incubation time, transfection of the reporter plasmid in K562 cells was seen but at a level of expression that was insufficient to be of value since it was less than the level of expression seen with transferrin-polycation conjugate mediated DNA delivery to the same cells which is reported in the next chapter.

CHAPTER 7 TRANSFERRINFECTION.

7.1. INTRODUCTION

Transferrinfection is a method of transfecting cells first reported in 1990 by the Vienna group [48] and it has been discussed in detail in Chapter 1.6. The method is dependent on the coupling of transferrin to polylysine to create a transferrin-polylysine conjugate (TFPLCo). The polylysine part of this conjugate is then able to bind the plasmid DNA which is to be used in transfection. These conjugates have now been made available commercially by Serva-Feinbiochemica Heidleberg, Germany - UK agent: Universal Biologicals. The TFPLCo-DNA complex is able to bind to cell membrane transferrin receptors which after internalisation pass to the endosomes and the DNA is then made available for translation. This was summarised in diagrammatic form in Chapter 1 FIGURE 1.2. This process is dependent on the upregulation of the transferrin receptors on the target cells and the release of the DNA, intact, from the endosomes. These processes can be modulated by use of iron chelators [53] and chloroquine respectively [53]. The role of the iron chelator is to decrease the free iron available to the cells which in turn due to Iron Response Elements (IRE) [54] increases the expression of the TF-R on the cells. IRE have been identified in the 3' untranslated region of human transferrin receptor messenger RNA. Maximising the expression of transferrin receptors on the target cells will optimise the delivery of the TFPLCo-DNA to the cells. The rationale for examining the effect of Cp 94 as well as DF, which was the chelator used in the original experiments of Cotten et al, was the observation that a period of incubation in Compound 94 leads to cell cycle synchronisation [104]. This is probably due to arrest of the cells at the G_1 -S border of the cell cycle in the presence of the chelator associated with reduced ribonucleotide reductase activity and cessation of DNA synthesis

and growth [104]. Removal from the chelator results in the rapid efflux of the chelator from the cells allowing them to return synchronously into S phase, this rapid efflux is probably related to the high lipid solubility of Cp 94 [105]. In these experiments I have therefore compared DF and Cp 94 in parallel with analysis of TF-R expression and cell cycle kinetics. I have also examined the application of this technique to haemopoietic cells other than K562, which hitherto has been used for most experiments, in particular the growth factor dependent cell line TF1. The potential advantages of this delivery system centre on its "physiological", non-toxic nature and therefore the efficiency of this system compared to electroporation was examined. The Vienna group who originally published this protocol have more recently extended the technique to using inactivated adenovirus or Influenza Haemagglutinin related polypeptides conjugated with the TFPLCo (as discussed in Chapter 1.6.) but these were not available for use in this project.

7.2. SPECIAL METHODS.

7.2.1. Transferrinfection: Transfection with Transferrin - Polycation conjugates (TFPLCo.)

Transferrin - Polycation conjugates (TFPLCo.) were obtained from Serva-Feinbiochemica Heidleberg, Germany - UK agent: Universal Biologicals. The method used was based on that of Wagner et al [48] with modification as per the Serva instruction sheet recommendations. Briefly, the TFPLCo. were saturated with iron by the addition of 5 µl of 10mM Ferric Citrate buffer to 1 ml (1mg) of TFPLCo. In the iron loaded form the TFPLCo. were stored at - 80 ^{o} C after rapid initial freezing in liquid nitrogen. The original method [48] describes the method by which the conjugates are synthesised by ligation of transferrin to polylysine through disulphide bonds after modification with the bifunctional agent succinyl 3-(2-Pyridyldithio) propionate (SPDP).

A complex of TFPLCo. and DNA was made by the addition of a known amount of DNA (6-20 µg) in 330µl Hanks balanced salts (+ HEPES 20 mM, pH 7.3) to the TFPLCo. which were also diluted in the same medium (170 µl). These two solutions were mixed to make a total volume of 0.5 ml and incubated for 30 minutes at room temperature.

The cells to be transfected were grown in cell culture at a density of 1-4 x 10^5 /ml. At 18-24 hours before transfection cells were transferred to fresh medium at a density of less than 2 x 10^5 / ml in the presence of an iron chelator at the concentrations specified in the text of the results section. The chelators used were either Desferrioxamine (Serva) or Compound 94, a N-alkyl-3-hydroxypyridin-4-one (supplied by Prof. RC Hider, Kings College, London). The iron chelator was also present for the 4 hour period of

incubation of the cells with the DNA -TFPLCo and in addition chloroquine (Serva) was added for the period of the incubation at a concentration of 50-100 μM.

The cells were resuspended in a total volume of 2 ml RPMI/FCS 10 % and the pre-incubated DNA and TFPLCo were added in a total volume of 0.5mls. The dose of DNA and TF-Polycation conjugate used were as specified in the results section. At the end of the incubation the cells were washed and resuspended in either new growth medium with Penicillin and Streptomycin or in filtered (through a 0.22 μm membrane) conditioned medium and then incubated for a further period of time as specified in the results section. The cells were then harvested, counted, washed three times in PBS and assayed as per the CAT Assay method, or a cytospin preparation of the cells was made for APAAP staining (see below).

7.2.2. Transferrin receptor (TF-R) assay:

Transferrin receptor expression was analysed by flow cytometric (Epics Elite, Coulter) analysis using a fluorescein conjugated mouse monoclonal antibody (CD71,BERT9 Dako, High Wycombe, Bucks) directed against the human TF-R, CD71. A mouse anti-IgG_1 antibody (Dako X927) was used as a control. 0.5 x 10^6 cells were used per data point. After incubation in chelator the cells were split into two samples , pelleted and resuspended in chelator-containing medium at 1 x 10^6 /ml. To these was added either 10 μl of anti TF-R antibody (200 μg/ml) or 10 μl of control antibody (100 μg/ml), incubated on ice for 30 minutes and then fixed in 100 μl of 1 % paraformaldehyde. The fluorescence at 488nm was then analysed by flow cytometry and expressed as the percentage increase in TF-R expression compared to control cells. Each control sample was used to set the cursor at 5% to remove the background fluorescence.

7.2.3. Cell cycle profile determination.

Cell cycle profile determination was also by flow cytometric analysis. Cells were fixed by the addition of 1 ml of 70% Ethanol (-20 °C) to pelleted cells for 1 hour after the cells had been drained by inversion of the tube after centrifugation. The cells were stained prior to analysis with 1 ml of stain added to the pelleted cells (For each 5 ml of stain : O.5ml RNAase, 4.5 mls PBS, 100 µl Propidium iodide from 1mg/ml stock.) The samples were analysed by flow cytometry (EPICS Elite, Coulter) and the percentage of cells in each phase of the cell cycle was determined. Control cells were used as a diploid standard to establish a consistent location (channel number) of the G_0-G_1 peak, with the G_2 + M peak being twice this number. The proportion of cells in each cell cycle phase was obtained from planimetric analysis of the DNA histograms, assuming a Gaussian distribution of the G_1 and G_2 + M peaks and attributing the remaining area to cells in S phase as described [55] [104] [106].

