Examining the Transferrin-Transferrin Receptor System as a Possible Mechanism for Cell Specific Targeting with Liposomes

Thesis submitted in partial fullfillment

of the Masters of Science degree

in Chemistry

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February 20, 1989

Abstract

This research focuses on targeting capabilities which may be applied for delivery of liposomes to T cells. Two methods of targeting were investigated. The first involved IgG purified from goat antisera to a portion of the viral envelope of Human Immunodeficiency Virus (HIV). The IgG was derivatized with the N-hydroxysuccinimide ester of palmitic acid (NHSP) to increase its hydrophobicity so that it could be incorporated into the lipid bilayer of egg phosphatidylcholine liposomes.

The second method used transferrin to coat liposomes made from egg phosphatidylcholine and egg phosphatidylethanolamine, 5:1 weight ratio, respectively. In this method the transferrin receptor (TfR) serves as a target for the delivery of transferrin (Tf) coated liposomes to cells.

From previous observations made at the City of Hope as well as data from a resent paper[1], HIV infected cells appear to have an increased affinity for Tf. This increased affinity is most likely associated with an increase in cell surface TfRs. The H-9 cell line, which can be infected with HIV, has been characterized for the number of TfRs per cell and for the binding of Tf.

Attaching IgG or Tf to a liposome will alter the protein structure. Thus, the receptor-ligand affinity characteristics of IgG as well as the affinity of iron for Tf and of Tf-Fe for TfR will change. As a result, the type of association which links IgG or Tf to the liposome will affect the targeting ability of the IgG or Tf coated liposomes. Many methods of associating IgG with liposomes have been studied[2,3]. The method of choice is one of the most successful ways for the use of liposome delivery. It involves the attachment of palmitoyl chains to the IgG followed by insertion of the now hydrophobic IgG into the lipid membrane. Only a few procedures for attaching Tf and liposomes have previously been tried. I have examined a new method for attaching Tf to liposomes, involving the use of water soluble carbodiimide. The crosslinking of Tf and liposome has been observed with a maximum efficiency of 1 Tf molecule for 4 liposomes. For both IgG and Tf coated liposomes, an average diameter of 45-50nm was determined.

I. Introduction

A major problem in medical treatment is directing drugs or other compounds to diseased cells. As a result, a large quantity of drug may have to be administered so that a small percent will be effective. The excess drug may kill healthy cells, and the final result for many drugs may be more detrimental than it is beneficial.

The ability to recognize and deliver drugs to specific cells is of great scientific interest for *in vivo* recognition of cells and has enormous potential for increasing drug efficiency and reducing side effects. Specific cell types have antigens and receptors on their surface which help distinguish them from other cells in the body. Any compound which recognizes these antigens or receptors should provide a certain degree of specific target ability to the cell. Such compounds include antibodies to the antigens or receptors as well as ligands specific for the receptors. Once specific cells can be targeted, one could envision exploiting this targeting ability to deliver drugs or other chemicals such as antigens, antibodies, nucleic acids, hormones or growth factors.

Liposomes provide a vesicle for such delivery. Liposomes are spherical shells made by lipid bilayers. Compounds such as drugs or peptides can be encapsulated inside the liposome. This provides cells

with some protection from direct interaction with the compound. These liposome encapsulated drugs must then be directed specifically to target cells and be taken up in such a way that the drug is effective.

Although the ability to effectively deliver drugs encapsulated in liposomes specifically to target cells has tremendous potential for medical therapy, there exist many problems which must be addressed in order to enhance its effectiveness. The problem to be addressed here is that of specific targeting. For delivery to target cells, the liposomes must specifically recognize those cells. *In vivo* this becomes difficult since many of the antigens and receptors on cell surfaces are common to a number of different types of cells. The studies which have been done are *in vitro*, but the possibilities for recognition by other cells which may be encountered *in vivo* were kept in mind.

The initial system studied was that of IgG antibody recognition of HIV infected cells. Antisera from a goat injected with a portion of the HIV envelop glycoprotein gp160 amino acid sequence was provided by Dr. Ravi Potavil (Hofmann LaRoche, Nutley, N.J.) through Dr. John Zaia (City of Hope, Duarte, CA). Gp160 is eventually cleaved into an outer gp120 and a transmembrane gp41[4,5]. Regions of the aminoterminal portion of gp120 associate in a non-covalent manner with the gp41. Together gp120 and gp41 regulate the binding and fusion of the virus to the target cell. As

expected, the antibodies most easily and universally detected are those to certain regions of the gp120 or gp160. Furthermore, gp120 is present on the surface of infected T cells[4,5]. Hence, an antibody to active recognition regions of gp120 or gp160 seems a likely candidate for specifically targeting to HIV infected cells.

