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Liposome encapsulation of interferon : effects on anticellular activity

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by

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ABSTRACT

Liposome encapsulation of interferon was performed to investigate its effects on the anticellular properties of interferon.

Purified mouse interferon was incorporated into positively charged multilamellar liposomes. The growth response of mouse leukemia L1210 cells to liposome encapsulated interferon was examined.

Comparison with free interferon showed no significant increase in anticellular activity by liposome encapsulation.

Cells resistant to the antiviral and anticellular effects of free interferon were incubated with liposome encapsulated interferon. Significant growth inhibition was demonstrated by liposome encapsulated interferon, evident at 24 hours but more pronounced at 48 hours. The implications of these results for mechanisms of interferon resistance is discussed.

2-5A, an oligonucleotide believed to be an intracellular mediator of interferon activity, is unable to penetrate intact plasma membranes. Liposome encapsulated 2-5A was incubated with cells to see if intracellular transfer of the oligonucleotide, with subsequent growth inhibition, could be achieved. Growth inhibition by liposome encapsulated 2-5A was not demonstrated. Liposome encapsulation of 2-5A does not effectively introduce the oligonucleotide into L1210 cells.

Future research applications of liposome encapsulated interferon are discussed.

LIPOSOMES

Liposomes are microscopic structures consisting of one or more concentric lipid bilayers enclosing an equal number of aqueous compartments. They have gained enormous popularity as models for biological membranes. Liposomes are versatile structures which have been investigated from many perspectives. Liposomes have also been used as tools for the investigation of a wide variety of biological phenomena, including the molecular details of membranes, interactions of biological membranes, membrane active compounds (ionophores, anesthetics, etc.), cell receptor activity, and antibody-antigen interactions.

The ability to encapsulate biologically active agents has created an exciting area of research. The use of liposomes as carriers of biologically active materials has given them a significant role in pharmacology.

PREPARATION

Liposomes are surprisingly simple to prepare. Dried films of phospholipids, alone or mixed with other lipid soluble compounds, are shaken with aqueous media. This results in a milky colored preparation of multilamellar vesicles (MLV) of

heterogeneous size. These structures, originally referred to as "myelin figures", are composed of concentric lamellae. Each lamella consists of a lipid bilayer (Bangham, 1968, Bangham, 1972, Bangham, etal, 1974) separated from adjacent bilayers by aqueous spaces.

Ultrasonication of these multilamellar liposomes transforms them into much smaller unilamellar liposomes, consisting of a single lipid bilayer. These sonicated unilamellar vesicles (SUV) are more homogeneous in size, and their transformation is heralded by clearing of the original vesicle preparation. The limiting size of these vesicles is approximately 200 angstroms, defined by the constraint of the small radius of curvature on the packing of lipid molecules within the vesicle membrane.

Liposomes can be fractionated according to size by centrifugation or molecular sieve chromatography.

Other methods have been described for the preparation of liposomes of differing structure. (Batzri and Korn, 1973, Szoka and Papahadjopoulos, 1978, Deamer and Bangham, 1976)

COMPOSITION

The unique amphipathic properties of phospholipids make them an obligatory component of the lipid bilayers of liposomes. A variety of other lipids or lipid soluble compounds can be incorporated into the bilayer, which enables investigators to

change the physical properties of the liposome.

Most laboratories employ a basic mixture of phospholipid, cholesterol, and a charged amphiphile in varying molecular ratios (6:3:1 to 5:5:0.5). Changes in liposome composition provide an useful variable in liposome investigation, especially in studies of liposome- cell interaction.

Cholesterol is an important component of liposome membranes. It increases bilayer stability and reduces leakage of entrapped materials (Papahadjopoulos, etal, 1973).

Liposomes are often given a net surface charge by including charged amphiphiles as components of the bilayer. Commonly used amphiphiles include stearylamine (positive) and dicetyl phosphate (negative). Inclusion of these charged species on the liposome surface increases the aqueous space volume within the liposome, allowing greater amounts of material to be entrapped.

The choice of charged amphiphile depends on the material to be encapsulated. If the material is not charged, either positive or negative amphiphiles can be used. If the material does bear a charge, then two schools of thought exist.

Using an amphiphile of similar charge will minimize electrostatic binding of entrapped material to the lipid bilayer. Such binding could theoretically decrease the pharmacologic activity of the encapsulated drug.

The use of a charge opposite that of the entrapped species also has several utilities. The amount of soluble material encapsulated is markedly enhanced. Absorption to both internal

and external surfaces can occur, as demonstrated with cytochrome C (Kimelberg, etal, 1970). This is especially useful if exposure of the material to the external medium is acceptable. If such exposure is not acceptable, material associated with the external surface can be removed by washing, or treatment with enzymes or high ionic strength.

Papahadjopoulos (1974) has shown that cyclic AMP and actinomycin D (1976) could both be encapsulated in oppositely charged vesicles without a substantial decrease in pharmacologic activity.

The presence of charged amphiphiles exerts an important influence on the mechanism of incorporation into cells.

FLUIDITY OF LIPID MEMBRANES

An important physical property of liposomes is the fluidity of the lipid bilayer. Phospholipid species undergo a first-order thermal phase transition from a solid to a liquid crystalline phase at a characteristic temperature. This transition temperature (T_c) differs for individual phospholipid species. Below the T_c the lipids are in a solid-like state, with their hydrocarbon chains relatively ordered. Above the T_c the chains are more disordered, with greater freedom of motion, and are considered to be in a fluid state. Bilayers in a fluid state have decreased thickness.

The characteristic T_c for each phospholipid species is defined by its molecular structure. In general, T_c is lowered by decreased chain length, by unsaturation of acyl chains, by bulky side groups (e.g. cyclopropane rings), and by branching of acyl chains. A characteristic T_c for each phospholipid makes it possible to construct vesicles that are either solid or fluid at the required experimental temperature. Such manipulation of liposomal properties has value in targeting of liposomes to specific intracellular sites. Cholesterol added to lipids above their T_c decreases fluidity, concomitantly decreasing permeability (Poste, etal, 1976).

PERMEABILITY

Liposome permeability has been studied by several methods, including efflux of radiolabeled material, efflux of non-radiolabelled materials (where appropriate assays exist), changes in optical density of liposome suspensions, and changing osmolarity of the external aqueous environment.

Liposomes composed of unmodified phospholipids are extremely impermeable to cations (Na^+, K^+), but are generally more permeable to anions (Cl^-) (Bangham, etal, 1965) The ions fluxes are considered quite low compared with most natural membranes. Liposomes show considerable permeability to water, comparable to natural membranes.

The effects of protein on liposome permeability are important to understand. The presence of proteins is the principle difference between liposomes and natural membranes. Plasma proteins, present both in both in vitro and in vivo systems, are rapidly absorbed by liposomes (Black and Gregoriadis, 1976).

Binding of proteins to liposomes may increase permeability to encapsulated materials (Kimelberg and Papahadjopoulos, 1971). Cell culture media are full of serum components and released cellular proteins, which may become a more significant problem with long incubations. Liposomes also confront this problem in vivo; body fluids are full of proteins capable of interacting with liposomes and increasing their permeability.

The inclusion of cholesterol in the liposome bilayer will substantially reduce the permeability to entrapped materials, even in the presence of protein (Papahadjopoulos, etal, 1973).

INTERACTION WITH CELLS IN VITRO

Cultured mammalian cells show a great affinity for liposomes. Incorporation of up to one million liposomes per cell has been demonstrated without effect on cell viability or growth rates. A variety of cell types can incorporate liposomes of widely varying lipid compositions without cytotoxic effects.

Different cells exhibit similar kinetics of liposome uptake.

At 37' C there is rapid uptake of liposomes within the first two hours, which levels off, reaching a plateau after 3-8 hours (Poste, etal, 1976).

Mechanisms of liposome-cell interaction include stable adsorption, endocytosis, fusion, and lipid exchange. Vesicle surface charge and the physical state of the vesicle lipids are important determinants of the mechanism of vesicle incorporation (Poste and Papahadjopoulos, 1975)

Stable adsorption implies association of liposomes with the cell surface, remaining intent on the surface without internalization. Scanning electron microscopy of fibroblasts incubated with liposomes demonstrates a characteristic bumpy surface, wheareas untreated cells have a smooth surface (Pagano and Takeichi, 1977). The diameter of the bumps ranges from 300-1000 angstroms, consistent with adsorption of vesicles to the cell surface. While repeated washings do not remove adsorbed liposomes from the cell surface, trypsin can remove them, suggesting an important role for cell surface proteins in this interaction.

Endocytosis is traditionally classified as pinocytosis (uptake of fluid) or phagocytosis (uptake of particles). Either of these processes can theoretically mediate cellular uptake of liposomes. Evidence for endocytosis of liposomes is abundant. Papahadjopoulos (1974) demonstrated that radiolabelled unilamellar vesicles with a different radiolabelled component trapped inside the vesicle are taken up as intact structures.

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Liposome components have been demonstrated within cells by electron histochemistry (Magee, etal, 1974) and electron radioautography (Huang and Pagano, 1975). Liposomes have been found in lysosomes (Gregoriadis, etal, 1971). Endocytosis was reduced in fibroblasts by cytochalasin B or appropriate metabolic inhibitors of respiration or glycolysis.

The appearance of liposome contents in the cytoplasm is the strongest evidence supporting the mechanism of liposome-cell fusion. The presence of such material in the cytoplasm has been established by many investigators. Tissue cells were incubated with liposomes containing cyclic AMP (Papahadjopoulos, etal, 1974) and actinomycin D (Poste and Papahadjopoulos, 1976) and biological activity of these compounds indicated their presence in the cytoplasm. Lymphocytes incubated with liposomes containing ^{3}H-inulin showed random distribution of the radiolabeled marker throughout the cytoplasm (Huang, etal, 1978). Evidence for fusion has also been provided by studies employing ultrastructural histochemistry (Weissman, etal, 1977) and fluoresence microscopy (Weinstein, etal, 1977).

Exchange of radiolabelled phospholipids has been observed between various cell and liposome types (Huang, etal, 1978) Radiolabelled phospholipids moved from vesicles to cells, and vice versa. The molecular mechanism of this exchange is yet undetermined, but involves interaction of the lipids of the outer liposome monolayer and the cell surface. Lipid monomers may be transferred, with possible mediation by lipid exchange proteins

present in the cell surface or in the medium. Intermediate structures may also be formed, with transient, reversible merging of the outer monolayers of liposomes and cell plasma membranes Lipid exchange is probably not responsible for significant incorporation of vesicle of lipid into cells at 37' C, but may predominate at low temperatures (Pagano and Huang, 1975).

LIPOSOME IMMUNOLOGY

Immune responses to liposomes are important to understand and minimize for optimal in vitro and in vivo use of liposomes.

Complement can mediate lysis of liposomes. Immune damage of liposomes involves the same classical complement sequence responsible for the antibody-induced lysis of cells (Haxby, etal, 1969). Cunningham (1979) found that liposomes can also activate the alternative pathway of human complement. A positive surface charge on the liposome was required for C_3 conversion. Activation was also influenced by liposomal cholesterol concentration, phospholipid fatty acyl chain length, and degree of saturation, all suggesting membrane fluidity as an important determinant of complement fixation. Thoughtful liposome design can thus minimize complement-mediated lysis of liposomes, which would adversely affect their carrier function. Heat-inactivation of serum used with liposomes has also been advocated, since heat-inactivation presumably destroys complement factors.

The ability of liposomes to induce humoral or cell-mediated immunity is of great interest because of their potential as carriers of biologically active agents in vivo.

One theoretical advantage of liposome entrapment of potentially immunogenic substances (e.g. foreign proteins) is prevention of antibody formation, which could inactivate the substances. Though theoretically attractive, full prevention of immunogenicity of entrapped substances has not fully materialized. Allison and Gregoriadis found that mice produced more antibodies against diphtheria toxoid when immunized with the antigen encapsulated in negatively charged liposomes. (Allison and Gregoriadis, 1974). However, they also showed a marked decrease in allergic reaction (i.e. Arthus reaction, serum sickness) when animals were subsequently challenged with liposome encapsulated diphtheria toxoid (Gregoriadis and Allison, 1974).

The incorporation of any drug or protein into liposomes increases the risk of immunogenicity. Immune responses to purified naturally occuring lipids are not significant, and liposomes composed solely of these substances would have minimal immunogenicity (Rapport and Graf, 1969).

DISTRIBUTION AND INTERACTIONS OF LIPOSOMES IN VIVO

The in vivo interactions of liposomes are important to understand for guiding in vivo research and developing clinical applications of liposome technology. The fate of liposomes varies with their route of administration.

Liposomes injected intravenously interact with a variety of substances. Serum proteins, lipoproteins, and other plasma components are encountered. Binding to erythrocytes has been documented (Kimelberg, 1976), and binding to leukocytes and platelets probably occurs.

The ability of liposomes to reach target tissue cells depends on their ability to cross capillaries into the tissues. Intact liposomes can pass from capillary lumen to interstitial space by endocytosis by endothelial cells, or by passage through intercellular spaces. However, even the smallest liposome could be too large for passage by these mechanisms. Fusion of liposomes with endothelial cells would allow liposomal contents to traverse the cell and emerge in the interstitial space.