7.2.4 APAAP (Alkaline Phosphatase Anti Alkaline Phosphatase) Staining.

Cytospin preparations were made using a Shandon cytospin 3 at 770 rpm for 10 minutes. The cell pellets were allowed to dry under ambient conditions, fixed for 10 minutes in ice cold methanol, rehydrated with TBS and then stained according to the protocol described below with the slides washed in TBS at least three times between each step. All incubations were in a wet chamber. The first incubation was with 30µl of a Rabbit anti-CAT antibody (Serotec, 5'-3' Inc. Boulder, Colorado, USA) at an optimum dilution of 1/500 of the original 8.5 mg / mg solution for 1 hour. After washing a Mouse anti-rabbit monoclonal antibody (Dako) was added at 1/20 of the original 0.09 g / l solution for further 1 hour. After a further washing step a Rabbit anti-mouse polyclonal antibody was added (Dako

Z259) at 1/20 of the original 3.2 g/l solution for a further 1 hour. After washing Alkaline phosphatase / anti alkaline phosphatase complex (APAAP) at 0.09 g / l was added (Dako D651) and incubated for 1 hour and then washed. The slides were then flooded with filtered staining solution for 20 minutes (10 ml of staining solution: 5mg Napthol AS-BI phosphoric acid (Sigma), 200 µl of Dimethyl formamide (Sigma), 5mg of Levamisole (Sigma) and 10ml of Veronal Acetate buffer (pH 9.2). (500ml of Veronal Acetate buffer -Sodium acetate trihydrate (BDH) 1.944g Sodium Barbitone (BDH) 2.944 g, 0.1N HCl 5 ml and 495 ml of Distilled water). Finally slides were washed and counter stained with Mayer's Haemalum (BDH, Poole, Dorset) and mounted with Aquamount (BDH).

7.3. RESULTS

7.3.1. Optimisation of TFPLCo dose.:

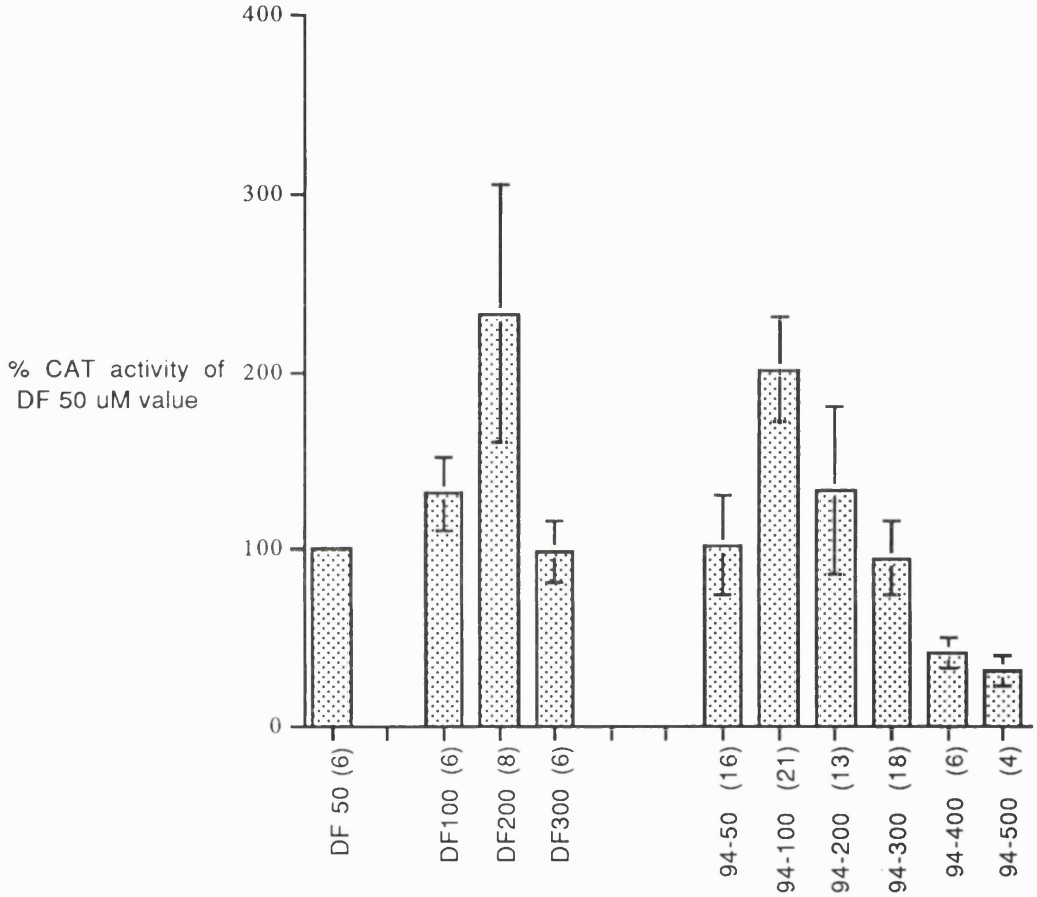
The ability of the TFPLCo. to transiently transfect haemopoietic cells when combined with plasmid DNA was investigated initially in K562 cells following the method originally described by Wagner et al [48]. Aliquots of 0.5 x 10^6 K562 cells were transiently transfected, the CAT activity assayed at 44 hours and expressed as the increase of CAT activity detected over that of control untransfected cells. With a dose of TFPLCo. of 5 µg /10µg DNA the CAT activity was 16.3 +/- 5.3 pg /200µl of cell lysate, at 10µg/10µg DNA the CAT activity was 18.2 +/- 5 /200µl of lysate and at 18/20 µg per 10µg of CMV CAT DNA the CAT activity was 76.3 +/- 29 /200µl of lysate. The maximum CAT activity detected at the optimum TFPLCo. ratio was much less than the value of > 300 µg CAT activity /200µl lysate seen with electroporation of an equivalent number of cells. Higher doses of TFPLCo. did not further increase the CAT activity detected and therefore 18-20 µg/10 mg of DNA was initially chosen as the optimum TFPLCo./DNA ratio.