IgG was purified was purified from goat antisera[6]. Palmitoyl chains were attached to the IgG by derivatization with an N-hydroxysuccinimide ester of palmitic acid in order to increase the hydrophobicity of IgG[7,8]. Using detergent dialysis, the palmitoyl-IgG was incorporated into unilamellar liposomes made from egg phosphitidylcholine (also known as egg -lecithin). Since neither the unfractionated antisera nor the purified IgG antibody fraction showed any ability to bind to H-9 HIV infected cells[9], further cell studies with this IgG antibody and with the liposome-antibody complexes were discontinued.

The second system of cell recognition and possible liposome delivery to be studied involved transferrin (Tf) and the cell surface transferrin receptor (TfR). Tf is a well characterized protein with several features which may be beneficial for cell targeting. For example, cells recycle Tf and TfR by a unique intracellular pathway. This quality may prove useful for intracellular delivery. Being an iron transport protein, Tf is vital for the growth of a cell. Highly proliferating cells,

such as those which are malignant, often have significantly more TfRs than normal cells[10-12]. A liposome with attached Tfs may be proferentially bound and taken up by these infected cells.

From initial studies done in conjunction with the City of Hope as well as recent literature[1], it appears that HIV infected cells also have increased levels of TfR on their surface. The H-9 cell line is relatively new (1984)[9] and has not been characterized with respect to Tf and TfR. Characterization of the H-9 cell line with respect to the number of cell surface TfRs per cell and the binding of Tf will set some of the groundwork for future studies of Tf with HIV infected cells as well as begin a basis for the use of liposome targeted delivery of drugs to HIV infected cells. It may also provide further groundwork for the targeting and delivery of drugs encapsulated in liposomes to metastatic cells.

This project has acheived four main goals. First is the insertion of palmitoyl derived IgG into liposome bilayers. Second, a new method of covalently attaching Tf to liposome has been examined. The advantages and disadvantages of this method are discussed. Third, a range of values for the number of TfRs on a H-9 cell has been established. Fourth, a priliminary value for the dissociation constant (k_d) of Tf with the TfR of a specific cell line (H-9) has been obtained.

II. Major Components of this Study

The initial system studied used IgG to recognize of HIV infected cells. Dr. Potavil, who provided the antisera from which the IgG was purified, claims that the antisera is from a goat immunized with the amino acid sequence 487 - 511 of the HIV gp160 and that this antisera binds well to HIV infected cells. All of the HIV infected studies included here have been done at the City of Hope in the laboratory of Dr. Zaia with the assistance of Delilah Stevas. The T cell line being used is H-9. Unfortunately, neither the antisera nor the purified IgG have shown specific binding to HIV infected H-9 cells. These cells, however, are bound by a different Ab which was also provided by Dr. Potavil to Dr. Zaia. This latter Ab is to a combined selection of HIV envelop and gag regions.

The motivation for the second portion of this project was the possiblity of using the Tf-TfR as a system for cell targeting and delivery. The specific recognition between Tf and the TfRs on cells may be able to be exploited for specifically targeting liposomes with attached Tfs to cells. The Tf-TfR system has some unique qualities which make it appealing for cell targeting and delivery. The number of TfRs tends to be upregulated on some types of infected cells[1,10-12], for example, many malignant cells[10-12]. Hence, one could envision more effective and less

detrimental chemotherapy against cancer by liposome targeting of chemotherapeutic drugs to the cancerous cells. This type of liposome therapy could certainly reduce many of the side effects of chemotherapy and increase the length of time one person may be able to handle the treatment. A second advantage of the Tf-TfR system is that TfR is expressed on T4 cells and macrophages, both of which are of great importance in immune regulation and of particular interest in immune deficient diseases. If specific targeting can be achieved with this system, an increase in the control of the immune system can be achieved. Finally, Tf and TfR have their own unique intracellular processing (figure 1). Hence, uptake by cells via the Tf-TfR may provide a special pathway for intracellular delivery.

To examine the feasibilty of the use of this system, two cell lines, both expressing TfRs, were being used. The promyelocytic leukemia cell line HL60 has been well characterized for the binding and uptake of Tf[13-16]. It served as the control cell line. Initial characterization for the specific binding of Tf to the T-4, lymphoma cell line H-9[9] was achieved. The results of this project set the groundwork for future studies characterizing the Tf binding and uptake by HIV infected H-9 cells and studies examining the role of Tf in the HIV retroviral infection. Future studies may provide knowledge concerning the use of Tf for

specific targeting to cells such as those infected by cancer.