Distribution studies of radiolabelled liposomes show they are cleared from blood and taken up by tissues after intravenous injection. Both composition and size affect the rate of clearance. Larger liposomes are cleared more rapidly than smaller ones (Kimelberg, 1976). Liposomes negatively charged before injection are cleared more rapidly than positively charged or neutral ones (Kimelberg, 1976, Juliano and Stamp, 1975).

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Black and Gregoriadis (1976) demonstrated that in the presence of serum, all liposomes acquire a negative charge. Therefore, differences in interaction with blood proteins or differences in final net negative charge density could be responsible for the differential clearance rates observed between liposomes with different charges before injection.

The liver takes up the largest amount of an intravenous dose of liposomes. Its large size and blood supply, as well as a high specific uptake by the reticuloendothelial system, are probably responsible for this. Up to 50% or more of labelled lipid or liposomal contents can be present in the liver within hours after administration (Gregoriadis, 1973).

Other tissues take up liposomes (Gregoriadis, 1973, Kimelberg, etal, 1976) with kidney, lung, and spleen taking up significant amounts. Gastrointestinal tissues do not take up liposomes very strongly.

Liposomes taken up by the liver can be found within both hepatocytes and Kupffer cells, suggesting endocytosis as the method of uptake by liver. Liposomes have also been found in lysosomes, as demonstrated by fractionation studies (Gregoriadis and Ryman, 1972, Segal, etal, 1976).

Intraperitoneal administration of liposomes shows similar tissue distribution, with liver, spleen, lung, and kidney as major sites of tissue uptake. Intestinal uptake of intraperitoneally injected liposomes is also low. Distribution is generally more even, with diminished liver and plasma levels

compared to intravenous injection.

Many drugs are ineffective when administered orally, and liposome encapsulation has allowed such drugs to show activity. Intragastric administration of liposome encapsulated insulin resulted in marked decreases in blood glucose levels in diabetic and normal rats (Patel and Ryman, 1977) compared to free insulin and empty liposomes. Similar effects were seen with liposome encapsulated glucose oxidase. Liposome encapsulation protected these substances from inactivation in the gut and facilitated absorption into the bloodstream. This technique offers great practical advantages to allow oral administration of otherwise ineffective agents.

CARRIER POTENTIAL OF LIPOSOMES

The use of liposomes as carriers to introduce biologically active materials to cells was first demonstrated by Magee and Miller (1972). Treatment of mouse L cells with multilamellar vesicles containing IgG with a high neutralizing titer to Coxsackie virus A-21 protected the cells against subsequent infection by that virus. The IgG-containing liposomes were 3000-10,000 times more effective in protecting cells than the equivalent titer of free antibody.

The use of liposomes for introducing material directly into the intracellular environment was demonstrated by Gregoriadis and

Buckland (1973), who used liposomes to introduce invertase into invertase-deficient mouse macrophages and human fibroblasts.

Since these pioneering experiments, a staggering array of substances have been encapsulated in liposomes for delivery to cells, including hormones, enzymes, plasma proteins, antibiotics, lymphokines, bacterial products, immunoglobulins, nucleotides, polynucleotides, steroids, chemotherapeutic agents, vitamins, chelating agents, and viruses (Gregoriadis, 1976).

Advances in liposome technology have made possible construction of different types of liposomes with diverse physical properties. Investigators have thus been able to modify liposomes to meet a variety of experimental demands. The ability to construct vesicles over a wide size range (200 A to 10 um) enables materials of widely differing molecular weights to be incorporated into liposomes.

Gregoriadis (1977) has reviewed approaches for targeting drugs. A variety of materials have been employed as carriers of drugs, and liposomes have many properties which make them the most promising of carriers. An ideal carrier should be non-toxic, biodegradable, and physically versatile enough to accomodate a variety of drugs. It should be stable, protecting its contents from the external environment (and vice versa), with the ability to cross cellular and anatomic barriers. Liposomes fulfill these criteria, and have demonstrated ability to function as effective carriers of biologically active materials.

liposomes

TARGETING OF LIPOSOMES

Specificity is an important quality for successful application of pharmacologically active agents. Pharmacologists have traditionally improved specificity of drugs through creation and fashioning of molecular structure. The use of liposomes as versatile carriers of drugs introduces an exciting alternative approach to the problem of drug specificity. The specificity of the liposome carrier itself can be altered.

Investigators have taken several approaches to improve the specificity of liposomes. Targeting of liposomes to specific regions of the cell has been attempted by influencing the mode of liposome-cell interaction. Liposomes designed to be incorporated by endocytosis can deliver their contents to lysosomes. While the phospholipid surface of liposomes is not a strong endocytic stimulus into the lysosomal system (deDuve, 1969), aggregated immunoglobulins constitute a strong stimulus for such endocytosis. Vesicles designed to enhance fusion with cellular membranes will deliver a greater proportion of their contents to the cytoplasm.

Altering liposome specificity for target tissue has also been studied. In vivo studies show the majority of intravenously introduced liposomes are taken up nonspecifically by fixed macrophages of the reticuloendothelial system (Gregoriadis, 1973). This clearly hinders effective delivery of drugs not destined for the RES system. However, this pattern of

distribution can be modified.

Varying the size and charge of liposomes affects their in vivo behavior. Changes in lipid composition also alters their tissue distribution.

Sonication of liposomes can affect tissue distribution. Using negatively charged liposomes containing radiolabelled bleomycin, Gregoriadis and coworkers (1977) demonstrated that increasing sonication time not only decreased uptake by liver and spleen, but also increased uptake by solid and ascitic tumors.

Pretreatment of animals with large doses of empty liposomes can effectively reduce RES uptake of subsequently administered liposomes (Haynes and Kang, 1978). The empty liposomes temporarily saturate the reticuloendothelial system, leaving it with diminished capacity to take up subsequent doses of liposomes.

A more versatile approach for increasing liposome specificity uses molecules inserted at the liposomal surface, which can bind liposomes selectively to appropriate cells. A variety of ligands have been associated with liposomes, attached by nonspecific forces or by one of the ligand's binding sites. These ligands can interact with specific determinants on the surfaces of cells, making the liposomes more selective in their cellular interactions.

Immunoglobulins offer the most refined targeting potential for liposomes. By attaching IgG immunoglobulin to liposomes through their F_c portion, the F_{ab} moiety extends into the

external environment, and can theoretically recognize and associate with the respective antigens on the target cell surface. Immunoglobulins raised against a particular antigen could be incorporated onto the liposomal surface, rendering those liposomes selective for a specific target. The potential targets would be limited only by the ability to raise antibodies against them.

Immunoglobulin-coated liposomes have been used to introduce enzymes into deficient cells. Weissman and coworkers have used this method to introduce purified hexosaminidase A into Tay-Sachs leukocytes (Cohen, etal, 1976). Horseradish peroxidase has been introduced by immunoglobulin-coated liposomes into smooth dogfish phagocytes, which lack endogenous peroxidase in their lysosomes (Weissman, etal, 1975). These experiments demonstrate the potential use of liposomes to deliver enzymes to cells of patients with deficiency diseases.

Cell specificity of liposomes was demonstrated by raising IgG against whole HeLa cells (Gregoriadis and Neerunjun, 1975) Liposomes containing ^{111}In-labelled bleomycin had ^{125}I-labelled anti-HeLa IgG attached to their surface. These liposomes were incubated with both HeLa cells and human skin fibroblasts. The uptake of both bleomycin and IgG radioactivity by HeLa cells was 25 times greater than uptake by fibroblasts. When the same liposomes had labelled anti-fibroblast IgG on their surfaces instead, the uptake of both radioactivities by fibroblasts was five-fold greater than in HeLa cells. Both cell types shared

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similar rates of uptake when incubated with liposomes bearing nonspecific IgG.

Heath and coworkers (1980) obtained remarkable cell specificity through antibody targeting liposomes. They conjugated antibody to human erythrocytes to liposomes, and found a 200-fold increase in binding of vesicles to human erythrocytes when compared to controls. 80% of vesicle lipid and contents were associated with the cells.

Cell-specific IgG on the liposomal surface mediates selective uptake of both carrier and its drug contents. IgG raised against whole cells effectively confers cell-specific uptake of liposomes. Raising antibodies to cell-specific antigens may be unnecessary. However, this may remain a useful method for targeting liposomes containing chemotherapeutic agents to cancer cells via antibodies raised against specific tumor antigens.

Interferon was discovered in 1957 by Alick Isaacs and Jean Lindenmann (Isaacs and Lindenmann, 1957a, 1957b). Investigating influenza viruses at the National Institute for Medical Research in London, Isaacs and Lindenmann were both interested in viral interference, a phenomenon in which infection by one type of virus seemed to inhibit subsequent infection by other types of virus.

They incubated chick chorioallantoic membrane with heat-inactivated influenza virus. The cells resisted further infection by other viruses added to the culture. Supernatants from these cultures were able to protect fresh cultures of membrane from infection by live virus.

The substance confering protection against further viral infection, presumed responsible for the 'viral interference' observed, was named interferon.

Since this discovery, much has been learned about interferon. Though originally described as an antiviral substance, many other properties have been attributed to interferon, including antiproliferative and immunoregulatory properties.

Early interferon research was complicated by the relative impurity of available interferon preparations. However, progress in interferon purification techniques has permitted the

production of interferon of extremely high purity.

Human interferon is very expensive and difficult to obtain. The major source of human interferon has been Dr. Kari Cantell of the Central Public Health Laboratory in Helsinki, Finland. Large scale production of human interferon by recombinant DNA techniques would be a great advance for interferon research.

CLASSIFICATION OF INTERFERONS

At least three types of interferon with different antigenic specificities have been described. Thus, rather than a single substance, the interferons represent a family of glycoproteins.

Type I, or classical interferon, is the same type discovered by Isaacs and Lindenmann. It can be produced by a wide variety of cell types, with production induced by a wide variety of agents, both viral and non-viral (Ho and Armstrong, 1975). The newest interferon classification system recognizes the classical interferons independantly as alpha interferon (leukocyte interferon) and beta interferon (fibroblast interferon). Human lymphoblastic interferon is a mixture of alpha (leukocyte) and beta (fibroblast) interferons, with beta interferon predominant.

In 1965, Wheelock stimulated human leukocyte cultures with phytohemagglutin (PHA) and found that the supernatant contained an antiviral, interferon-like substance (Wheelock, 1965). This has since been called gamma, type II or immune interferon. Gamma

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interferon shares many physical, chemical, and biological properties with Type I, or classical interferon. However, differences in stability exist at extremes of pH and heat.

Epstein (1979) has comprehensively reviewed the comparative biology of the major classes of interferons.

PRODUCTION OF INTERFERON

Induction of classical interferon (alpha and beta interferons) can be accomplished by a wide variety of agents, including intracellular microorganisms, bacterial products, polymers, and many low molecular weight substances (Ho and Armstrong, 1975). Grossberg (1979) has developed a modified classification of interferon inducers.

Class A inducers are relatively potent and contain nucleic acid. These include RNA and DNA animal viruses, plant and insect viruses, mycophages and bacteriophages, double stranded RNA from normal cells, and synthetic double stranded RNA.

Class B inducers are relatively weak compared with Class A inducers, and include intracellular microbes (e.g. Bordetella, Brucella, Franciscella, Hemophilus, Listeria, and Serratia), rickettsia, chlamydia, mycoplasma, and protozoa. Certain microbial products, such as lipopolysaccharides, as well as certain polymeric chemicals and various low molecular weight substances also induce interferon.

Certain human tumor lines (colorectal carcinoma, melanoma, rhabdomyosarcoma, and cervical carcinoma) interestingly induce interferon production by allogeneic human lymphocytes. The interferon produced has the physical properties of type I interferon (Trinchieri, etal, 1977).

Gamma, or immune interferon, is produced by the lymphocyte. Both human T and B lymphocytes can produce interferon in vitro in response to the mitogens PHA and PWM (Epstein, etal, 1974). Interferon production by lymphocytes is markedly enhanced in the presence of macrophages. This enhancement is probably related to macrophage presentation of mitogen or antigen to the lymphocytes.

Inducers of gamma interferon generally induce relatively low levels of interferon. The immune status of the lymphocyte donor is an important factor. Immune interferon inducers comprise those exerting nonspecific mitogenic effects, and those showing an immune specific antigenic effect on the cells to be induced.

Mitogens able to induce gamma interferon production include phytohemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A (ConA), streptolysin O (SLO), and staphylococcal enterotoxin A (SEA). Antilymphocyte serum (ALS) and Corynybacterium parvum can also induce gamma interferon.

Antigens capable of inducing gamma interferon in sensitized lymphocytes in vitro include bacterial antigens, purified protein derivative (PPD), tetanus toxoid, and diphtheria toxoid. Viral antigens from vaccinia, herpes simplex, and varicella zoster are also able to induce immune interferon.

In addition to herpes simplex antigen, herpes simplex virus-antibody complexes can stimulate interferon production (Fujibayashi, etal, 1975). Thus, interferon may still be induced in sensitized cells, even if viral antigenic sites are masked by antibody.