7.3.2. Optimisation of chelator Concentration and Type:

It has been suggested that efficient transferrinfection requires the up regulation of transferrin receptor expression of the target cells by pre-incubation in an iron chelator [53]. K562 cells were again used for this experiment. The CAT activity of transfected cells was assayed after pre incubation in 2 iron chelators (Desferrioxamine (DFO) and the 3-hydroxypyridin-4-one: Compound 94 (Cp94)) at a variety of concentrations. Concentrations are expressed as Iron Binding equivalents (IBE) since a given concentration of the hexadentate ligand DFO is equivalent to three times that of the bidentate ligand Cp94. Concentrations between 50 µmol IBE and 300 µm IBE of desferrioxamine and between 50 µM IBE and 500

μM IBE for Compound 94 were used in a total of 16 experiments comparing the transfection activity in terms of CAT activity that was detectable by ELISA after collection and lysis of the treated cells. All data was compared with the CAT activity of Desferrioxamine 50 μM IBE (the optimum concentration reported by [53]) with the CAT activity in any given experiment for the value for DF 50 taken as 100%. The results of all these experiments are shown in FIGURE 7.3.1. and show that for both chelators there is an optimum concentration for transfection but that concentrations greater or lesser than this result in lower transfection activity. The optimum observed concentrations for the two compounds were 200 μM IBE for DF and 100 μM IBE for Compound 94. The ratio of the CAT activity compared with the untransfected cell background for DF (200 μM IBE) was 7.0 ± 1.0:1 and 6.1 ± 0.9: 1 for Cp94 (100 μM IBE). Comparison of transferrinfection with electroporation starting with the same number of cells (1×10^6) and with the same amount of DNA (6 μg) showed that transferrinfection using the optimal Conjugate to DNA ratio (3 : 1) gave rise to 16.8 ± 3 % of the value for CAT activity seen with electroporation with 200 μM IBE of DF and 9.8 ± 1 % of the value for CAT activity seen with electroporation with 100 μM IBE of CP94 .

FIGURE 7.3.1.: Transfection activity according to chelator type and concentration: % of value for DF 50 mM IBE.



NOTE: Data pooled from 11 Experiments.

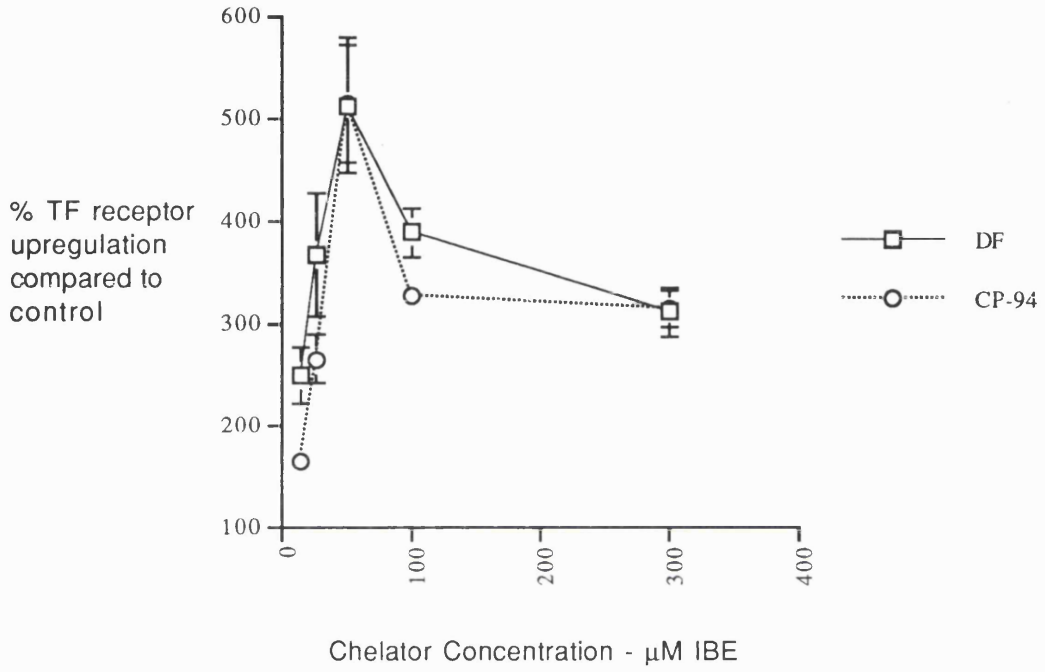
CAT activity of DF200/ background = 7.0 ± 1.2

(DF 200/Electroporation) x 100 = 15 ± 2.1%.

7.3.3.: Relationship between Transfection activity, Transferrin receptor expression and cell cycle status:

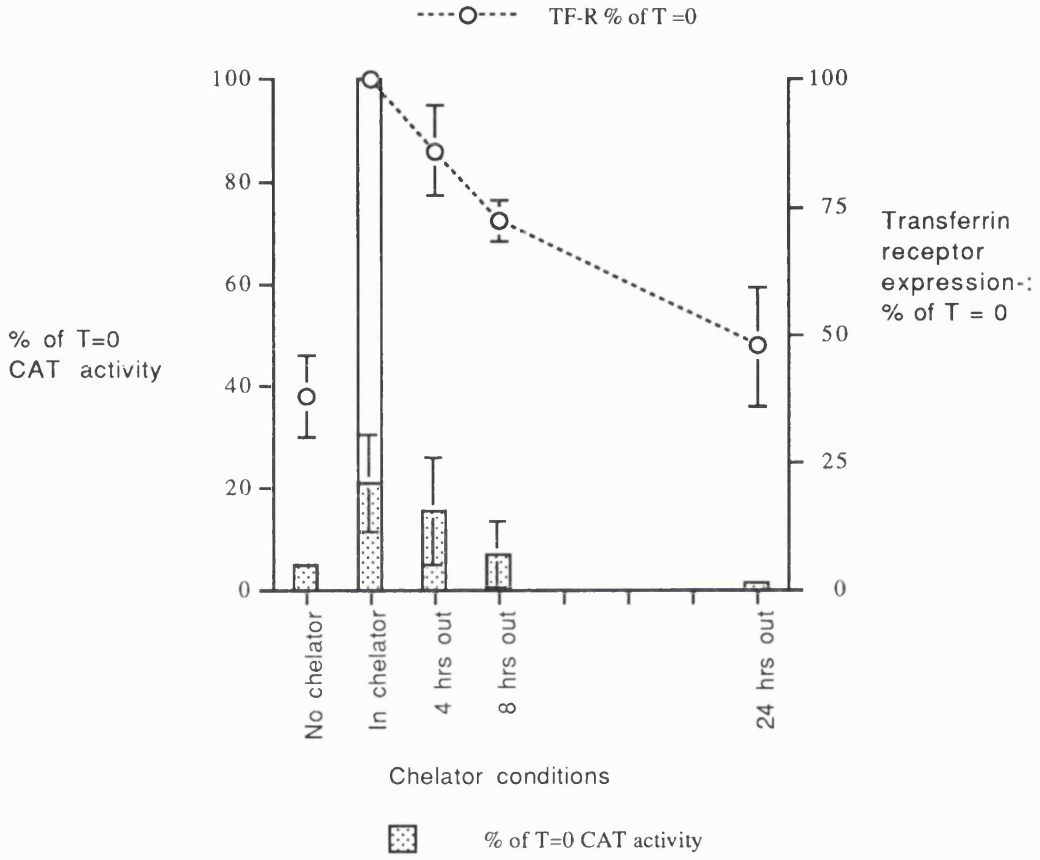
The effect of these two iron chelators was then examined on the target cells firstly in terms of the up regulation of transferrin receptor expression seen after a 24 hour pre incubation with 5 different concentrations of each chelator. Cp 94 was used because of the observation that a period of incubation in Compound 94 leads to cell cycle synchronisation due to the rapid efflux of the chelator from the cells when it is removed from the culture medium allowing them to return into cell cycle [104]. This experiment showed a very similar degree of up regulation for both chelators up to approximately five times the baseline value for each chelator with the maximum effect seen at 50 μM IBE for both chelators. This data, pooled from 3 experiments is shown in FIGURE 7.3.2.