IV. Experimental Methods

A. Antibody Purification

To purify the IgG from the antisera, the common purification method using Protein A was found to be ineffective since the source of the antisera was goat. Goat Ab, unlike mouse or human, binds very poorly to Protein A. Instead, the double precipitation procedure of McKinney and Parkinson was used[6]. Albumin and other non-IgG proteins are precipitated out of solution at room temperature with caprylic acid. At this point, an acrylamide gel indicated the sample free of any non-IgG protein. To ensure its purity, ammonium sulfate was used at 4^{o}C to precipitate IgG out of solution. This was followed by dialysis for 10 hrs, heating at 50-55^{o}C for 20 minutes, and centrifuging for 20 minutes near 5000xg. The supernatant was then stored at -20^{o}C. The purity of the final product, as well as the products after various steps throughout the procedure, was evaluated using acrylamide gels. To further ensure that the product was IgG, the acrylamide gels were run both with and without the reducing agent 2-mercaptoethanol. As expected, gels with 2-mercaptoethanol showed products with two bands, corresponding to the IgG heavy chain at approximately 50kD and the IgG light chain at approximately 30kD. The gels without 2-mercaptoethanol showed only one band, corresponding to

the whole IgG complex of approximately 160kD.

B. ^{125}I Iodination of Antibody and Transferrin

Labeling of IgG and Tf with ^{125}I (Amersham, Inc), was accomplished using either the chloromine T or Enzymobead (Bio-Rad) method. For the chloromine method, the following amounts of reactants were used per mg of IgG or Tf: 0.1 to 0.13 mCi of Na^{125}I, 93ug chloramine T, 205ug sodium meta-bisulfite. Na^{125}I was first added to the IgG solution. A fresh 10mg/ml solution of chloramine T in PBS was prepared and the necessary amount of chloramine T added. After 5 minutes with occasional mixing, the necessary excess of sodium meta-bisulfite was added from a fresh 4mg/ml PBS solution. After 10 minutes of occasional mixing, the sample solution was concentrated with an Amicon Membrane and washed once with PBS to remove a large amount of the unincorporated ^{125}I. The solution was then placed in a spectrum 2 dialysis bag and dialyzed for 48 hours against 4 changes of 2 L PBS or until the remainder of the unincorporated ^{125}I was removed.

The Enzymobead iodination was done as instructed by the supplier with some alterations. Briefly, Enzymobead reagent was rehydrated in 0.5ml of distilled water for one hour and stored at 4^{o}C. Rehydrated Enzymobead reagent was diluted 1-fold with 0.2M Phosphate buffer. IgG

was added to solution as well as a 480uCi or less of Na-^{125}I. A 1.5% D (+) glucose solution was made and a volume added which was 1/2 that of the rehydrated Enzymobead reagent being used. The solution was left to react for 35 minutes. To remove the enzymobeads as well as excess ^{125}I, the solution was put through a 2.5-3 ml G-25 (fine) spin column by centrifuging at 1500xg for 3-5 minutes. The sample solution was then placed in a spectrum 2 dialysis bag and dialyzed against 300ul PBS until unincorporated ^{125}I was removed (~30 hrs with 3 changes).

The concentrations of the final ^{125}I-IgG and ^{125}I-Tf solutions were analyzed using the Bio-Rad Protein Assay Kit which is based on the Bradford assay[17].

C. Experimental Discussions

Crosslinking

There are many methods for crosslinking a protein to a liposome[2,3]. Each has its advantages and disadvantages. The best method depends on the special characteristics of the lipid(s) (if any specific lipids are required for the system) and on the protein to be used. For example, if either the lipid or the protein are unstable, the process should be gentle and completed in as short a period of time as possible. Another concern is

the reactive site(s) of the protein. When crosslinking, the effect on reactive sites should be minimized and the exposure of these sites in the final complex should be maximized. Only repeated studies comparing various crosslinking methods will reveal the best crosslinker for any specific system. Studying the various crosslinkers and their effects on proteins and lipids will enable better decisions to be made in the future when choosing a crosslinking method.

A number of general methods for protein-liposome crosslinking have been described. A common method involves covalent attachment by both the protein and liposome. Most methods exploit the free amino headgroup of phosphadylethanolamine by reacting the lipid with an amino reactive crosslinker. Crosslinkers which have different reactive components on each end are particularly nice in order to reduce the problem of homocoupling. Hence, a disulfide linkage that can be reduced by a thiol is often desired for the attachment of a thiolated protein to the crosslinker or the use of a maleimido protein derivative. An example of these type of bifunctional crosslinkers is N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP) and maleimiobenzoyl-N- hydroxysuccimide ester (MBS)[2,3].