GENETICS

Interferon sensitivity is dependent on human chromosome 21. Tan (1973), studied mouse-human hybrids, and found that the presence of human chromosome 21 enabled the hybrids to respond to the antiviral effects of human interferon. If human chromosome 21 was lost from the hybrid, the response to interferon was also lost.

The response to interferon varies in cells containing different numbers of chromosome 21. Cells from humans with trisomy 21 are more responsive to exogenous interferon than cells from normal individuals, and cells monosomic for chromosome 21 are less responsive (Tan, etal, 1974, Chany, 1975, De Clercq, etal, 1975)

The gene responsible for expression of antiviral effects of interferon (AVG) was localized to the distal portion of the long arm of chromosome 21 by Epstein and Epstein (1976). Normal fibroblasts and fibroblasts monosomic and trisomic for chromosome 21 were incubated with interferon. Cells with trisomy 21

demonstrated an antiviral effect three times that of normal cells. Monosomic cells had an antiviral response one-fifth that of normal cells. A cell line trisomic only for the distal half of the long arm of chromosome 21 permitted the more precise localization of AVG.

Interferon production is determined by human chromosome 5 (Tan, 1977), as shown by studies on human-Chinese hamster hybrid populations.

Leukocyte and fibroblast interferon share many similarities. They are both glycoproteins with similar molecular weights. They both show immunomodulatory activity, can activate natural killer cells, have promising antiviral and antitumor activities, and are sufficiently similar to be recognized by the same receptor (Paucker, 1977). Their syntheses appear to be under similar control. However, several differences suggest they are encoded for by different genes, including different target cell specificities, different dose-response curves for antiviral activity, and different degrees of growth inhibitory action. Antibodies directed against leukocyte interferon do not neutralize fibroblast interferon, and vice versa.

Nucleotide and amino acid analysis of the cloned genes of human leukocyte interferon and human fibroblast interferon show significant homologies (Taniguchi, etal, 1980). Homology between these two genes was 45% at the nucleotide level and 29% at the amino acid level, strongly suggesting derivation from a common ancestral gene.

Recombinant DNA technology has permitted cloning of the human fibroblast interferon gene. (Derynck, etal, 1980) Isolation and sequencing of the gene has allowed the amino acid sequence to be deduced. The protein contains 166 amino acids and is preceded by a 21 amino acid signal sequence. As interferon is a secretory protein, this sequence probably represents a signal peptide, cleaved off as or after the nascent protein crosses the endoplasmic reticulum. While mature fibroblast interferon is a glycoprotein, precise description of the carbohydrate moieties on the polypeptide has not been achieved.

SPECIES SPECIFICITY

An interesting aspect of interferon biology is its species specificity. Though species specificity exists to a relatively high degree, it is not complete. For instance, one form of human leukocyte interferon is equally active on human and rabbit cells (Stewart and Desmyter, 1975).

Some components of the interferon system are not species specific. Cells rendered resistant to viral infection by homologous interferon can transfer resistance to heterologous cells insensitive to that interferon. Blalock and Baron (1977) cocultivated human amnion and baby hamster kidney cells with mouse L cells in the presence or absence of mouse interferon. When challenged with vesicular stomatitis virus (VSV), transfer

of viral resistance to the human and hamster cells was observed, presumably by a product of the interferon treated mouse cells.

Transfer of viral resistance was also observed between cells of the same species. Interferon treated L cells were washed free of interferon, then treated with excess antiserum to mouse interferon. Untreated L cells were added , and viral resistance was transferred to the newly added, untreated cells. (Blalock and Baron, 1977).

Isolation of the factor(s) responsible for this intercellular transfer of viral resistance will be an important step in understanding amplification of the interferon system.

Bourgeade (1974) examined the role of cell membrane receptors in the species specificity of interferon. Receptors for monkey and mouse interferon are distinct in somatic monkey-mouse hybrid cells. The two types of receptors exhibited differential sensitivity to trypsin. By examining the antiviral response of the hybrid cells to both monkey and mouse interferon, Bourgeade concludes that only the interferon membrane receptors govern the cell species specificity of the different biological effects of interferon.

Carter (1979) examined the species specificity of interferon, and found carbohydrate-altered leukocyte interferon could exert effects on heterologous cells. Porcine and bovine carbohydrate-altered interferons from leukocytes demonstrated increased efficacy in protecting human cells from viral challenge. Carter proposes that, as a glycoprotein, interferon's

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ability to cross or not cross species lines lies in the carbohydrate moiety, and cross species biological activity is a property of the polypeptide. If Carter's theory is correct, carbohydrate-altered animal interferons could conceivably be effective in experimental systems using human cells. This would be a tremendously useful contribution to interferon research.

ASSAY OF INTERFERON

Interferon has a very high specific activity, greater than one billion units per milligram of protein. One unit of interferon is defined as equal to the amount required to reduce viral yield by 50%.

The assay methods used to measure interferon's antiviral activity are many, and have been reviewed by Grossberg (1979). They can be summarized as 1) inhibition of generalized cell specific cytopathic effects in a monolayer of cells, 2) reduction in the number and size of viral plaques, 3) reduction in yield of either infectious virus or viral hemagglutinin, 4) reduction in yield of a virion-associated structural enzyme, 5) reduction in quantitative hemadsorption, 6) changes in relative cellular metabolism, 7) activity of cells surviving virus-induced cytolysis to take up a vital dye, and 8) inhibition of RNA synthesis.

Interferon plays an important role in recovery from viral infections. Interferon appears early in the course of a viral infection, with rapidly rising titers within 24 hours of infection. Interferon limits viral replication at the portal of entry, reduces viremia and subsequent dissemination, and protects target organs against infection. Its appearance preceeds formation of antibodies. This has been demonstrated by numerous studies in both animals and man (Baron, 1973).

Anti-interferon antibodies have been employed to examine the extent to which interferon plays a role in fighting viral infection. When anti-interferon antibodies were injected simultaneously with virus, death occurred earlier than in animals injected with virus alone (Fauconnier, 1970). Virus-infected mice treated with sheep anti-mouse interferon serum demonstrated severly limited ability to fight the infection (Gresser, etal, 1976a). Higher titers of virus were found in visceral organs, onset of disease was accelerated, and death occurred earlier in animals receiving the anti-serum.

Other evidence dramatically illustrates the importance of interferon in combating viral infection (Gresser, etal, 1976b). Mice were given herpes simplex virus (HSV) type I subcutaneously. Anti-mouse interferon globulin treatment resulted in death in all mice. Only 5% of the virus-infected control mice died.

Virus-induced tumors are also affected by interferon

(Gresser, 1977). In mice treated with anti-interferon globulin, MSV-induced tumors appeared earlier, in a greater proportion of mice, and were larger in size (Gresser, etal, 1976b). The development of Friend leukemia is inhibited by interferon (Gresser, etal, 1967). Interferon treatment also decreased the incidence of lymphoid leukemia and significantly increased the survival time of AKR mice, who develop spontaneous virus-induced leukemia (Gresser, etal, 1969b). Interferon plays an important role in the prevention of virus-induced tumors.

ANTICELLULAR ACTIVITY

The anticellular effect of interferon has been established by many investigators, and has attracted several explanations. The inhibition of cell growth by interferon could result from 1) inhibition of tumor virus replication and reduction of viral cell transformation, 2) direct inhibitory effects of interferon on cell growth, 3) growth inhibition mediated by interferon-induced changes in the immune system, or a combination of these effects (Friedman, 1978).

The first evidence of interferon anticellular activity was gathered by Paucker, Cantell, and Henle (1962), who found that crude interferon preparations inhibited the multiplication of mouse L cells in suspension cultures.

The growth of several transplantable tumors in mice was

inhibited by repeated injections of interferon preparations (Gresser, etal, 1969a, 1970a). Interferon-treated mice had increased survival, and were often without obvious sign of tumor.

Interferon treatment of mouse leukemia L1210 cells in vitro significantly decreased their subsequent oncogenicity in DBA/2 mice and their colony forming capacity in agarose (Gresser, etal, 1971a).

Interferon preparations can also inhibit the growth of normal cells. Lindahl-Magnusson (1971) showed that interferon inhibited the growth of mouse embryo and weanling mouse kidney cells. Interferon inhibited the regeneration of the liver in partially hepatectomized mice (Frayssinet, etal, 1973). Newborn mice treated with interferon exhibited weight loss and diffise hepatic cell degeneration (Gresser, etal, 1975). Drasner and coworkers (1971) studied the antiproliferative effects of interferon on mouse embryo development, and found responsiveness to interferon was achieved in mouse embryos by 10 days. Interestingly, this stage corresponds developmentally with the time of maximal sensitivity to teratogenic effects of congenital viral infections in human embryos.

Gresser and coworkers have examined the effect of interferon on the growth of L1210 cells in vitro. Interferon inhibits the growth of L1210 cells; inhibition was directly proportional to interferon titer (Gresser, etal, 1970a, 1970b). Inhibition was evident at 24 hours, but was more pronounced at 48 hours.

As interferon purification methods became more refined, it

became apparent that interferon itself was responsible for the anticellular effects observed (Gresser, etal, 1972a).

Studies of macromolecular synthesis showed that growth inhibition of L1210 cells by interferon was accompanied by an inhibition of total RNA and protein synthesis, and inhibition of polyribosome formation (Brouty-Boye, etal, 1973). No effect on the formation of free ribosomal subunits was observed.

Examination of the cell cycle suggests that interferon decreases the doubling potential of each L1210 cell (Macieira-Coelho, etal, 1971).

Study of interferon's antitumor activity showed noncycling tumor cells to be more sensitive to the antiproliferative effects of interferon than cells rapidly multiplying (Horoszewicz, etal, 1979). Enhanced interferon activity might be seen when combined with drugs that impair cell-cycling activity, resulting in a population with a higher proportion of resting tumor cells. Several studies have shown interferon to be of particular benefit in combination with other antitumor drugs (Gresser, etal, 1978, Chirigos and Pearson, 1973). Chemotherapy and interferon in combination may be superior to either drug alone.

The presence of a membrane-bound receptor system for interferon has been established for some time. It plays an important role in the expression of the antiviral effects of interferon. Binding of interferon to specific cell surface receptors is required for its antiviral action (Friedman, 1967). Evidence for the presence of an interferon receptor system comes from a variety of studies.

Ouabain, which blocks membrane bound ATPase, inhibits the antiviral action of interferon without effect on its synthesis or release from the cell (Lebon, etal, 1975), implying a membrane-bound free energy requirement for interferon action. Similar inhibition occurs when interferon-producing cells are treated with specific antisera to interferon (Vengris, etal, 1975). This suggests interferon must interact with the external cell membrane is required for its antiviral action.

Interferon covelently bound to Sepharose is capable of inducing an antiviral state (Ankel, etal, 1973). The Sepharose beads used had diameters several times those of the cells, which precluded internalization of the interferon-Sepharose construct. Increasing concentrations of interferon linked to a fixed number of beads yielded a sigmoidal dose-response curve with a sharp vertical slope. This curve suggests that a critical concentration of interferon molecules in contact with the receptors is needed to induce the antiviral state, which is thus

a cooperative process.

Bourgeade and Chany (1976) postulated that cooperative activation could involve individual receptors or a number of them, and that the mobility of these receptors could be important in cellular response to interferon. Cytochalasin B, colchicine, and vinblastine are known to disrupt microtubule and microfiliment systems, which have been associated with mobility of membrane bound proteins. When these substances were given simultaneously with interferon, they inhibited the antiviral response to interferon, while having no effect on RNA or protein synthesis. Integrity of the cytoskeletal components of the cell is necessary for activation.

Interferon receptors contain both a glycoprotein and a ganglioside or ganglioside-like component. An oligosaccharide moiety of this ganglioside or ganglioside-like component is a critical feature for receptor function (Besancon, etal, 1976). Human KB-3 cells, unresponsive to interferon, have nearly absent levels of membrane gangliosides, especially G_{M2} and G_{M3}; these gangliosides are present in high levels in membranes of sensitive mouse L cells.

Binding and activation of antiviral mechanisms seem to be independent events. Binding of interferon to a ganglioside or ganglioside-like receptor is insufficient to establish an antiviral state. Human KB-3 cells, which are unresponsive to both mouse and human interferon, can bind both (Kohn, etal, 1976).

Cholera toxin and TSH also interact with cell surface receptors believed to contain a ganglioside as a functional component (Kohn, 1977). Recent experiments show that cholera toxin and TSH inhibit the antiviral action of interferon (Kohn, etal, 1976). This inhibition is related to action of these agents on the plasma membrane.

Grollman and coworkers (1978) examined the effect of interferon on certain membrane characteristics, especially ion fluxes. Interferon causes an increase in the interior free calcium ion content of the cell by releasing calcium ions from a membrane-bound state. Interferon interaction with the membrane also induces a rapid efflux of protons from the cell and alter sodium and glycine uptake into the cell. A change in intracellular pH and an increase in net outside-to-inside Na^+ flux result. These both influence the electrical potential across the membrane. These membrane effects are specific, being absent in interferon-resistant cells, and are not induced by other agents binding the cell (cholera toxin or TSH). The relationship of these specific interferon-induced membrane changes and the establishment of the antiviral state is still unknown, and continues to be the subject of intense investigation.