FIGURE 7.3.2: % Upregulation of transferrin receptor expression after incubation of cells in chelator according to chelator type and concentration.



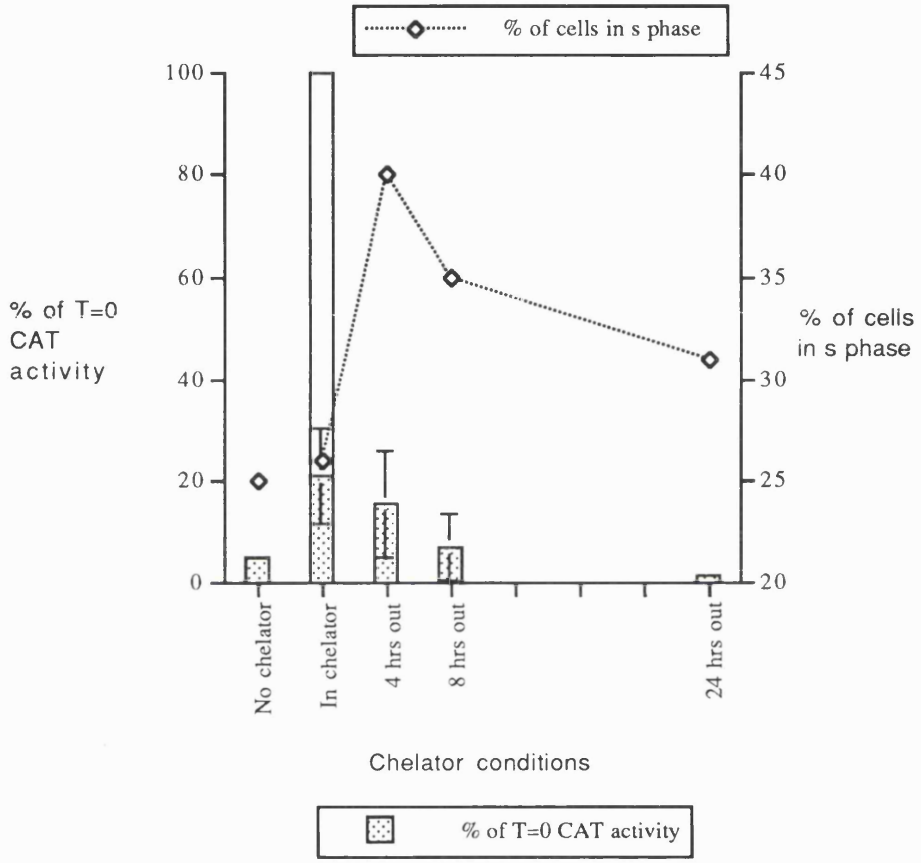
In the next series of three experiments the effect of a period out of chelator, when using 100 μM IBE of Compound 94, was examined with reference to Transferrin receptor expression, cell cycle profile and transfection activity with all three of these parameters measured in parallel. The results of these experiments are shown in FIGURE 7.3.3a & b. These show that transfection activity seen is not directly related to either the transferrin receptor up regulation since the optimum concentration for TF receptor up regulation (50 μM IBE) was not the same as the optimum concentration for transfection activity (100 μM IBE). In addition the cell cycle profile of the target cells, expressed as the proportion of cells in s phase, did not directly relate to the transfection seen since the transfection activity decreased rapidly once the cells were removed from chelator whereas the proportion of cells in s phase increased till between 4 and 8 hours later. In particular for the cells in chelator at the time of mixing with the TFPLCo/DNA complexes the continued presence of the chelator during the incubation was needed for the maximum CAT activity to be seen.

FIGURE 7.3.3a. % Transfection activity and Transferrin receptor expression after removal of cells from chelator.



" In chelator" shaded area shows value when cell + TFPLCo/DNA complex were not incubated with chelator.

FIGURE 7.3.3b. % Transfection activity and % cells in s phase after removal of cells from chelator.



NB " In chelator" shaded area shows value when cell + TFPLCo/DNA complex were not incubated with chelator.

7.3.4. Detection of CAT protein in Cytospin Preparations.

In addition to the total CAT activity of transfected cells in a lysate made from an aliquot of cells it is valuable to know the proportion of cells that are expressing the transfected protein. Therefore an analysis of intact cells, after transfection, was made by the APAAP method to look for the presence of CAT protein. Cell cytospin preparations were made after transferrinfection or electroporation of the cells had been performed. The whole, fixed cells were then examined using the APAAP technique for expression of CAT protein. Examples of K562 cells transfected with CMV-CAT DNA by transferrinfection are shown in FIGURE 7.3.4a & b. These studies showed that for both electroporation and transferrinfection, the proportion of transfected cells in a cytospin preparation is low (<1%) however the individual cells which have been transfected may express very high levels of CAT protein. Toxicity to the cells after transferrinfection was much reduced compared to electroporation but these results are not presented in detail because as the proportion of transfected cells is low the great majority of surviving cells represent untransfected cells.