The method that was used for attaching the HIV IgG to liposomes is well established. The hydrophilic antibody is modified to increase its

hydrophobicity so that it will insert into the lipid bilayer[7,8]. The hydrophobic modification is achieved by using the N-hydroxysuccinimide ester of palmitic acid (NHSP) to derivitize the IgG[7,8]. The palmitic acid ester and an amino group on the IgG react to form a strong amide bond. The hydrophobicity of the palmitoyl chain allows the modified antibody to insert into the lipid bilayer. IgG is preferentially modified in the Fc region[2,3,7,8]. Hence, the Fab region will protrude from the bilayer, enabling recognition of the antigen.

The crosslinking method of an imide had never before been studied for the crosslinking of Tf to liposomes. The water soluble carbodiimide 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDCI) has been shown to attach IgG antibodies effectively to the surface of liposomes[18]. The carbodiimide bond of EDCI is used to produce a peptide bond between a carboxylic group of Tf and the amine headgroup of phosphatidyl ethanolamine[18-25]. Some of the advantages of this method are its experimental simplicity and rapidity. Liposomes are formed prior to crosslinking. This saves Tf from the unnecessary exposure to the procedures of making liposomes. Furthermore, all components can be added at one time with sufficient crosslinking complete in 2 hours. Many other linkers require time consuming procedures to prepare both the lipid and the protein for the crosslinking with further time required for the

actual crosslinking.

Liposomes

Large unilamellar vesicles (LUVs) range in size from roughly 100 to 500nm in diameter. Small unilamellar vesicles (SUVs) are less than 100nm in diameter. Multilamellar vesicles (MLVs) have multiple bilayer membranes. The studies included here involve SUVs.

For the IgG studies, liposomes were formed from a 1:1 molar ratio of egg phosphatidyl choline (egg PC) and cholesterol. This lipid composition was choosen for two primary reasons. The phospholipid PC head group has no charge. Hence, charge effects by the lipid are eliminated. Secondly, these egg PC - cholesterol liposomes are very stable.

For the Tf studies, the lipid composition used was a 5 to 1 weight ratio of egg phosphatidyl choline (egg PC) to egg phosphatidyl ethanolamine (egg PE) with a 2 to 1 molar ratio of phospholipid to cholesterol for the sake of liposomal stability. Egg PC with this proportion of cholesterol makes very stable liposomes[26]. The amine headgroup of egg PE is the active portion of these stable liposomes.

All phospholipids were purchased in chloroform from Avanti Polar Lipids, Inc.. The appropriate quantities of each lipid in chloroform solution were mixed. To some a trace of hexadecyl [^{3}H] cholesterol ether

($1x10^6$ cpm/ug lipid) was added. The chloroform solvent was removed by placing the mixture under a steady stream of argon followed by lyophilizing to remove the final traces of solvent. The lipid was rehydrated in PBS to make a 16mg/ml solution. To form SUVs, the lipid solution was bath sonicated (Lab Supplies Co., Model G112SPIT) for 3 to 5 15 minute cycles or until the solution became opalescent, indicating the formation of liposomes. A Spectra-Physics Series 2000 light scatterer was used to evaluate the size of the liposomes after sonication as well as after crosslinking and after elution from a Sepharose 4B column (see next page). Electron microscopy studies were carried out to confirm the data obtained from the light scattering experiments. A 1% solution of phosphatidyl tungstic acid was used to negatively stain the liposome samples.

D. Crosslinking of IgG and Tf to Liposomes

Deoxycholate (DOC) Purification

A supersaturated solution was made by desolving DOC (Sigma, 99+) into a heated ethanol/water mixture. The solution was vacuum filtered and let cool to either 4^oC or -20^oC. The crystals were vacuum filtered out of solution. Recrystallization procedures were repeated until white

crystals of DOC were formed in a colorless ethanol/water solution. Following the final vacuum filtration, the crystals were dried overnight under vacuum.

Drying of Ethyl Acetate

Phosphorous (V) oxide was added to ethyl acetate and mixture left to sit for 2-3 hours. The ethyl acetate was then distilled into a flask containing molecular sieves.

Synthesis of N-hydroxysuccinimide ester of palmitic acid (NHSP)

NHSP was synthesized by the procedures of Lapidot,et al.[27]. Briefly, a 1:1:1 molar ratio of N-Hydroxysuccinimide, palmitic acid, and dicyclohexylcardodiimide was added to dry ethyl acetate with a final concentraton of 0.75 M. The mixture was stirred for 15-30 minutes and then let sit at room temperature for 8 hours. The mixture was vacuum filtered to remove the dicyclohexylurea. NHSP precipated out of the filtrate when cooled. NHSP then underwent ethanol recrystallization until it was deemed pure by Thin Layer Chromatography (TLC). It was then dried overnight under vacuum and stored at $4^{o}C$.