The antiviral activity of interferon can be blocked by inhibition of RNA or protein synthesis, suggesting that the antiviral state is dependant on expression of certain genetic information.

Binding of interferon to receptors triggers the synthesis of at least three proteins which act to prevent viral replication. These proteins remain inactive until viral infection or exposure to double-stranded RNA occurs. This requirement may be protective, sparing host protein and nucleic acid synthesis in the absence of viral infection.

The three proteins known to be produced in response to interferon include a protein kinase, an oligonucleotide synthetase, and an endonuclease activated by that oligonucleotide. The oligonucleotide formed, known as 2-5A, is believed to mediate some of the antiviral and anticellular effects of interferon. 2-5A is discussed in greater detail in a later section of this thesis.

INTERFERON RESISTANCE

A very useful development in the study of interferon systems has been the discovery of L1210 cells resistant to the antiviral and growth inhibitory effects of interferon.

These interferon resistant cells (L1210 R) were found among wild or cloned populations of interferon sensitive L1210 cells (L1210 S) at a rate of 0.004 - 1% (Gresser, etal, 1974) It seems that a small number of these cells are present in populations of sensitive cells. Treatment of the parent population with interferon selects for resistant cells. Resistant cells grow

exponentially in the presence of interferon, and an interferon-resistant cell population results.

Fluctuation analysis tests show this interferon resistance results from spontaneous random change rather than induction from interferon (Gresser, etal, 1974).

The mechanism of interferon resistance was investigated vigorously. No intracellular transfer of resistance could be demonstrated. L1210 S and L1210 R cells were cultivated together in suspension culture for six weeks, and had the same ratio of sensitive to resistant cells as when mixed initially. The generation times of both interferon sensitive and resistant cells were shown to be identical. Ultrasonic extracts of sensitive or resistant cells were unable to confer sensitivity or resistance when incubated with resistant or sensitive cells. No evidence was found to suggest the release of an interferon-inactivating factor by L1210 R cells.

Interferon resistant cells are resistant to both the antiviral and anticellular effects of interferon. It is still unknown if the fundamental change is a gene mutation or a stable shift in phenotypic expression. Sensitivity to interferon appears to be the only difference detectable between these cell lines. L1210 S and L1210 R cells have the same generation time, the same chromosome pattern, the same colony-forming efficiency in agarose, and the same tumorogenicity in DBA/2 mice.

Interferon was recovered from extracts of interferon-treated L1210 S cells, but was not found in extracts of interferon-

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treated L1210 R cells. The relevance of this finding to interferon resistance is unclear. However, other investigators (Stewart, etal, 1972) have demonstrated a correlation between recovery of interferon from various types of mouse cells and their sensitivity to the antiviral effects of interferon.

Relative resistance to interferon has been noted in transformed cells. Brouty-Boye and colleagues (1979) transformed C3H/10T1/2 cells in vitro using x-irradiation or methylcholanthrene treatment. The transformed cells were found to be 10 - 20 times less sensitive to the antiviral effects of interferon. A significant decrease in sensitivity to the anticellular effects of interferon was also noted.

Earlier studies showed that in vitro transformation of cells by viruses (Bravilovsky, etal, 1969) or by chemical carcinogens (Rotem, etal, 1964) results in decreased sensitivity to interferon when compared to control, untransformed cells.

Examination of sensitivity of several human cell lines to interferon showed that established aneuploid cell lines were much less sensitive to interferon than diploid cell lines (Moehring and Stinebring, 1973). However, other studies suggest that decreased sensitivity to interferon is not a characteristic of transformed cells. Freeman and colleagues (1970) examined normal rat embryo cells and found a wide variation in interferon sensitivity. They found no correlation between interferon sensitivity and transformation, leukemia virus infection or pretreatment with carcinogens.

Cell surface changes are common in transformed cells. Whether cell surface modification was associated with this particular transformation, and whether there is any role of cell surface changes in interferon sensitivity was not established.

In vivo studies show interferon can protect mice inoculated with interferon resistant L1210 R tumor cells (Gresser, etal, 1972), suggesting host-mediated inhibition of tumor growth may be triggered by interferon. Interferon may still be of value in vivo against tumor cells found to be resistant to its effects in vitro.

Recent investigations with L1210 R cells have shown the antiviral effects of interferon to be decreased in the presence of fatty acid cyclooxygenase inhibitors (Pottathil, etal, 1980). Several anti-inflammatory drugs with known fatty acid cyclooxygenase inhibiting activity were used. The prevention of development of interferon antiviral protection correlated with the degree as well as the duration of cyclooxygenase inhibition for the various inhibitors tested. Other anti-inflammatory compounds which lack cyclooxygenase inhibiting activity did not affect antiviral protection by interferon. Exogenous prostaglandins did not replace the need for functional cyclooxygenase. In some cases, addition of exogenous prostaglandin actually diminished the antiviral effect of interferon.

L1210 R cells were subsequently found to lack any fatty acid cyclooxygenase activity (Chandrabose, etal, 1981). A diffusible

enzyme inhibitor could not be demonstrated. Fatty acid lipoxygenase activity was found to be similar in both interferon sensitive and resistant strains of L1210 cells. The significance of fatty acid cyclooxygenase activity to the expression of interferon sensitivity is not yet fully understood.

Further research must be conducted to completely understand the mechanisms of interferon resistance.

2-5A

The mechanisms of interferon action have been the subject of intense investigation. One result of this research has been the isolation of a group of 2'-5' linked oligoadenylic acid triphosphate inhibitors, known collectively as 2-5A. 2-5A is believed to play a crucial role in the induction of interferon action.

2-5A inhibits cell-free protein synthesis. It is synthesized by an enzyme activated in interferon-treated cells in response to double-stranded RNA (Hovanessian, etal, 1977, Kerr and Brown, 1978). This enzyme, 2-5A synthetase, utilizes ATP as its substrate.

Treatment of mouse L cells with 2-5A resulted in inhibition of protein, RNA, and DNA synthesis. While these inhibitory effects are transitory at low 2-5A concentrations (<20 nM), higher concentrations of 2-5A inhibit protein, RNA, and DNA

synthesis sufficiently to measurably affect cell growth. Protein synthesis is noticeably inhibited after 30 minutes of incubation with 2-5A, before any effect on RNA synthesis is appreciated, suggesting independent mechanisms for protein and RNA inhibition by 2-5A. DNA synthesis is inhibited by 2-5A in a dose-dependent fashion 24 hours after treatment.

Antiviral effects are also evident with 2-5A. Cells infected with vesicular stomatitis virus (VSV) show inhibition of viral RNA replication when treated with 2-5A (Hovanessian and Wood, 1980). Virus production is inhibited by 90-100% after treatment of infected cells with 10-100 nM of 2-5A. Antiviral activity seems to be mediated by a 2-5A dependent nuclease which degrades viral RNA.

The 2-5A system may mediate the antiviral and anticellular effects of interferon.

Interferon treatment enhances the levels of 2-5A synthetase activity. In the presence of double-stranded RNA and ATP, 2-5A is formed. 2-5A, in turn, activates a nuclease which degrades both polyadenylated and ribosomal RNA (Hovanessian, etal, 1979). Extracts from cells treated with 2-5A show enhanced nuclease activity in vitro when incubated with exogenous RNA (Williams, etal, 1979). This nuclease activity is transient, and the cell survives after the period when mRNA species are degraded. This provides an effective mechanism for eliminating undesired messages.

The effect of 2-5A on intact cells is dependent on its

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penetration into cells. Treatment of cells with hypertonic medium or by adsorption of 2-5A onto the cell membrane will facilitate penetration into cells. Several investigators have obtained good results with a calcium chloride coprecipitation technique (Hovanessian, etal, 1979).

Preliminary studies indicate viral RNA synthesis can be reduced to background levels after 2-5A treatment. This suggests that the 2-5A activated nuclease may mediate the antiviral effects of interferon.

Lengyel and coworkers have isolated 2-5A synthetase from mouse Ehrlich ascites tumor cells treated with mouse interferon (Dougherty, etal, 1980). They have studied the enzyme and its activity extensively, and have described the kinetics, stoichiometry, and equilibrium of the 2-5A synthetase reaction in detail (Samanta, etal, 1980).

Kimchi and colleagues (1981) studied the antimitogenic effect of 2-5A on mouse spleen lymphocytes stimulated with Concanavalin A (ConA). Mitogens increased the intracellular levels of 2'-phosphodiesterase, which degrades 2-5A. Ratios of 2-5A synthetase to 2'-phosphodiesterase were examined. They propose a model of synthesis and degradation of 2-5A as a regulator of cell growth in the presence of interferon and mitogens. The ratio of 2'-phosphodiesterase to 2-5A synthetase was ten times higher in fast growing monkey kidney cells than in serum-starved, quiescent cells arrested in G_0. They propose that the growth-dependent changes in 2'-phosphodiesterase activity

regulate the intracellular level of 2-5A, affecting cell proliferation. The model assumes constant production of 2-5A with rapid degradation by phosphodiesterase preventing accumulation and consequent growth inhibition.

Cells of all vertebrates have a basal level of 2-5A synthetase activity (Stark, etal, 1979), which probably facilitates constant synthesis of 2-5A.

Interferon specifically induces synthesis of 2-5A synthetase at the transcriptional level, increasing the enzyme level 10 to 100-fold. Interferon treatment also increases 2'-phosphodiesterase levels 3 to 4-fold (Schmidt, etal, 1979). The precise mechanisms controlling the variation of 2'-phosphodiesterase levels with rate of cell growth are not clear. The ratio of synthetase to phosphodiesterase remains much higher in interferon-treated cells than in untreated cells. Net synthesis of 2-5A results in inhibition of protein, RNA, and DNA synthesis, and ultimately cell growth. Mitogens reverse this enzymatic relationship, and phosphodiesterase activity exceeds synthetase activity, leading to net degradation of 2-5A. The inhibitory effects of 2-5A diminish, with accelerated cell growth the final result.

This model for the role of 2-5A in the control of cell proliferation is attractive, and further research is being conducted to substantiate it.

LIPOSOME ENCAPSULATION OF INTERFERON

Liposomes have proven to be powerful tools, especially as carriers of biologically active agents. Their ability to prolong the half-life of entrapped species in vivo, to reduce the immunogenicity of entrapped species, and the developing ability to target liposomes to specific anatomic and intracellular sites make liposomes ideal carriers with enormous potential. The growing interest in interferon and its antiviral and anticellular effects make it an attractive candidate for liposome encapsulation.

The advantages of liposome encapsulation seem well suited for interferon. Rapid in vivo clearance and degradation of interferon necessitate large and repeated doses of interferon. Encapsulation of interferon in liposomes would conceivably increase its in vivo activity.

LaBonnadiere (1977) reported the association of mouse interferon with liposomes. Interferon was associated with liposomes composed of phosphatidylcholine, stearylamine or dicetyl phosphate, and cholesterol in a 7:2:1 molar ratio. Both sonicated and unsonicated liposomes were prepared. Sonication inactivated a large proportion of interferon activity, but the liposomes were associated with 9.6% (anionic) and 20% (cationic) of initial interferon activity. The unsonicated liposomes were associated with appreciable amounts of initial interferon

activity: 18% was associated with anionic liposomes.

Liposome encapsulated interferon preparations were treated with trypsin. Both anionic and cationic liposomes contained a fraction of interferon which was simultaneously resistant to trypsin and still biologically active in cell culture. This fraction represented 4% of the total interferon activity in anionic liposomes, and 5% of the total in cationic ones.

Triton X-100, a detergent, has been widely used to demonstrate the latency of various molecules in liposomes. Its action on liposome associated interferon induced release of interferon from liposomes. The interferon activity so released was probably located internally. Multilamellar vesicles contain a large proportion of entrapped species in their internal aqueous spaces.

Liposome encapsulated interferon was then used in its first known in vivo study. Liposome encapsulation enhanced the antiviral effect of interferon on mice infected with hepatitis virus (LaBonnadiere, 1978). This was presumed to be a result of the greater accumulation of the liposomes in the liver and the spleen.

Anderson and colleagues (1981) successfully demonstrated entrapment of human leukocyte interferon in multilamellar liposomes. Liposomes were created from phosphatidylcholine, dicetyl phosphate, and cholesterol (7:2:1). The encapsulated interferon was resistant to inactivation by trypsin, which completely inactivated free interferon. A trapping efficiency of

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11.0 $\pm$ 1.9% is reported. Little evidence was found for significant electrostatic or hydrophobic interactions with the outer bilayer. Evidence suggested that liposomes capture interferon by passive entrapment.

The association of interferon with liposomes probably modifies its intrinsic antiviral and anticellular activity, since liposome-cell interactions are the dominant interaction. Any molecules bound to the liposomes will be dependent on the liposome-cell interactions.

Liposome association of interferon could increase its concentration in the vicinity of membrane bound receptors. Such association might allow a given amount of interferon to have a higher intrinsic activity.

Liposome encapsulation of interferon could also allow detailed study of responses to interferon presented intracellularly. Many details of the membrane bound receptor system for interferon have been elucidated. However, it is not known if an intracellular receptor system for interferon exists. Other mechanisms might be available to allow interferon to express its antiviral and anticellular activity. The ability to introduce interferon directly into cells via liposomes will help elucidate the mechanism of action of interferon at steps distal to its receptor binding.