FIGURE 7.3.4a. K562 cells transfected by Transferrinfection demonstrating the presence of CAT protein by an APAAP technique. (x 450)

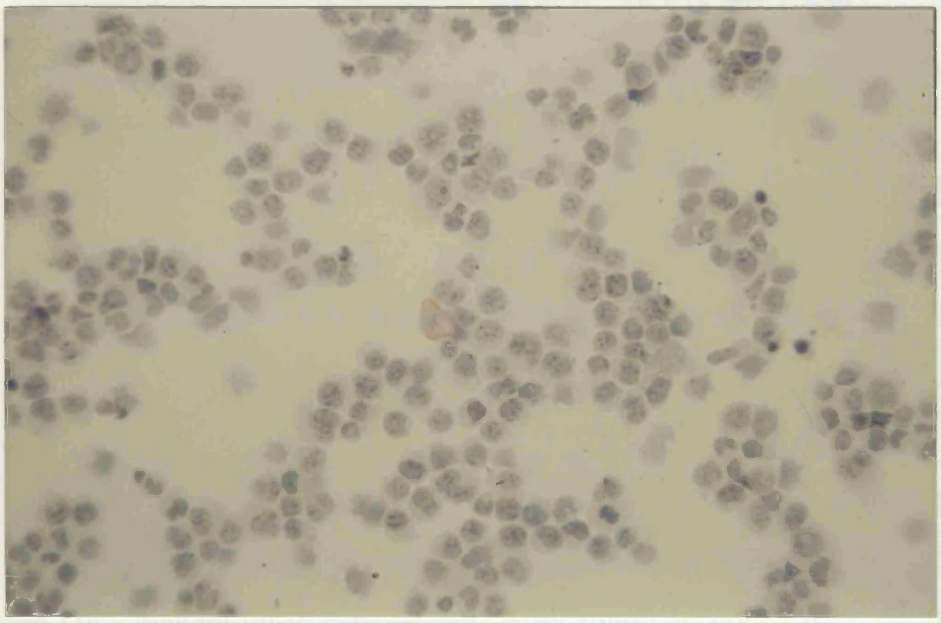
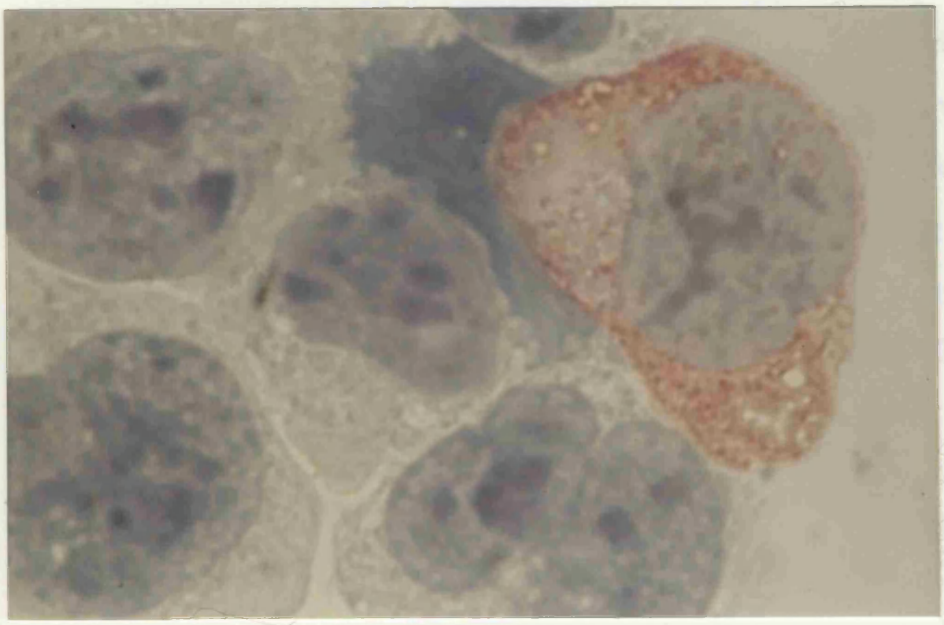


FIGURE 7.3.4b. The same K562 cells as in 7.3.4a. seen at x 4500 magnification.

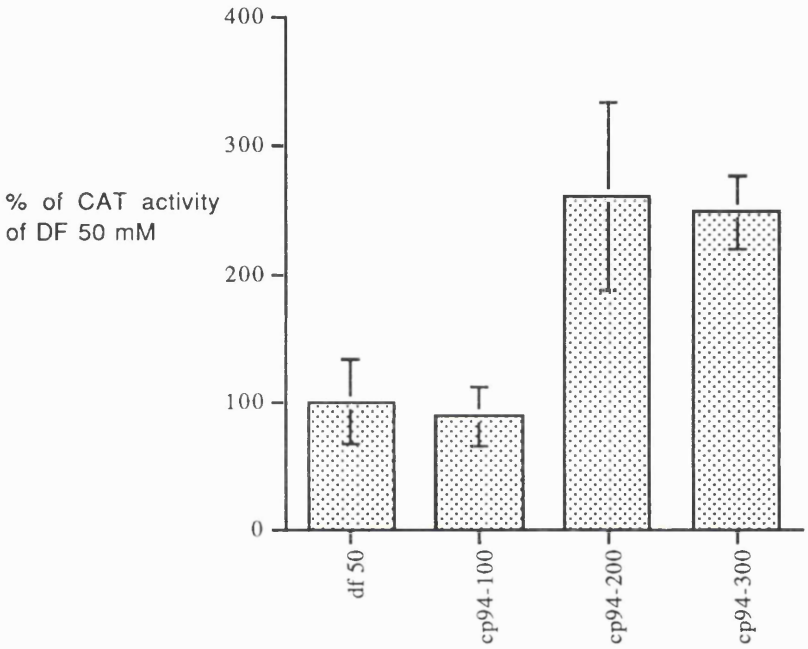


7.3.5. Transfection efficiency of TFPLCo in other cells ;

The transfection efficiency of the transferrinfection method was also examined in the growth factor dependent cell line TF-1. This cell line had been noted (see chapter 3) to be more difficult to reliably transfect by electroporation, in particular requiring frequent treatment to exclude Mycoplasma contamination. The results of transferrinfection also showed this tendency with some experiments resulting in no transfection while others were more successful. The two experiments of which the results are shown in FIGURE 7.3.5. were done in parallel with electroporation to demonstrate that the cells used were suitable for transfection. The cell background activity detected by the ELISA CAT assay was higher with TF1's than K562 and this in part explains the lower ratio of 4.5 ± 1.0 : 1 for the CAT activity after transfection under optimum conditions compared with the background CAT expression in untransfected cells. The CAT activity detected after incubation with 4 different chelator conditions are shown in FIGURE 7.3.5. with the optimum IBE (200 μM Cp 94) comparable, but possibly higher, to that seen for K562 cells. The comparison with electroporation was also similar to K562 cells with the value for transferrinfection under optimum conditions being 23.5 ± 8.5 % of that for electroporation of the same number of cells with the same amount of DNA.

The toxicity of the protocol for HL60 cells (even at low concentrations of chelator such as 10 μM IBE of DF or 30μM IBE of CP94) and for Daudi cells (50 μM IBE of DF) prevented the testing of the use of TFPLCo in these cell lines.