For TLC, the samples were dissolved in absolute ethanol, spotted onto TLC plates and run in a 1:3 petrolium ether : ethyl ether solution. The

plates were stained by Haines-Ischerwood solution, iodine, and 30% sulfuric acid followed by heating. The latter technique proved the most inclusive, but all were necesary for a complete analysis of the final product and possible byproducts.

Modification of IgG

Palmitic acid was coupled to the IgG, using the published procedures of Huang, et al. (`82) with minor changes[9]. Varying amounts of NHSP were added to 1-2 mg of ^{125}I-labelled or unlabelled Ab in a 2%DOC PBS solution so that the molar ratio of NHSP to IgG varied from 0 to 30. A 10-fold molar excess of palmitic acid was added to the control solution in which no IgG was present. The reaction was incubated at 37^{o}C for 9-15 hours. A Sephadex G-75-100 or G-75 column (30ml, 1.5x26cm) eluted with PBS containing 0.15% DOC was used to remove the unattached palmitic acid, which underwent spontaneous hydrolysis in the water based buffer. The IgG solutions were then concentrated (Amicon). For comparison, dialysis against three or more changes of a 200ml minimum 0.15%DOC PBS solution was also used to remove the unincorporated material. Raidoactivity from samples containing ^{125}I labelled IgG was counted using a Beckman Biogamma II gamma counter.

Incorporation of IgG to Liposome

Liposomes were made as discussed earlier. Varying amounts of palmitoyl Ab was added to preformed SUV liposomes to achieve ratios of lipid:IgG, ranging from 0 to 40 (w/w). Enough 5% DOC was added to reach a 0.7% DOC solution. The mixtures were placed in Spectra/Por 2(Spectrum) dialysis tubing and dialyzed against 3 changes of 2 L of PBS for 45 hours. A Sepharose 4B column (30ml, 1.5 x 26 cm) was used to remove the unincoporated IgG. Radioactivity from samples containing ^{125}I labelled IgG and/or ^{3}H labelled lipid was counted using gamma (Beckman Biogamma II) and liquid scintillation (Beckman LS7500) counters.

Crosslinking Tf to preformed liposomes

As explained above, SUVs were made by bath sonication of rehydrated lipids. A sample of 0.2-200ug of ^{125}I-Tf was added to 100-200ul of SUVs (200ul = 1.67mg PC, 0.33mg PE, 0.517mg cholesterol). Sufficient PBS (pH7.4) was added so that all samples were of equal volume. A small aliquot of PBS pH7.4 was reduced to pH1.5-3.5 with hydrochloric acid. A pre-determined amount of this low pH solution was added to each sample so that the pH~4.7 needed for crosslinking[18] was reached. Appropriate amounts of fresh 20%, 40%, or 80% aqueous EDCI

solution were added to the various samples. After 2 hours with occasional mixing, ^{125}I-Tf-liposomes (and ^{125}I-Tf-Tf complexes) were separated from free ^{125}I-Tf with a 30ml (1.5x22.5cm) Sepharose 4B column. Elution aliquots of ~1ml were collected and the radioactivity counted with a Beckman Biogamma II gamma counter. For controls, ^{125}I-Tf and ^{3}H-liposomes were separately carried through the same reaction conditions. ^{3}H was counted with a Beckman liquid scintillation counter.

E. Cell Binding and Uptake Experiments

HIV infected Cells

Binding studies to HIV infected cells were done with the assistance of Delilah Stevas in the cell culture facility equipt for AIDS work in the laboratory of Dr. John Zaia at the City of Hope. Initial studies have been completed. These studies use H-9 cells. This cell line is a clone from the HT line derived from an adult with lymphoid leukemia, and although the cell line is not infected, HIV may be grown and virus produced in it[22].

The affinity of IgG and Tf for HIV infected H-9 cells was compared to that for uninfected H-9 cells. ^{125}I iodinated protein was being used to track the amount of protein associated with cells. ^{125}I-protein and HIV

infected or uninfected cells ($2x10^6$) were incubated at 37°C in the presence of CO_2 for 2 hours. The cells were centrifuged (Beckman TJ6, 21,000 RPM for 5 min.) and the pellets washed once. The samples were then counted using liquid scintillation (Beckman LS7500)

Various amounts of ^3H-liposomes with attached antibody were incubated with HIV infected and uninfected H9 cells by the same procedure as explained above. The ^3H associated with the cells was counted by liquid scintillation (Beckman LS7500).