Liposomes designed for fusion with cells would allow introduction of interferon into the cytoplasm. However, if endocytosis were significant, liposome encapsulated interferon

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would be at greater risk for degradation by lysosomal enzymes. If the endocytic vacuole did not fuse with lysosomes, its contents could be either released slowly into the cytoplasm, or secreted back into the extracellular environment.

Liposome encapsulation of interferon would prove an interesting subject for in vivo studies. The half-life of free interferon in plasma is known to be very short. Liposome encapsulated interferon should have a plasma half-life far greater than the free species. The other advantages of liposome encapsulation would also be conferred to interferon for in vivo investigations.

EXPERIMENTS

The experiments for this project were conducted in two phases. Phase I was conducted in the laboratory of Dr. Lois B. Epstein of the University of California San Francisco Cancer Research Institute, during the summer of 1980. Research could not be continued in Dr. Epstein's laboratory due to the need to return to New Haven. The remainder of experiments, Phase II, was conducted in the laboratory of Dr. Thomas R. Tritton of the Department of Pharmacology, Yale University School of Medicine, in close collaboration with Dr. Joseph R. Bertino, Department of Pharmacology and Medicine, and Dr. Peter Lengyel of the Department of Molecular Biology and Biophysics.

DESCRIPTION

Liposome Encapsulated Interferon and Free Interferon

Free interferon is known to exert antiviral and anticellular effects. Gresser has studied the anticellular effects of interferon on the growth of L1210 cells in depth (Gresser, etal, 1970b, 1970c, 1972a, Macieira-Coelho, 1971). It is of interest to see if and how liposome encapsulation affects the anticellular effects of interferon.

L1210 cells were chosen for study because of extensive literature discussing the effect of interferon on their growth. They are easy to grow in suspension cultures and can be readily

counted with cell counting machines.

Jansons (1978) studied the in vitro interaction of L1210 cells with liposomes. Uptake of liposomes by cells was temperature dependent, and greater in young cultures than in stationary cultures. Uptake of positively charged liposomes by cells was significantly greater than that of either neutral or negatively charged vesicles. However, about one-half of the cell-associated radioactivity seen with positively charged liposomes could be removed by cell surface treatment with trypsin, neuraminidase, or high-ionic-strength treatment, suggesting a significant degree of adsorption of liposomes to the cell surface.

To maximize the cellular uptake of liposomes, a positive charge was given to the liposomes used (Phase II only). Liposomes were made using phosphatidylcholine, cholesterol, and stearylamine in a 4:3:1 molar ratio. Use of a charged liposome also enhances the capture rate of the entrapped species.

Unsonicated multilamellar liposomes were used to encapsulate interferon. This type of vesicle is associated with an increased rate of entrapment. Experience with this vesicle in encapsulation of interferon (LaBonnadiere, 1977, Anderson, etal, 1981) also made its use desirable.

Initially, both cell growth and DNA synthesis were to be measured, the latter by examining the incorporation of ^{3}H-thymidine. However, Brouty-Boye and Tovey (1978) found that, although interferon reduced ^{3}H-thymidine uptake, the uptake of

^{3}H-deoxyadenosine and ^{3}H-deoxy-D-glucose was not inhibited. They conclude that reduced ^{3}H-thymidine incorporation does not reflect a decrease in DNA synthesis, but rather a change in exogenous nucleoside utilization. Accordingly, measurement of DNA synthesis by ^{3}H-thymidine incorporation was not pursued.

Liposome Encapsulated Interferon and Interferon Resistance

Interferon resistant L1210 cells (L1210 R) were discovered and intensely studied by Gresser (1974). The actual mechanisms of interferon resistance have remained elusive. Though deficiency in the ganglioside content of interferon membrane receptors (Besancon, etal, 1976) and lack of fatty acid cyclooxygenase activity (Chandrabose, 1981) are differences identified in interferon resistant cells, their precise role in interferon resistance is unknown. The fundamental differences between sensitive and resistant cells are yet undefined.

If interferon acts through a membrane bound receptor system, this system seems an appropriate place to search for differences between interferon sensitive and resistant cells. Differences in the ganglioside content of receptors have been noted (Besancon, etal, 1976).

Liposome encapsulation of drugs has been used to overcome drug resistance. Papahadjopoulos and colleagues (1976) used liposomes to introduce actinomycin D into resistant tumor cells. Drug resistance in those cells was due to a decreased membrane permeability to the drug, with reduced ability to incorporate

actinomycin D intracellularly. Liposome encapsulation allowed the drug to be introduced intracellularly, with marked inhibition of RNA synthesis and cell growth at concentrations that had little or no effect extracellularly. Liposome encapsulation was clearly able to overcome drug resistance due to decreased membrane permeability.

Because the precise mechanisms of resistance to interferon in Ll210 R cells are not known, the success of liposome encapsulation of interferon in overcoming interferon resistance cannot be predicted. However, liposome encapsulated interferon could be a useful tool in studying the mechanism of interferon resistance in these cells.

Liposome Encapsulation of 2-5A

The oligonucleotide 2-5A, believed to be a mediator of the antiviral and anticellular effects of interferon, cannot penetrate intact plasma membranes. It exerts its effects intracellularly, so that entry into the cell is essential for its activity. Entry of 2-5A intracellularly has been facilitated by permeabilizing cells in hypertonic medium, and with a calcium phosphate coprecipitation technique (Hovanessian, etal, 1979).

Since liposomes have been employed successfully to introduce a variety of agents into cells, including nucleotides (Papahadjopoulos, etal, 1974), it is reasonable to attempt to use liposomes to facilitate entry of 2-5A into cells, allowing

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expression of its anticellular effects.

Preliminary experiments were conducted to estimate the encapsulation rate of 2-5A. Since 2-5 A is expensive and difficult to obtain, ^{3}H-ATP was used to estimate encapsulation rate because of similarity in structure to the oligonucleotide. Its relatively low cost and ready availability were also factors favoring its use in estimation of 2-5A encapsulation rate. It was felt that 2-5A would exhibit liposome encapsulation behavior similar to that of ^{3}H-ATP. An encapsulation rate of 1.6% in sonicated liposomes was estimated.

Study of permeabilized cells was considered, to compare liposome encapsulation of 2-5A with previously employed methods of introducing 2-5A into cells. However, such comparison was not pursued. If liposome encapsulation successfully introduces 2-5A into cells, a decrease in the growth rate of cells would presumably reflect the intracellular action of 2-5A.

MATERIALS AND METHODS

Phase I (UCSF):

Interferon

Purified mouse interferon was kindly provided by Dr. Kurt Paucker. Interferon was added to cell cultures at final concentrations of 0, 10, 30, 100, 300, and 1000 u/ml.

Cells

L1210 cells were obtained from UCSF Cell Culture Facility frozen stock. Cell were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum (Gibco), with 1% penicillin/streptomycin at 37' C in a 5% CO_2 humidified atmosphere. Experiments were conducted in plastic microwell dishes (Falcon). Cells were counted with a hemocytometer using the Trypan blue dye exclusion test for cell viability.

Cell concentrations were measured after 48 hours of incubation, as anticellular effects of interferon are more pronounced at this time (Gresser, etal, 1970b).

L1210 S and L1210 R cells were kindly provided by Dr. Ion Gresser. However, an unfortunate mycoplasma contamination precluded meaningful studies of these cells during Phase I.

Liposomes

Liposomes were prepared by the reverse evaporation phase (REV) method (Szoka and Papahadjopoulos, 1978) using phosphatidylglycerol and cholesterol in a 1:1 molar ratio. Interferon for liposome encapsulation was added to the lipid mixture in a concentration of 25,000 u/ml in TES/Histidine/NaCl buffer pH 7.4. Separation of free from encapsulated interferon was achieved by centrifugation in a Ficoll-Hypaque gradient.

The concentration of liposome encapsulated interferon is reported as the estimated interferon concentration of the preparation. This was estimated by preliminary assay of liposome

encapsulated interferon activity by a virus plaque reduction method (Epstein, 1976). The concentration of empty liposomes is reported as the dilution of the original liposome preparation added to cell cultures. The lipid concentration of the 1:1 empty liposome preparation is equivalent to the lipid concentration of the 1000 u/ml liposome encapsulated interferon preparation.

Phase II (Yale):

Interferon

Purified mouse interferon was kindly provided by Dr. Peter Lengyel. Interferon was added to cells at final concentrations of 0, 5, 50, and 500 u/ml.

Cells

L1210 S and L1210 R cells were kindly provided by Dr. Peter Lengyel. Four cell lines were maintained: RF 21 and SWA were interferon sensitive, and RF 3 and RMI were interferon resistant. These lines were assayed for sensitivity to the anticellular effects of interferon, and their reported interferon sensitivities were confirmed. Cells originated from the laboratory of Dr. LeBleu.

Cells were grown in Fischer's medium supplemented with 10% heat-inactivated horse serum (Gibco) at 37' C in a 5% CO_2 humidified atmosphere.

Cells were counted on a Coulter Counter.

2-5A

Purified 2-5A (oligonucleotide of composition $pppA(2'p5'A)_n$ was kindly provided by Dr. Peter Lengyel.

Liposomes

Liposomes were prepared from phosphatidylcholine, cholesterol (both from Sigma Pharmaceuticals) and stearylamine (ICN K&K Laboratories) in a 4:3:1 molar ratio. Lipids were dissolved in chloroform and dried to a thin film under nitrogen. Purified mouse interferon, 2-5A or control buffer was added to the lipid film. Mechanical agitation (5 minutes on a Vortex mixer) was then applied, resulting in the formation of multilamellar vesicles.

Sonicated unilamellar liposomes were prepared for encapsulation of 2-5A. After mechanical agitation, as described above, the liposome preparation was sonicated for 2 hours in a bath type sonicator (Laboratory Supplies Co., Inc.).

Separation of free from encapsulated species was achieved by passing the mixture through Sephadex G-50 in a 1.5 x 25 cm BioRad column. Fractions were measured and collected by an LKB fraction collector. The elution profile of liposomes was determined by producing ^{14}C-phosphatidylcholine:cholesterol:stearylamine (4:3:1) liposomes and measuring radioactivity of sequential fractions.

The liposome preparation was added to cells at a concentration of 0.2 mg lipid per million cells. 1:10 and 1:100 dilutions of the liposome preparation were also added to cells.

The concentration of interferon in the liposome encapsulated preparation was estimated to be 500 u/ml, based on encapsulation rates of interferon in multilamellar vesicles as reported by Anderson and colleagues (1981). 1:10 and 1:100 dilutions of liposome encapsulated interferon, with estimated interferon concentrations of 50 and 5 u/ml, were also incubated with cells.

The encapsulation rate of 2-5A was estimated by encapsulating ^{3}H-ATP in ^{14}C-phosphatidylcholine:cholesterol:stearylamine liposomes (4:3:1) and measuring radioactivity of sequential fractions. Based on an encapsulation rate of 1.6% in sonicated unilamellar vesicles, the concentration of 2-5A in the liposome encapsulated 2-5A preparation was estimated to be 100 nM.

DATA

Phase I (UCSF)

Table 1

Effect of Liposome Encapsulation of Interferon on the Growth of
Ll210 Cells
(First Trial)

(cell concentration x 10^6 cells/ml)

Cell concentration at 0 hrs, 0.232

Condition		Cell Concentration at 48 hours	% control
Control		2.925	100.00
Empty Liposomes	1:1	2.797	95.62
	1:3.33	2.770	94.70
	1:10	2.805	95.90
	1:33.33	3.040	103.93
	1:100	2.780	95.04
Free Interferon	1000 u/ml	1.485	50.77
	300 u/ml	1.668	57.03
	100 u/ml	1.948	66.60
	30 u/ml	2.573	87.97
	10 u/ml	2.335	79.83
Liposome	1000 u/ml	2.600	88.89
Encapsulated	300 u/ml	2.678	91.56
Interferon	100 u/ml	2.913	99.59
	30 u/ml	2.775	94.87
	10 u/ml	2.018	68.99

Table 2

Effect of Liposome Encapsulated Interferon on the Growth of
L1210 Cells
(Second Trial)

(cell concentration x 10^6 cells/ml)

Cell Concentration at 0 hours 0.282

Condition		Cell Concentration at 48 hours	% control
Control		3.090	100.00
Empty Liposomes	1:1	2.170	70.23
	1:3.33	2.955	95.63
	1:10	2.450	79.29
	1:33.33	2.695	87.22
	1:100	3.278	106.08
Free Interferon	1000 u/ml	1.660	53.72
	300 u/ml	1.962	63.50
	100 u/ml	2.120	68.61
	30 u/ml	2.582	83.56
	10 u/ml	2.250	72.82
Liposome	1000 u/ml	0.760	24.60
Encapsulated	300 u/ml	2.038	65.95
Interferon	100 u/ml	2.498	80.84
	30 u/ml	2.628	85.05
	10 u/ml	2.065	66.88

Table 3

Effect of Liposome Encapsulated Interferon on the Growth
of L1210 S and L1210 R cells.