FIGURE 7.3.5. Transferrinfection efficiency in TF 1 cells according to chelator type and concentration.



7.4. DISCUSSION.

Thesedata confirm that the process of transferrinfection is an effective means of transiently transfecting haemopoietic cells in culture by utilising the process of receptor mediated internalisation to introduce DNA in to the cytoplasm of the target cells, at least in K562 cells and possibly in TF-1 cells. The process does not require physical disruption of the plasma membrane in the way that electroporation does but, like electroporation, only a small proportion of the cells are transfected. This work has examined possible parameters that might effect the efficiency of transfection.

The first step in the process is the binding of the transferrin-polylysine DNA complex to the transferrin receptor located in the cell membrane. The number of such receptors can be upregulated by the use of iron chelating drugs such as Desferrioxamine and the novel 3-hydroxypyridin-4-one: Compound 94 which act by causing a relative deprivation of free iron to the cell. This up regulation of the TF-R was reported [53] to be important in achieving efficient transferrinfection. A detailed examination of the relative efficacy of these two compounds shows them to be relatively similar. However the effect of TF-R up regulation does not entirely explain their effect since by analysing TF-R up regulation in parallel with transfection it has been shown that the optimum concentration for TF-R up regulation does not correspond to that for expression of the reporter protein by the transfected cells. It seemed possible that cell cycle kinetics might have a role in the susceptibility of the cells to be transfected and so this was examined by parallel measurement of the cell cycle profile and the expression of the transfected CAT protein after pre incubation with CP94. Compound 94 was used because of cell cycle synchronisation but this did not prove to be significant since the amount of transfection observed

decreased rapidly after the chelator was removed even if it was present up to the time when the TFPLCo/DNA complexes were added, so that the postulated increase in transfection when the proportion of cells in S phase increased did not occur.

The next step required following internalisation of the receptor is for the introduced DNA to escape from the endosome. In this work this has been achieved by the use of chloroquine which it is postulated has an effect on the survival of the DNA in the endosome and this appears to be particularly marked in K562 cells [53] which explains why this method is successful in this cell type. It will be important to determine for other haemopoietic cells whether this is a useful delivery method as in K562 cells or whether it is limited by constraints such as the probable toxicity of the chelator as has been observed here for HL60 cells. Cotten et al [66] have reported a list of cell lines which have been successfully transfected but, apart from K562, include only one of human monocyte or myeloid lineage that is the monocytic cell line U937 and this is only under the heading of "cells that work poorly". Citro et al [70] have reported successful delivery of antisense oligonucleotides to HL-60 cells using this method. In this report the preincubation step in iron-chelator was omitted without loss of efficacy of delivery, though it is possible that the constraints on expression of introduced DNA maybe different from those on the downregulation of the targets of antisense oligonucleotides.

In addition to the amount of activity of the reporter plasmid in transfected cells an important parameter of the success of a transfection procedure is the proportion of cells that are expressing the introduced gene. In these experiments I found that with transferrinfection as with electroporation only a small proportion of the cells express the transfected gene when examined by the use of an APAAP technique on a cytospin preparation of

cells. Cotten and co-workers [66] initially examined expression of a β-galactosidase (β-gal) gene product *in situ* but found the system to be "insensitive and unreliable". In a second series of experiments [66] they utilised a plasmid with the capacity to undergo episomal replication in combination with a flow cytometric method of detecting the β- galactose substrate and after a week detected nearly 100% expression of the transfected gene in K562 cells. It is likely that this system is much more sensitive than the one which I utilised, because of the episomal replication step, and this almost certainly explains the difference in the proportion of transfected cells identified compared with the experiments reported here. In other reports of *in situ* β gal expression they suggest that HeLa cells require the addition of replication deficient adenovirus particles and mouse hepatocytes require the adenovirus to be linked with the TFPLCo-DNA in a ternary complex in order for a high proportion of cells to express the β-gal gene following transferrinfection [63, 64, 66]. Use of an N-terminal fragment of the influenza virus haemagglutinin molecule [65] may be able to replace the use of defective adenovirus with this synthetic peptide resulting in a theoretical improvement of safety from adverse virus related events. Until recently data to suggest that this method would be useful in the creation of stable transfectants was limited since only the cytoplasm is targeted [50] but the use of a Chicken Adenovirus (CELO), which is replication deficient in mammalian cells, has recently allowed much increased efficiency of stable transfection by transferrinfection to be reported in HeLa cells and a myoblast cell line, but not in haemopoietic cells [67]. More generally the concept of the receptor-ligand pathway being used to deliver DNA has been broadened to the use of the asialo-glycoprotein and its receptor pair to deliver DNA to hepatocytes [107]

In summary this method of transferrinfection has been confirmed to effectively deliver plasmid DNA to target cells without the physical disruption of the cell membrane which is a prerequisite of most currently used transfection protocols. The expression of the reporter plasmid under comparable conditions to electroporation is lower but by less than one order of magnitude. The full potential of this delivery system to transfect haemopoietic cells without significant toxicity will require a higher proportion of the treated cells to be transfected.

CHAPTER 8 CONCLUDING REMARKS.

This work was started with the premise that in order to study mechanisms of differentiation in the haemopoietic system novel, non toxic transfection protocols would be required. The reasoning for this was that current methods of transient transfection of non-adherent haemopoietic cells are problematic because of the toxicity observed and the small proportion of the cells transfected within a treated population. The role of transfection in the study of haemopoietic cells is that, by the up or downregulation of the expression of specific intermediary macromolecules thought to be implicated in signal transduction pathways, the outcome of such manipulations on cellular behaviour can be studied. However, it can be reasoned that, if only a small proportion of cells being studied survive and successfully express the factor under study, then the interpretation of the behaviour of the whole population will not allow meaningful conclusions to be reached. One possible solution to this problem is the cotransfection of a selectable marker, such as the antibiotic resistance gene *neo*, and the use of a period of culture under selective conditions after transfection to increase the proportion of transfected cells. This approach has been successfully reported recently by Visvader et al [22] in their study of the GATA binding family of transcription factors in a myeloid leukaemia cell line (416B), however, the criticism can still be made that the selection of a small proportion of surviving, transfected cells cannot be representative of the *in vivo* processes that I wished to study. For these reasons I opted to try and optimise non toxic methods of transfection of haemopoietic cells initially for studies in cell lines but with the intention that such a technique, if successful, could also be used in the study of haemopoietic cells in primary culture. The possibility that haemopoietic stem cells, derived from patients, might be transfected without the use of retroviruses, would have had major implications for the application of gene therapy to the clinical arena

because as I have discussed at length in Chapter 1 there are significant reservations about the widespread use of retroviruses in clinical gene therapy protocols.