Noninfected Cells and Tf

HL60 cells were a gift from Tom Amatruda, at the California Institute of Technology. H-9 cells were a gift from Delilah Stevas of Dr. Zaia's laboratory at the City of Hope. Marie Krempin graciously grew the cells at California Institute of Technology in the cell culture facility in the basement of Crellin. HL60 cells are grown in -modified Eagles media (MEM) supplemented with 10% fetal calf serum (FCS). H-9 cells are grown in RPMI 1640 media supplemented with penicillin, streptomycin, and 10%FCS. Both cell suspensions were grown at 37°C with 5% CO_2.

A commonly used method of distinguishing the binding of Tf from its uptake is to conduct experiments at both 0-4°C and 37°C[28]. At a temperature between 0 and 4°C Tf will only bind to cells and their TfRs.

No uptake is observed. At 37°C the normal procedures of uptake and intracellular trafficking commence. Temperature was used to distinguish between Tf binding and uptake by cells.

For most samples, a Hamilton syringe was used to measure 0.001 to 200ugs of ^{125}I-Tf into Eppendorf tubes. For binding experiments, the tubes were placed in ice and 1-2 x 10^6 cooled cells were added to each tube. Assays were usually carried out in triplicate. The ^{125}I-Tf / cell suspensions were incubated on ice. For both cell lines, a 30 min incubation time was sufficient for the binding equilibrium to be reached. After incubation, the suspensions were centrifuged for 3-5 minutes in the Eppendorf centrifuge (12,000 x g). The supernatant was removed and the cell washed with ~700ul PBS. Repeated washes yielded no difference in the data and no ^{125}I was detected in the supernatant of the repeated washes. The washed cell pellet was resuspended, transfered into a gamma counting tube, and counted. In some experiments, the cap of the eppendorf tube was cut off and the tubes placed directly into gamma counting tubes to count the ^{125}I associated with the washed cell pellet.

Initial uptake experiments were carried out in the same manner as the binding experiments with the exception of temperature. ^{125}I-Tf was added to Eppendorf tubes at room temperature. Cells kept at 37°C were added to the ^{125}I-Tf solutions and the ^{125}I-Tf / cell suspensions

incubated in a 37°C incubator with 5% CO_2 for a minimum of 20 minutes.

IV. Results/Discussion

Liposome Size

The liposome size was determined by light scattering (Malvern Submicron Particle Analyser with a Spectra-Series 2000 light source) and confirmed with electron microscopy studies. For both egg PC / cholesterol liposomes with associated IgG and egg PC/ egg PE / cholesterol liposomes with covalently attached Tf, the average diameter of the protein coated liposomes was 45 - 50 nm.

Attachment of IgG to Liposome

The first step of attaching the IgG to the egg PC SUV liposome was to make the IgG hydrophobic by the addition of palmitoyl chains through a reaction with NHSP. The NHSP/IgG molar ratio was varied from 0 to 30. To remove unincorporated NHSP/Palmitic acid, the solution was either run through a G-75-120 or G-75 Sephadex column eluted with 0.15% DOC in PBS or dialyzed against PBS (200ml or more) with 0.15% DOC . Two advantages of the method of dialysis over that of the G-75 column are that IgG is not lost on the column and that no concentrating is necessary of the final IgG-palmitoyl solution is necessary.

A Sepharose 4B column was used to separate unincorporated IgG

from liposomes. In all the collected samples past the void volume, ^{125}I was counted. Some affinity by the column for the IgG was indicated. However, running ^{125}I-IgG through the column alone as a control, showed that after collecting 25ml (~15ml beyond the void volume) 78% of the IgG had been collected. The elution peak of IgG corresponded to 76% of ^{125}IAb added. Hence, over 97% of the IgG collected was in the IgG peak and was distinguishable from the background.

Definite incorporation of IgG into liposomes was seen. A molar ratio of 1:10 for both IgG:NHSP and IgG:lipid gave good IgG-lipid incorporation. An important point to raise is that maximum IgG incorporation into liposomes is not necessarily the best IgG/lipid ratio for maximum targeting to cells. For example, while increased palmitoyl chains may increase the amount of IgG incorporated into the lipid bilayer, too many palmitoyl chains may significantly reduce the affinity of IgG for its antigen[2,8]. To account for this factor, binding of IgG-liposomes to cells must be studied.

The IgG-liposomes prepared in these studies did not show any specific binding to cells. This was not unexpected since ELISA and miniture Western blots showed no binding to H-9 infected cells by either the purified IgG or the original antisera.