L1210 Sensitive Cells
(control - cell concentration x 10^5 cells/ml)
(other conditions - % of control concentration)

Condition	0 hr	24 hr	48 hr	72 hr
Control	0.566	1.19	4.51	8.45
Empty Liposomes				
1:1	100.00	99.16	99.33	107.34
1:10	100.00	97.48	97.12	106.27
1:100	100.00	93.28	96.01	105.44
Free Interferon				
500 u/ml	100.00	87.39	63.19	73.49
50 u/ml	100.00	91.60	76.50	91.36
5 u/ml	100.00	96.64	100.67	100.71
Liposome Encapsulated Interferon				
1:1	100.00	96.64	88.69	97.28
1:10	100.00	100.84	96.67	102.49
1:100	100.00	96.64	94.01	96.57

L1210 Resistant Cells
(control - cell concentration x 10^5 cells/ml)
(other conditions - % of control concentration)

Condition	0 hr	24 hr	48 hr	72 hr
Control	0.485	1.38	4.68	8.17
Empty Liposomes				
1:1	100.00	97.10	102.35	87.88
1:10	100.00	96.38	105.34	92.04
1:100	100.00	95.65	104.27	87.39
Free Interferon				
500 u/ml	100.00	96.38	104.70	91.80
50 u/ml	100.00	92.75	105.56	92.17
5 u/ml	100.00	100.72	104.27	88.49
Liposome Encapsulated Interferon				
1:1	100.00	92.03	102.35	82.50
1:10	100.00	94.93	107.48	85.68
1:100	100.00	95.65	103.21	84.58

Table 4

Effect of Liposome Encapsulated Interferon on Sensitive and
Resistant L1210 Cells

RF3 - Resistant
(cell concentration x 10^5 cells/ml)
(% of control concentration indicated in parentheses)

Condition	0 hr	24 hr	48 hr	72 hr
Control	1.16	3.776	7.835	7.537
Empty Liposomes	1.16 (100.00)	3.460 (91.63)	7.677 (97.98)	7.377 (97.35)
Liposome Encapsulated Interferon	1.16 (100.00)	1.442 (38.19)	2.732 (34.87)	8.125 (107.80)

RF 21 Cells - Sensitive
(cell concentration x 10^5 cells/ml)
(% of control concentration indicated in parentheses)

Condition	0 hr	24 hr	48 hr	72 hr
Control	0.493	1.086	2.745	5.726
Empty Liposomes	0.493 (100.00)	1.027 (97.34)	2.370 (86.34)	7.671 (133.97)
Liposome Encapsulated Interferon	0.493 (100.00)	0.534 (49.17)	0.778 (28.34)	2.020 (35.28)

RMI Cells - Resistant
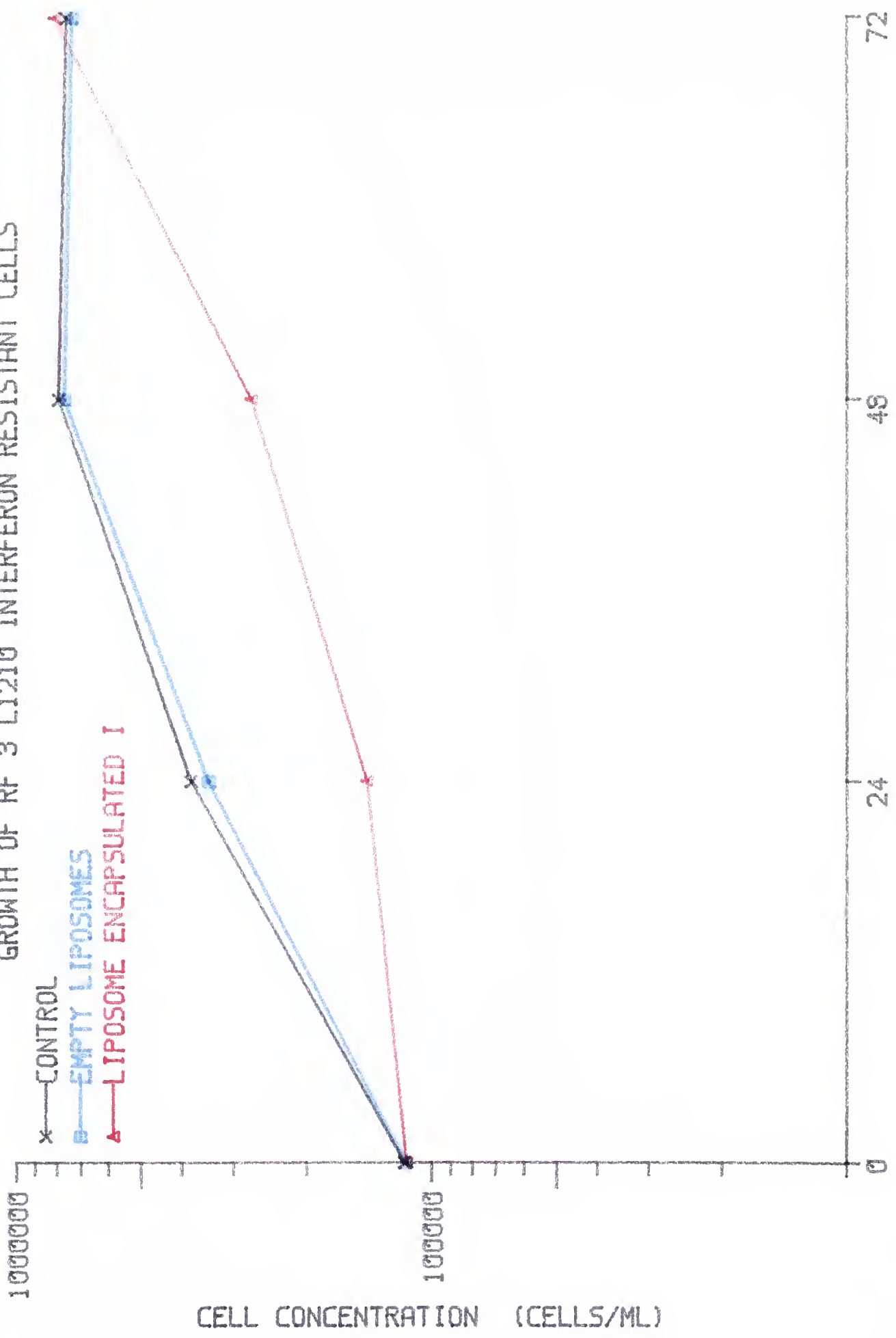(cell concentration x 10^5 cells/ml)
(% of control concentration indicated in parentheses)

Condition	0 hr	24 hr	48 hr	72 hr
Control	0.614	1.795	6.037	8.293
Empty Liposomes	0.614 (100.00)	1.756 (97.83)	5.868 (97.20)	8.889 (107.19)
Liposome Encapsulated Interferon	0.614 (100.00)	0.832 (46.35)	1.684 (27.89)	6.741 (81.29)

GROWTH OF RF 3 L1210 INTERFERON RESISTANT CELLS

CONTROL
EMPTY LIPOSOMES
LIPOSOME ENCAPSULATED I

CELL CONCENTRATION (CELLS/ML)

1000000

1000000

TIME (HRS)

0   24   48   72

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GROWTH OF RF 21 L1210 INTERFERON SENSITIVE CELLS

x —— CONTROL
■ —— EMPTY LIPOSOMES
▲ —— LIPOSOME ENCAPSULATED I

CELL CONCENTRATION (CELLS/ML)

TIME (HRS)
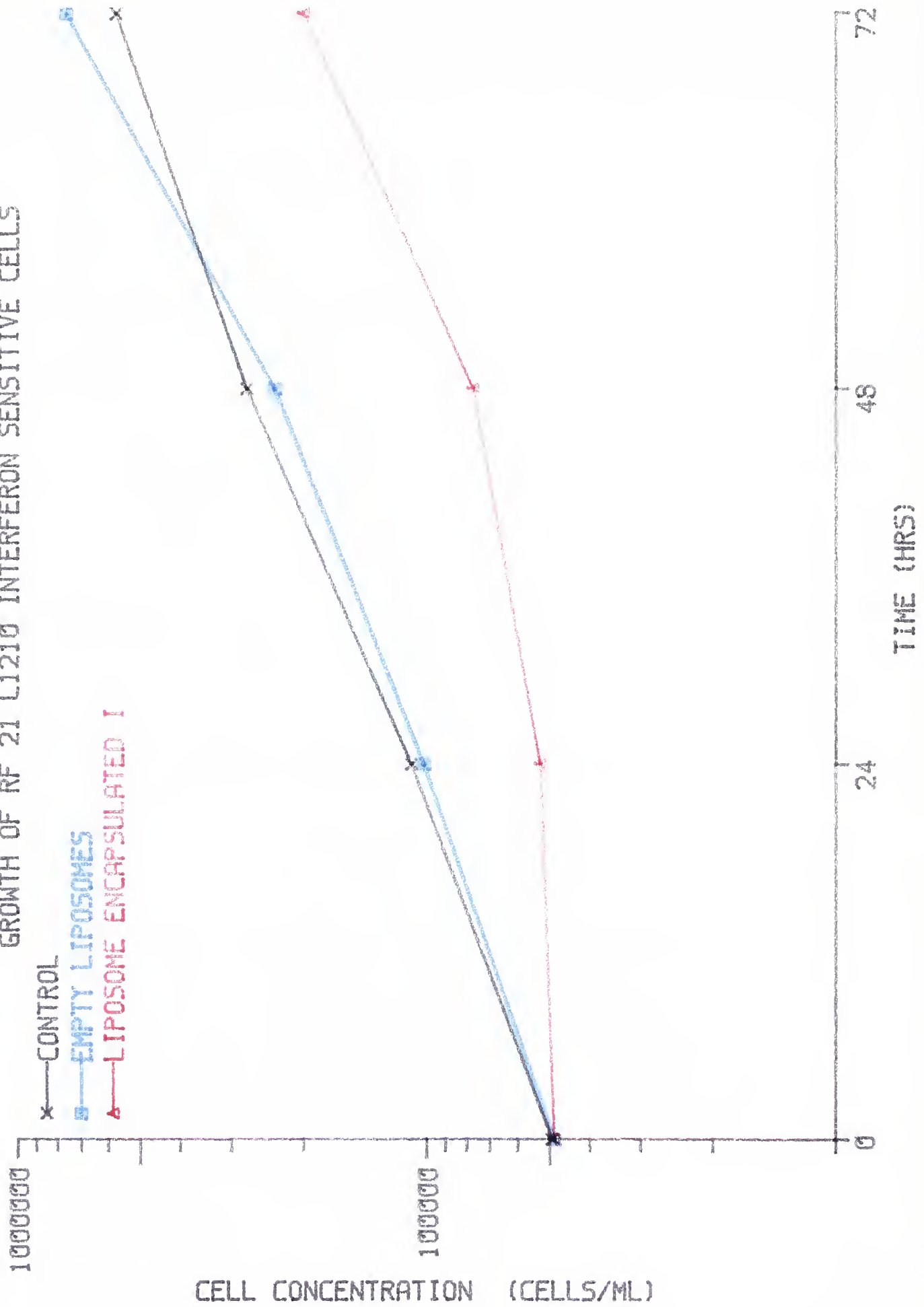
GROWTH OF RMI L1210 INTERFERON RESISTANT CELLS

CELL CONCENTRATION (CELLS/ML)