The first set of experiments, which I reported in Chapter 3, were those in which I used electroporation to transfect a number of haemopoietic cell lines with the reporter gene Chloramphenicol acyl transferase (CAT). Methods of transfection such as Calcium Phosphate precipitation and the use of DEAE dextran which have become established in the transfection of adherent cell lines have been shown to be not useful in transfecting haemopoietic cells [26]. Therefore the successful use of electroporation to transfect these cell lines allowed me to establish the value of the CMV promoter used with the CAT construct in these cells and to set up a reference system in terms of parameter's such as toxicity, efficacy and target cell spectrum against which novel transfection strategies could be compared. I identified that while more robust cell lines, such as K562, could be electroporated relatively easily the two cell lines that I was particularly concerned with, namely HL60 and TF 1 required careful attention to culture conditions and in particular the avoidance of Mycoplasmal contamination by frequent treatment with the anti mycoplasmal agent MRA (ICN-FLOW). I was particularly concerned with HL60 because of the property of differentiation towards a myeloid or monocytic phenotype that can be caused by the use of agents such as retinoic acid and vitamin D3 respectively [18] and with TF1 because of its growth factor dependence [19]. I established stably transfected K562 cells by electroporation with a *neo* containing construct but I was unable to establish stable transfection in HL60 cells, even with lower concentrations of the selecting antibiotic G418, therefore suggesting that if the HL60

differentiation pathways were to be studied then alternative transfection strategies would have to be pursued.

The next series of experiments, described in Chapter 4, involved the use of the fluorescent dye Carboxyfluorescein as a marker of the delivery of liposome encapsulated molecules by the liposome delivery systems under study. Though a small molecule compared to the DNA, which was to be used in transfection studies, CF has two important properties. Firstly it is hydrophilic and lipophobic, and so, once encapsulated, remains within the aqueous interior of the liposome and cannot escape by traversing the lipid lamellae. Secondly if it is encapsulated at a high concentration (e.g. 200 mmol) the fluorescence is quenched and this quenching is reversed with release to a lower concentration (dequenching) so that fusion with cells could be anticipated to be associated with a greater fluorescence than simple binding. Therefore this model system was used to investigate a range of liposome types which varied in terms of lipid composition, size and method of preparation chosen on the basis of a careful review of liposome based transfection strategies reported in the literature. In addition the pH sensitive dye SNARF was used to try and determine the pH of the environment to which any delivery was occurring. These experiments identified a range of liposome preparation protocols suitable for transfection studies. The range of methods used reflected differing methods used in the successful liposomal transfection methods that had been published.

Transfection studies with liposomes were then commenced with these selected protocols using CAT plasmids containing promoters of proven efficacy in the target cells when introduced by electroporation. In addition it was clearly demonstrated that the DNA was encapsulated in significant

amounts within the aqueous interior of the liposomes used in these studies. The results of these experiments were reported in chapter 5 and, disappointingly, failed to demonstrate any significant transfection activity in target cells inspite of the use of a very wide range of liposome types, incubation methods, conditions and other protocol modifications. This result was disappointing because of the utilisation, first in the CF studies reported in chapter 4 and then in these transfection experiments of the Haemagglutinin and Fusion proteins of the Human Sendai Virus as a means of achieving targeting to and fusion with the cells to be transfected. These proteins have been used in a number of successful transfection studies reported in the transfection of non haemopoietic or non-human cells and appeared to provide a promising method of ensuring binding and delivery of DNA encapsulating liposomes [25, 42, 43 & 46]. Targeting is valuable because the fusion of larger liposomes, which have the ability to encapsulate greater amounts of DNA, probably require such mechanisms. Smaller liposomes are more likely to be phagocytosed but have a markedly smaller encapsulation volume. The technique of preparation of chimerasomes [25] has the added advantage of incorporating these viral proteins in a hybrid particle without including the viral nucleic acids of the virus core (see Figure 4.3.5). The theory behind the use of these proteins is that the Haemagglutinin protein ensures rapid binding to sialic acid residues in the target cell membrane and this appeared to be achieved (see Figure 4.3.6) however this binding should then be followed by fusion mediated by the fusion protein (F protein) and this may not have occurred. An alternative virus to Sendai virus is the use of the membrane proteins of the closely related Orthomyxovirus, the Human Influenza virus, however there appeared to be no advantage over HVJ in the above reported studies and the theoretical disadvantage that fusion of Influenza protein containing

endosomes is triggered at acid pH as oppose to physiological pH as is the case with Sendai virus.

Therefore after the lack of successful transfection in the studies reported in Chapter 5, in Chapter 6 I reviewed the possible reasons for successful transfection not being achieved and adopted a number of strategies to try and overcome these problems. A number of methods to optimise DNA encapsulation, packaging and passage unchanged through an endosomal compartment were adopted without success but the two critical experiments were those demonstrating that failure of liposomal fusion with the target cells was the most likely reason for the failure of transfection to occur. These experiments were firstly, the failure to see fluorescence within the cytosol of cells mixed with CF containing chimerasomes when they were examined by con-focal microscopy (FIGURE 6.3.1) and secondly the demonstration that the toxic compound Hygromycin was not delivered to cells when encapsulated in intact chimerasomes but was when identical chimerasomes were disrupted by high energy sonication and the released Hygromycin incubated with the cells (FIGURE 6.3.2-5). Once these experiments established that failure of fusion was the most likely problem, two further experiments with PEG and electroporation were combined with the use of chimerasomes to try and overcome this failure of fusion with the target cells. The former was ineffective and the latter proved highly toxic and ineffective.

Therefore in conclusion of the experiments involving the use of liposomes it did not prove possible to demonstrate any useful transfection activity in the treated cells. This finding contrasts with the published protocols quoted above but even after the completion of these studies further review of the literature has not revealed any reports of significant transient transfection of

the human myeloid cell lines that I have studied either with the techniques that I have used or with the commercially available DNA binding lipid preparations which I reviewed in Chapter 6. The reason for this lack of successful transfection of human myeloid cell lines with liposomal methods remains obscure, however in as much that these cells represent maturation arrest at a early stage of myeloid differentiation it can be speculated that this situation mirrors the reported difficulty in transfecting Human haemopoietic stem cells by retroviral methods [72].