Aquisition of material which would be consistent with the cell

system being studied was unsuccessful. The study of transferrin as a model system for liposome targeting to cells was then begun. Incubation of Tf with infected H-9 cells indicated an upregulation of the Tf receptor.

Crosslinking Tf to liposomes

The carbodiimide (EDCI) was required for interaction between Tf and liposomes (figures 2,3). Since Tf has reactive amine groups, EDCI may cause self-crosslinking between Tfs (figures 4,5). Originally, small amounts (1.8%) of EDCI were used. After 2 hours, no appreciable self-crosslinking was evident, but crosslinking between Tf and liposomes was observed (figures 2,5). The efficiency, however, was poor. For targeting purposes, it is best if several proteins are attached to each liposome[7,8]. From 200ug of Tf, only 1ug was associated with the liposomes. Assuming on the basis of light scattering data an average liposome diameter of 47nm, the calculated Tf:liposome molar ratio is only 1:4.

Large amounts of EDCI (20% and 40% in the final sample solution) were used to increase the amount of crosslinking. After 2 hours, an appreciable amount of self-crosslinked Tf was observed. Tf-liposomes could not be distinguished from the Tf-Tf complexes by either their elution from the Sepharose 4B column or by light scattering. This latter

method showed more variation between repeated measurements of the Tf-Tf complexes than repeated measurements of liposomes, but the variation was not significant enough to use as a criteria for the composition of the sample.

A typical sample at the completion of the crosslinking experiment consists of $3x10^{13}$ liposomes each around 47nm in diameter. For a 1:1 Tf:liposome molar ratio, 3.5ug of Tf need to be associated with the liposomes. After 2 hours of 50ug Tf in a typical liposome solution with 40% EDCI, the amount of Tf associated with the combined Tf-liposome/Tf-Tf fractions was only 0.63ug. Even if these fractions did not consist of undoubtably large amounts of Tf-Tf complexes, the calculated Tf:liposome molar ratio of 1:6 is still low. It is concluded that binding Tfs to the PE headgroups of liposomes via carbodiimide is an ineffective method for coating liposomes with Tf.

Cell studies - Tf binding

The binding and uptake of Tf by HL60 cells have been previously studied[13-16]. These cells served as a control of experimental technique and viability. From Scatchard analysis[29-36] of 2 different studies, the dissociation constant (k_d) of Tf and the TfR of HL60 cells was calculated to be $2\text{-}7x10^{-9}$M. This is consistant with the literature values of $1x10^{-8}$

- $9x10^{-9}$ M[13-16].

Four sets of binding experiments for Tf to the TfR of H-9 cells were done. Data was converted to Scatchard plots and analyzed[29-36](figure 6). Subsequent calculations of the k_d varied from $1x10^{-8}$ - $4x10^{-10}$ M. The majority of the studies indicated a k_d of $1\text{-}5x10^{-8}$M.

From Scatchard plot analysis the number of TfRs determined to be on the surface of both HL60 and H-9 cells varies for the different sets of experiments. This might be a result of different growth or activation states of the cells. The number of TfR determined to be on the surface of HL60 cell ranged from $5x10^4$ - $3x10^5$. Literature values of the TfR present on HL60 cells under similar conditions are on the order of $3x10^4$ [13]. For H-9 cells, the number of TfR determined to be on the surface of cells ranged from $3x10^4$ - $8x10^6$ with the majority of the data indicating values of $8x10^5$-$5x10^6$.

V. Comments

A promising method for crosslinking Tf to liposomes is a relatively new method[49]. SUVs of a 20:15:5 molar composition of eggPC:cholesterol: egg maleimido-4-(*p*-phenylbutyrate)PE (MPB-PE) are reacted for 2 hrs with Tf which has been thiolated with the heterobifunctional reagent Succinimidyl-S-acetylthioacetate (SATA). Capping of the reaction will follow by the addition of N-ethylmaleimide. The crosslinking of Tf and SUVs may then be analyzed by methods described earlier.