×——— CONTROL
■——— EMPTY LIPOSOMES
▲——— LIPOSOME ENCAPSULATED I

1000000

1000000

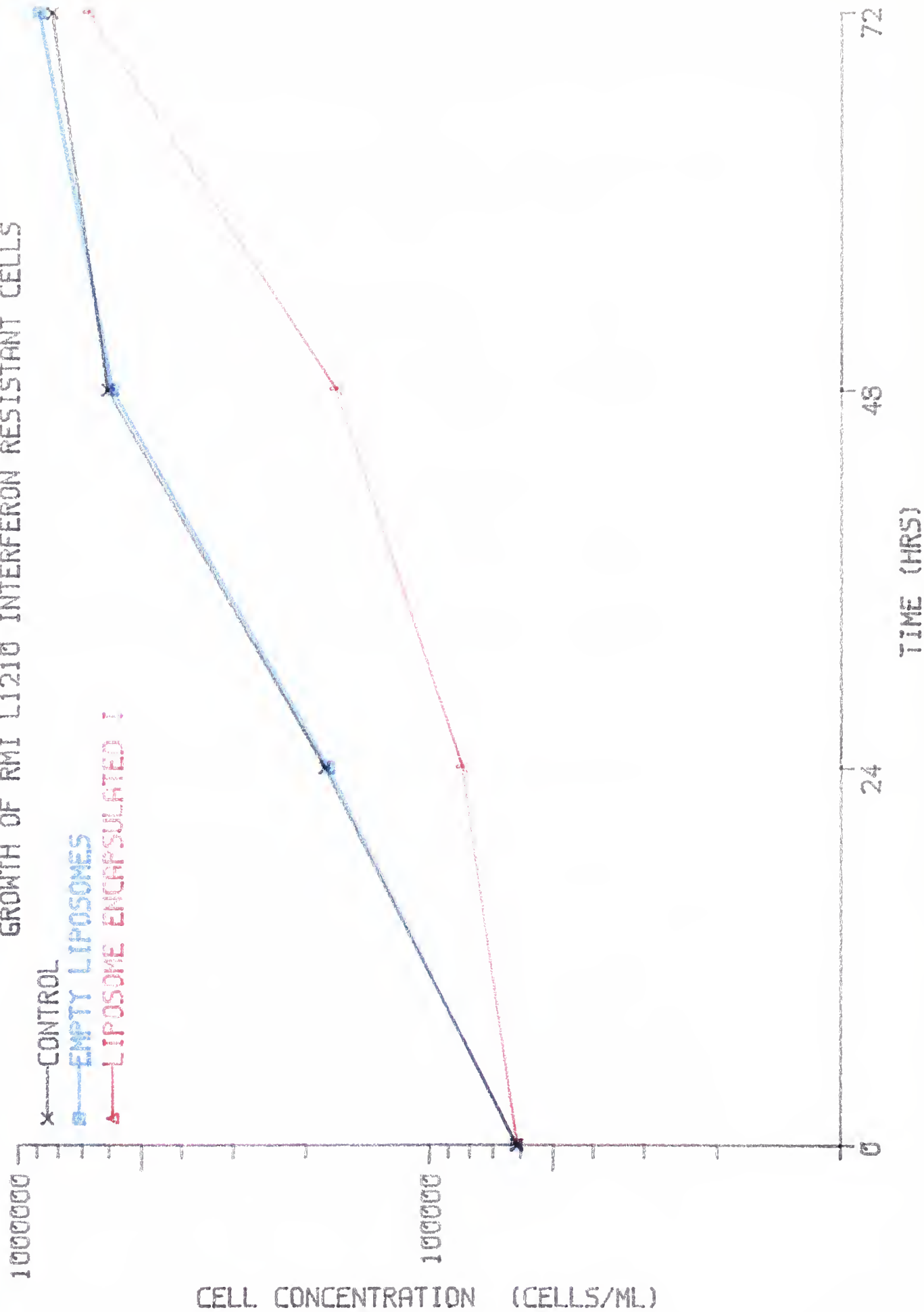0   24   48   72

TIME (HRS)

63

Table 5

Effect of Liposome Encapsulated 2-5A on the Growth of
L1210 S and L1210 R Cells

L1210 Sensitive Cells
(cell concentration x 10^5 cells/ml)
(% of control concentration indicated in parentheses)

Condition	0 hr	24 hr	48 hr	72 hr
Control	0.514	1.213	5.145	8.555
Free 2-5A (100nM)	0.514 (100.00)	1.214 (100.08)	5.080 (98.74)	9.499 (111.03)
Empty Liposomes	0.514 (100.00)	1.193 (98.35)	5.046 (98.08)	8.846 (103.40)
Liposome Encapsulated 2-5A	0.514 (100.00)	1.083 (89.28)	4.343 (84.41)	7.937 (92.78)

L1210 Resistant Cells
(cell concentration x 10^5 cells/ml)
(% of control concentration indicated in parentheses)

Condition				
Control	0.498	1.243	4.643	8.063
Free 2-5A (100nM)	0.498 (100.00)	1.226 (98.63)	4.360 (93.90)	7.343 (91.07)
Empty Liposomes	0.498 (100.00)	1.179 (94.85)	4.244 (91.41)	7.556 (93.71)
Liposome Encapsulated 2-5A	0.498 (100.00)	1.091 (87.77)	3.841 (82.73)	7.669 (95.11)

RESULTS

LIPOSOME ENCAPSULATION OF INTERFERON

1. Comparison with Free Interferon
Phase I (UCSF)

The results of the first trial with liposome encapsulated
interferon are summarized in Table 1. Free interferon showed
dose-dependent growth inhibition of L1210 cells, with growth
inhibition to 50.77% of control cell concentration seen with 1000
u/ml at 48 hours. Empty liposomes showed no effect on cell
growth at all concentrations. Liposome encapsulated interferon
did not demonstrate appreciable effect on cell growth. Liposome
encapsulated interferon at 10 u/ml did exhibit growth inhibition
(69% of control at 48 hours). However, such inhibition was not
consistent with the low estimated concentration of interferon.
Attempts to replicate this finding in were complicated as
described below.

Table 2 displays results from a second trial of liposome
encapsulated interferon. Free interferon again demonstrated a
dose-dependant growth inhibitory effect, with cell concentration
53.72% of control at 1000 u/ml. Surprisingly, both empty
liposomes and liposome encapsulated interferon showed significant
growth inhibition at their higher concentrations. Empty
liposomes reduced cell concentration to 70.23% of control in its
highest concentration. While marked growth inhibition was seen
with the liposome encapsulated interferon (24.60% of control

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concentration at 1000 u/ml, 65.95% of control concentration at 300 u/ml), these results are not valid. Inhibition by empty liposomes had not been seen previously, nor was it expected with the amount of lipid per cell used. The liposome preparation used demonstrated cytotoxic effects in the absence of interferon (Table 2). These findings significantly complicated data analysis, and these results were therefore not accepted as truly representing the effects of liposome encapsulated interferon.

Experiments did not indicate that liposome encapsulation significantly increased the anticellular effect of interferon. However, time limitations at Dr. Epstein's laboratory precluded adequate resolution of difficulties encountered in experiments examining the anticellular effects of liposome encapsulated interferon. Research was therefore continued in Dr. Tritton's laboratory at Yale.

Phase II (Yale)

Comparisons between free and liposome encapsulated interferon were repeated at Yale. However, several experimental conditions were necessarily changed as a result of working in different laboratories.

The sources of interferon and cells were different. The purity of currently available mouse interferon, and the low phenotypic variability of cultured L1210 cells made changes in these experimental conditions of minimal concern.

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The most significant change between phases I and II was in liposome preparation. In Phase I, neutral liposomes of phosphatidylglycerol and cholesterol (1:1) were prepared by the reverse evaporation phase (REV) method. While continued use of these vesicles would have been desirable, prospects for their creation at Yale were not favorable, since neither the proper experience nor equipment were available. At Yale, liposomes were prepared by the standard method of mechanical agitation of the phospholipid film with an aqueous phase. Unsonicated, multilamellar vesicles were employed for the encapsulation of interferon since the capture rate is higher with this type of vesicle. Additionally, several investigators had reported their experience with the encapsulation of interferon in unsonicated multilamellar vesicles (Anderson, 1981, LaBonnadiere, 1977). Liposome encapsulated interferon demonstrated curiously variable effects on cell growth as shown in Tables 3 and 4. While preparations of liposome encapsulated interferon used in Table 3 had no growth inhibitory effect, preparations used in Table 4 showed significant anticellular effects, evident at 24 hours, but more pronounced at 48 hours.

None of the preparations in phase II showed toxicity of empty liposomes, which had complicated an earlier experiment in phase I (Table 2). The growth inhibition seen in table 4 was due to the inhibitory effects of interferon. Differences in the interferon activity of the various preparations clearly existed. The availability of a quick, reliable assay for interferon

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activity of the liposome encapsulated interferon preparations would have been desirable, and would help explain the differing results obtained with the different preparations. Such an assay was, unfortunately, not available to this laboratory.

There are some aspects of liposome preparation which might have affected interferon activity. These factors may account for the variation in interferon activity observed with different liposome encapsulated interferon preparations.

1) Incomplete drying of lipid mixture before introduction of the interferon mixture could have left residual chloroform in the preparation vessel, which could have denatured the interferon.

2) Mechanical agitation is known to inactivate interferon. (Edy, etal, 1974) This step of preparation, however, was constant for all liposome preparations.

3) The chromatographic gel may have been contaminated by substances able to decrease the activity of interferon. Attempts were made to minimize such contamination by storing the column in sodium azide when not in use.

It is difficult to determine the precise reason for the differences in interferon activity observed. Future research efforts in this area must resolve such differences, and strive for uniformity.

2. Effect on Interferon Resistant Cells

Table 3 illustrates the effect of liposome encapsulated

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interferon on the growth of Ll210 sensitive and resistant cells. Results with this liposome encapsulated interferon preparation show no significant effect of liposome encapsulated interferon on the growth of Ll210 R cells.

The experiments summarized in Table 4 are most interesting. The effect of empty liposomes and of liposomes containing interferon were examined in three cell lines, two resistant and one sensitive to the effects of interferon. In all three cell lines, empty liposomes had very little effect on cell growth.

Liposomes containing interferon had marked inhibitory effects on cell growth with all three cell lines, two of which were interferon resistant lines. The effect was evident at 24 hours, but was greatest after 48 hours of incubation. Inhibition of sensitive cells remained significant at 72 hours (35.28% of control). However, the inhibitory effect on interferon resistant cells was markedly decreased by 72 hours of incubation, with the two resistant cell lines showing cell concentrations 107.80% and 81.29% of control.

This difference in effect at 72 hours may be related to the sensitivity or resistance of the cells to the anticellular effects of free interferon. Decreased effect of liposome encapsulated interferon after 72 hours of incubation could be expected if leakage or dissociation of interferon from liposomes, or breakdown of the liposomes themselves was significant. However, this does not explain the observed differential sensitivity between interferon sensitive and resistant cells.

L1210 cells resistant to the anticellular effects of free interferon exhibit growth inhibition when incubated with liposome encapsulated interferon. Anticellular effects are evident at 24 hours, but are more pronounced after 48 hours of incubation. These effects are decreased after 72 hours of incubation.

The mechanism of interferon resistance in L1210 R cells is unknown. Liposome encapsulation of interferon allowed expression of anticellular activity in cells resistant to free interferon. Liposome encapsulation may have altered the nature of interferon interaction with its membrane receptor, allowing expression of anticellular activity in normally resistant cells.

Alternatively, liposome encapsulated interferon may have gained access directly into the intracellular environment. Endocytosis and fusion would allow liposome encapsulated interferon to cross the plasma membrane. The anticellular activity seen in resistant cells may have resulted from interferon exerting its anticellular activity through an intracellular mechanism. If liposome encapsulated interferon exerted its effects intracellularly in L1210 R cells, its action was independent of the membrane receptor system. This raises new questions about alternative mechanisms of interferon action.

Concurrent incubations with interferon were not conducted during the experiments summarized in Table 4. Such incubations would have allowed direct comparison of an effective preparation of liposome encapsulated interferon with free interferon.

LIPOSOME ENCAPSULATION OF 2-5A

Neither free 2-5A (100 nM) nor liposome encapsulated 2-5A (estimated concentration 100 nM) were able to inhibit the growth of L1210 S or L1210 R cells. Hovanessian and Wood (1980) demonstrated significant inhibition of cell growth at 2-5A concentrations of >10 nM, using a modified calcium phosphate coprecipitation technique to allow 2-5A access to the internal cellular environment.

These experiments indicate that liposome encapsulation of 2-5A cannot facilitate its entry into cells sufficiently to produce measurable growth inhibition.

The failure of liposome encapsulated 2-5A to significantly inhibit cell growth could reflect several factors. 1) The estimation of encapsulation rate could have been erroneous. ^{3}H-ATP and 2-5A might exhibit significantly different behaviors when incorporated into liposomes. The amount of 2-5A actually encapsulated could have been much lower than expected. 2) Leakage of 2-5A out of liposomes might have occurred at a rate high enough to decrease the amount available for transfer into cells. 3) Inactivation of 2-5A might have occurred during some phase of liposome preparation. Nucleic acids have been inactivated by sonication during liposome preparation (Poste, etal, 1976). As with liposome encapsulated interferon, the availability of an assay for 2-5A activity would have been most valuable. 4) Liposome-cell interaction could have involved

mechanisms (e.g. stable adsorption) which might not have allowed maximal internalization of liposomes and their contents. 5) Endocytosis of liposomes could have occurred, with significant lysosomal fusion and subsequent enzymatic of degradation of 2-5A by nucleases before its growth inhibitory action could be manifested.

DIRECTIONS FOR FUTURE INVESTIGATION

Review of this project reveals several areas of concern for future research.

A major obstacle throughout the course of this project was the lack of a method for precisely determining the amount of interferon associated with liposomes. Constraints of facilities and time precluded development of methods for accurate determination of liposome associated interferon activity. Accurate determinations of encapsulation rates based on interferon activity will allow more meaningful comparisons between free and liposome encapsulated interferon. This is surely one of the most important tasks to be addressed in future investigations using liposome encapsulated interferon.

Assays of interferon presently available rely on measurements of its biological activity through the methods described earlier. Development of interferon assays which require less time for accurate determination of the presence of interferon would certainly have effects on interferon research. Radiolabelling of interferon, or the use of anti-interferon antibodies in assays (e.g. radioimmunoassay) might prove useful. However, while such assays might yield more rapid quantitative measurements of interferon, they would offer no indication of its biological activity.

Liposome encapsulated interferon could also be used to explore other areas of interferon biology. There are many areas

of future research in which liposome encapsulated interferon could play a central role.

The action of intracellular interferon is not known. Liposome encapsulated interferon could be used to examine such action, and to explore possible intracellular mechanisms for interferon activity that exist independant of the membrane receptor system.

Species specificity of interferon is governed by membrane receptors (Bourgeade, 1974). Liposome encapsulation of interferon might overcome species specificity through circumvention of the membrane receptor system.