It remains possible that there were technical reasons failure to reproduce the successful use of chimerasomes in non-haemopoietic cells in human haemopoietic cells but I attempted to rule out this possibility by discussing the methodology carefully on a number of occasions with the original authors who also provided me with a copy of their detailed laboratory methods manual in addition to updated published methods. In addition I demonstrated reconstitution of the intact viral membrane proteins on PAGE gel electrophoresis as suggested by Gould-Fogerite and Mannino (FIGURE 4.5) and effective encapsulation of plasmid DNA within the intact chimerasomes (FIGURE 5.3.1). I obtained the Phosphatidylserine from the USA from the same source as Gould-Fogerite and Mannino (AVANTI POLAR LIPIDS), used dialysis membrane from the same source (SPECTROPOR) and in all possible ways attempted to reproduce the original method as closely as possible. The department from which I obtained the Sendai Virus (Dept. of Virology, St Bartholomew's Hospital Medical School) has extensive experience in the laboratory use of this virus and its proximity to University College enabled rapid purification of the virus by differential centrifugation without delay prior to immediate storage at -70 °C in appropriate aliquots. Therefore I believe the failure to

demonstrate transfection activity shows that this method is probably not effective in these cells.

The lack of effective transfection activity achieved using liposome based methods meant that alternative methods were sought to achieve the original aims of the project. The methods of Birnstiel and co-workers published in a series of papers from Vienna from 1990 onwards offered a promising alternative strategy [48, 49, 53, 60, 62-67]. The method of transfection used, called Transferrinfection, is based on the conjugation of transferrin and polylysine with the polylysine having the property of binding DNA to form a Transferrin-Polylysine-DNA complex which is delivered to the cell by receptor mediated internalisation via the Transferrin receptor (TF-R). This process is in many ways similar to the process of liposome mediated transfection in that it is non -toxic and results in the delivery of the DNA to the cell without disruption of the continuity of the cell membrane. The process was summarised in diagrammatic format in FIGURE 1.2. The authors noted that effective expression of DNA in K562 cells required modifications of the original protocol, firstly by upregulation of TF-R expression by pre-incubation of the cells in an iron chelator and secondly by modification of the passage of the DNA through the endosomes using chloroquine. The system has since been further modified to increase the process of release from the endosomes, in the series of papers cited above, for use in other cell types such as HeLa and mouse hepatocytes. These modifications include the use of inactivated adenovirus, adenovirus conjugated to the TFPLCo-DNA complex or a synthetic peptide based on an N-terminal fragment of the Influenza haemagglutinin protein. The choice of which of these alternatives to use is based on two criteria. Firstly the adenovirus (ADV-R) receptor expression and TF-R expression of the target cells can be considered and secondly, if there is toxicity to the cells with free adenovirus, then the conjugated virus may be chosen because it can

be used at a much lower dose. Thus, for example, K562 expresses little ADV-R but plentiful TF-R and is well transfected with TFPLCo-DNA alone, HeLa requires the use of inactivated adenovirus for expression to be optimal, whereas mouse Hepatocytes are best treated with the conjugated Adenovirus because of the toxicity seen with the free virus. In the experiments reported in Chapter 7 I examined the role of the iron chelator in this system mainly with K562 cells. I had the opportunity to compare the use of the novel 3-hydroxypyridin-4-one iron chelator Compound 94 (Cp 94) with the iron chelator, Desferrioxamine, which was used in the original published methods. Cp 94 causes cell synchronisation following withdrawal of the chelator from the cell culture medium [104]. I was therefore able to examine in detail the relationship between TF-R expression, cell cycle phase and transfection efficiency. Cell cycle synchronisation did not lead to greater expression of the introduced DNA probably because of a rapid decrease in transfection efficiency seen if the cells are removed from chelator during the incubation of the cells with the TFPLCo (FIGURE 7.3.3a & b). Next, when cytospin preparations of transfected K562 cells were examined for expression of the transfected CAT protein by means of an APAAP technique it was found that only a small proportion of cells within the sample expressed the transfected protein, albeit at high levels (see FIGURE 7.3.4a & b). However, the use of an episomally replicating plasmid and a flow cytometric analysis reported by Cotten et al. [66] would suggest that DNA is delivered to a much higher proportion of K562 cells than can be detected to be expressing the reporter gene in the experiments that I have reported here. The pattern of expression of the reporter gene in a small proportion of cells, using APAAP to detect CAT protein expression, is also seen with cells transfected by electroporation and this means that, in my experience, the cells expressing the introduced gene in a transfected cell population will represent only a

small proportion of the cells present. I also looked at the efficiency of transfection in the growth factor dependent cell line TF1, in which transfection was recorded but at lower levels than that seen with K562 cells. Transferrinfection therefore appears to be a promising technique but one which will need to be further refined before the initial aim of this study, of achieving a high level transfection in a significant proportion of the cells without toxicity, can be achieved. The efficiency of the techniques using inactivated adenovirus conjugated to the TFPLCo-DNA complex may improve efficiency of transfection and remove the need for the use of chloroquine. The greater efficiency of endosomolysis afforded by the adenovirus based techniques suggests that this would be a logical direction in which to take studies of the use of transferrinfection of other haemopoietic cell lines or primary bone marrow cells. The use of the synthetic Influenza HA-like peptide allows the avoidance of the use any potentially infective virus particles but the efficiency achieved does not yet match that of the use of inactivated adenovirus. It is notable that TF-R is highly expressed by most dividing cells of the haemopoietic system so it is to be hoped that the system will prove to be widely applicable.

With regard to future studies, the concept of delivery via receptor mediated internalisation has already been extended to the use of the asialo-glycoprotein ligand receptor pair in Hepatocytes [107] and this raises the possibility that the concept of delivery by conjugation of DNA / polylysine to a ligand which then interacts with its cellular receptor could be extended to more or less any cell by choice of the correct ligand-receptor pair. This would also have the potential promise of a useful degree of selectivity in macromolecular delivery. This concept could also be applied to the delivery to selected target cells not only of DNA but also of antisense oligonucleotides, peptides or therapeutic synthetic compounds.

With references to the application of gene therapy to clinical practice, I believe that the questions raised about the safety of retroviruses in the discussion of this issue in Chapter 1 have to be answered, beyond reasonable doubt, if gene therapy is to be used in a wide range of clinical situations especially in children and young adults of reproductive age. However, in the absence of effective alternatives or clear evidence that the fear of recombination events giving rise to pathogenic viruses is justified, it is likely that work with retroviral gene transfer protocols will continue. It also seems likely that the use of lipid-DNA complexes as pioneered by Nabel et al. [79] may have a significant role to play in the direct transfection of cells that are easily transfected in *vivo* at accessible sites such as the skin or in the lung and GI tract epithelial surfaces. The search for alternative, non-toxic, non-retroviral alternative methods of transfecting stem cells should continue. For the present the transfection of haemopoietic cells with liposome-encapsulation methods seems ineffective but the use of transferrin-polylysine-DNA complexes, possibly conjugated with inactivated Adenovirus (or a synthetic equivalent) as an endosomolytic mechanism, may be an appropriate area for future study.

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