The mechanisms of interferon resistance are unknown. Liposome encapsulated interferon could be used to better understand these mechanisms. Results of this project indicate that cells resistant to the anticellular effects of free interferon will exhibit growth inhibition when incubated with liposome encapsulated interferon. Perhaps liposome encapsulation allowed interferon to bypass defective interferon receptors. Such results are only the first step in a long series of investigations needed to reveal the mechanisms of interferon resistance.

In vivo studies using interferon would benefit greatly by use of liposome encapsulated interferon. The many advantages of liposome encapsulation would be extended to interferon. Plasma half-life of free interferon, known to be short, would be greatly extended. Immunogenicity of interferon could be reduced.

Targeting of interferon to specific organs or intracellular sites could be achieved through the targeting methods described. LaBonnadiere (1978) conducted the first known in vivo study using liposome associated interferon, and found an enhanced antiviral effect on mice infected with hepatitis virus. There are no known in vivo studies examining the anticellular effects of liposome encapsulated interferon. In vivo experiments will certainly make clear the full potential of liposome encapsulated interferon.
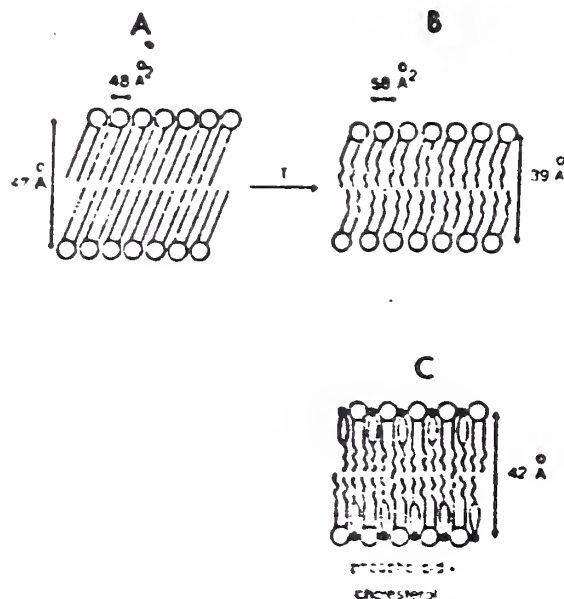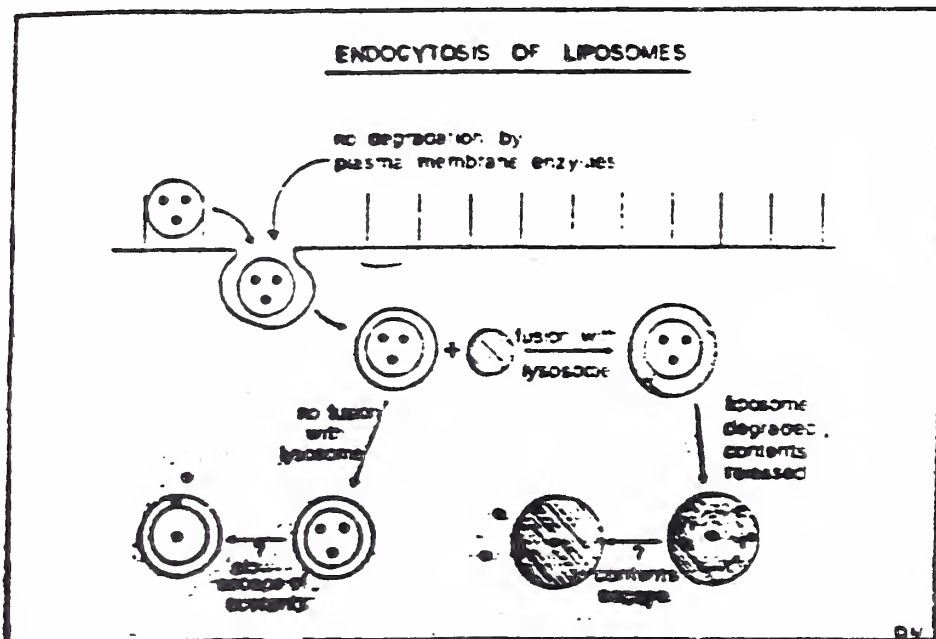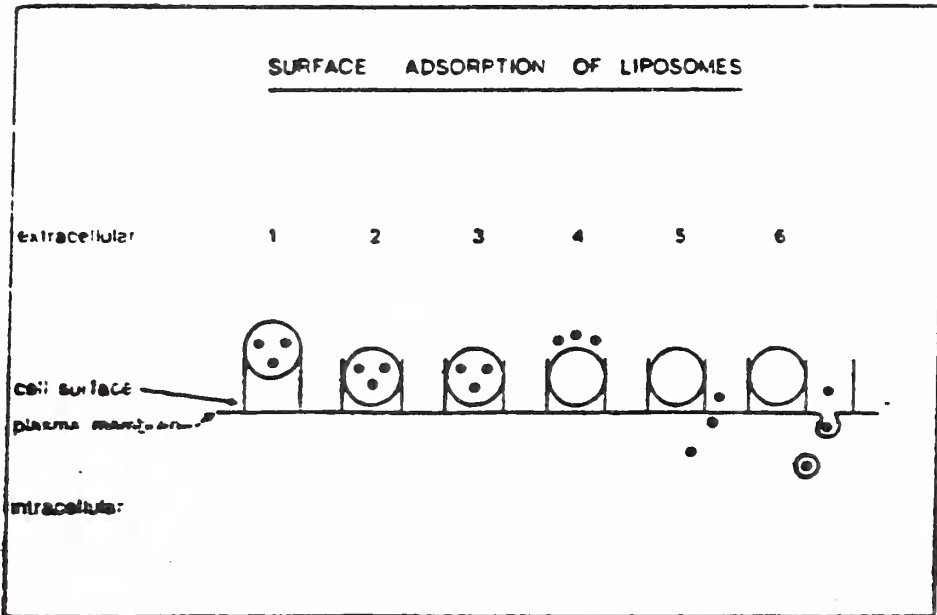
APPENDIX

Fig. 7. Schematic representation of molecular orientation in phospholipid bilayers below (A) and above (B) the phase transition. The head groups of the phospholipid molecules arranged as a bilayer are shown by the open circles, and the fatty acyl chains by solid lines. Below the phase transition, the motion of the acyl chains is inhibited as indicated by the straight lines. Above the phase transition (B) the acyl chains have greater motional freedom, and this is indicated by the curved lines. The effect of the introduction of cholesterol into a fluid bilayer is shown in (C). Cholesterol is represented by a small dark circle (hydroxyl), a larger circular area (the four-ring steroid skeleton), and a curved line (hydrocarbon tail). The dimensions for the thickness of the bilayer in (A) and (B) refer to dipalmitoyl phosphatidyl-choline bilayers and in (C) to a phosphatidylcholine–cholesterol bilayer. Full references to the

Table 1 Properties of phospholipids used in liposomes

Lipid type	Abbreviation	Charge	T_c (°C)
Single Components			
Egg phosphatidylcholine	egg PC	0	−15 to 7
Dioleoyl phosphatidylcholine ($C_{18:1}$)	DOPC	0	−22
Dilauryl phosphatidylcholine ($C_{12:0}$)	DLPC	0	~0
Dimyristoyl phosphatidylcholine ($C_{14:0}$)	DMPC	0	+23
Dipalmitoyl phosphatidylcholine ($C_{16:0}$)	DPPC	0	+41
Distearoyl phosphatidylcholine ($C_{18:0}$)	DSPC	0	+58
Diisostearoyl phosphatidylcholine	DIPC	0	—*
Bovine brain sphingomyelin	brain SM	0	+32
Egg phosphatidylethanolamine	egg PE	0	
Dimyristoyl phosphatidylethanolamine ($C_{14:0}$)	DMPE	0	+45
Dimyristoyl phosphatidylglycerol	DMPG	−	+23
Dimyristoyl phosphatidic acid, pH 6	DMPA	−	+52
Bovine brain phosphatidylserine	brain PS	−	+5
Dicetyl phosphate	DCP	−	
Stearylamine ($C_{18:0}$)	SA	+	
Common Mixtures			
PS/DSPC/DPPC (1:4.5:4.5)		−	~43
DPPC : cholesterol (1:1)		0	none

* Not observed over the temperature range −30 to +75°C (86); T_c probably < −30°C.

APPENDIX B – Mechanisms of Liposome-Cell Interaction
from Kimelberg and Mayhew, 1978

LIPOSOME MEMBRANE — PLASMA MEMBRANE FUSION

Liposomal lipid
associated with
plasma membrane

A. CONTENTS ESCAPE
TO CYTOPLASM
acted upon by soluble
enzymes only.

B. CONTENTS ESCAPE TO
EXTRACELLULAR SPACE -
uptake as for free drug.
Acted upon by plasma
membrane, lysosomal
and/or soluble enzymes.
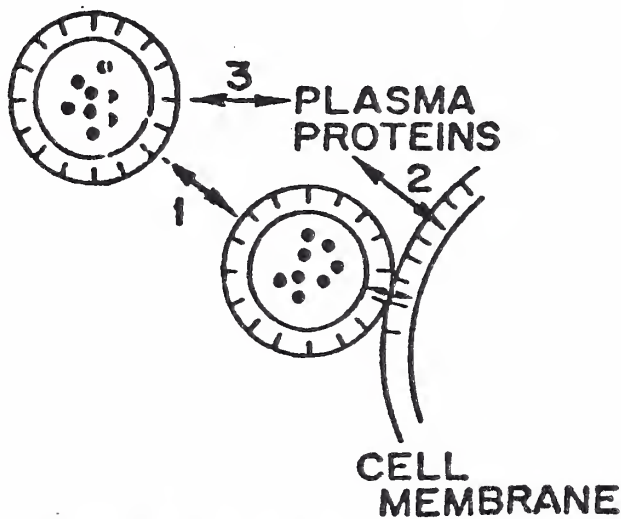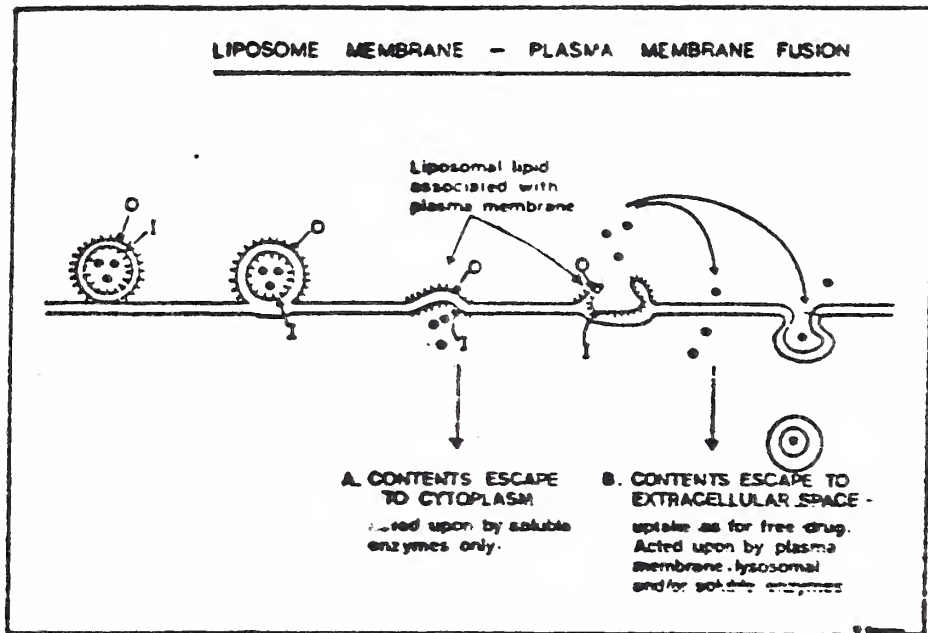

PLASMA
PROTEINS

CELL
MEMBRANE

FIGURE 7. Exchange reactions between cells, liposomes, and serum components. Diagram shows possibilities for exchange of lipid components, denoted as short lines within the lipid bilayer membrane, directly with membrane after surface adsorption (1) or indirectly via serum plasma proteins (lipoproteins) (2), (3).

APPENDIX C - In vivo Behavior of Liposomes

Percent of Injected ^{3}H-Phospholipid per Gram
Tissue 1 hr after Injection

	Route of Injection	
Tissue	I.V. (tail vein)	I.P.
Liver	22.4	9.8
Spleen	6.0	6.4
Kidney	9.6	7.6
Lung	8.6	5.2
Brain	1.3	0.5
Stomach	2.0	4.7
Small intestine	3.0	4.0
Large intestine	2.2	3.6
Testes	1.4	0.5
Mesenteric lymph nodes	1.1	2.2
Thymus	2.1	1.4
Plasma	11.5	4.1

Note: Unilamellar, sonicated liposomes composed of PC plus 10% PS labeled with [^{3}H] DPPC were injected into DBA/2 male mice (mean of determinations made on tissues from three mice).

From Mayhew, E. and Papahadjopoulos, unpublished data.

APPENDIX D - The First Clinical Trial of Interferon
from Time, March 31, 1980

First clinical use of interferon is shown in 1960 *Flash Gordon* comic strip as space medics inject it to save victim of an extraterrestrial virus

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