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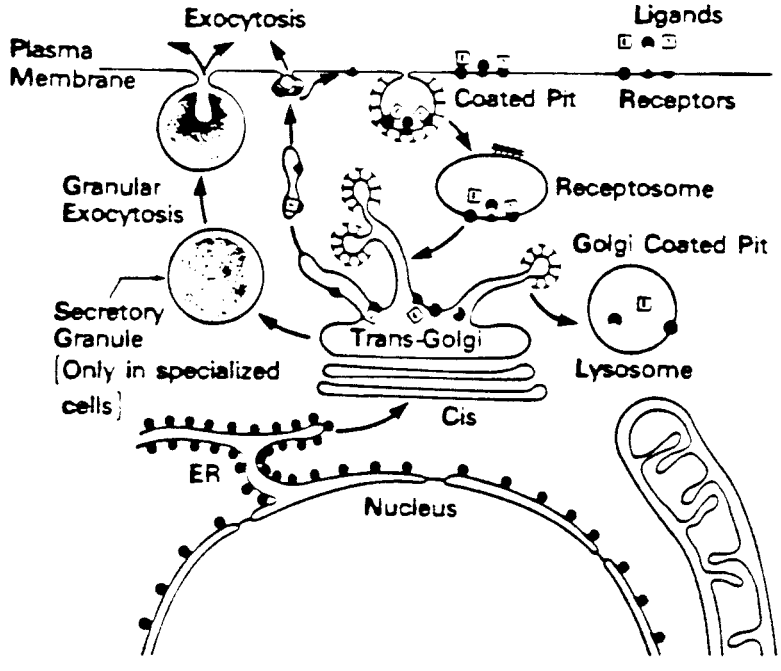


36 FIGURE 1. A diagrammatic summary of the morphological pathway of endocytosis and exocytosis in cultured cells. The morphological elements of the pathway of endocytosis and exocytosis are shown diagrammatically, but are not drawn to scale. The ligands shown as examples are (E) for EGF, (T) for transferrin, and (●) for α_2-macroglobulin. The respective receptors for these ligands are shown as (●) for the EGF receptor, (●) for the transferrin receptor, and (▲) for the α_2-macroglobulin receptor. EGF is shown as an example of a receptor system in which both the ligand and the receptor are delivered to lysosomes; transferrin is shown as an example of a system in which both the ligand and receptor recycle to the surface; α_2-macroglobulin is shown as an example of a system in which the ligand is delivered to lysosomes but the receptor recycles efficiently back to the cell surface. It is important to point out that, in some systems, the receptor may also be concentrated in coated pits in the absence of exogenous ligand and cycle in and out of the cell in a constitutive non-ligand-dependent manner.

For figures 2-5, the crosslinking procedures were carried out as explained in the text. The amount of EDCI, Tf, and liposomes (100ul ~= 3×10^{13} liposomes with an average size of 47nm and a 5:3:1 molar ratio of PC:cholesterol:PE) will be given for each figure. After a reaction time of 2 hrs (unless otherwise specified), the sample was passed through a 30ml (1.5x22.5cm) Sepharose 4B column. After collecting 8.5ml of the void volume, 1ml aliquots were collected and counted for the gamma decay of ^{125}I-Tf.

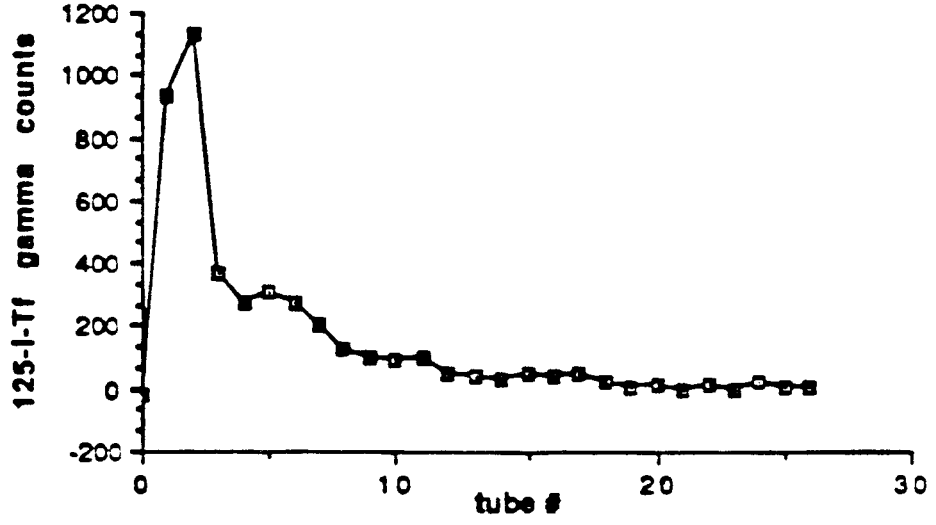


Figure 2. Tf-liposome binding experiments.
For both samples: 2ug ^{125}I-Tf, 1.77% EDCI.

TOP: In the presence of 200ul liposomes => All of the Tf appears to be coupled to liposomes

BOTTOM: In the absence of liposomes => The ^{125}I-Tf remains uncoupled. Only negligible amounts of ^{125}I-Tf are homocoupled.

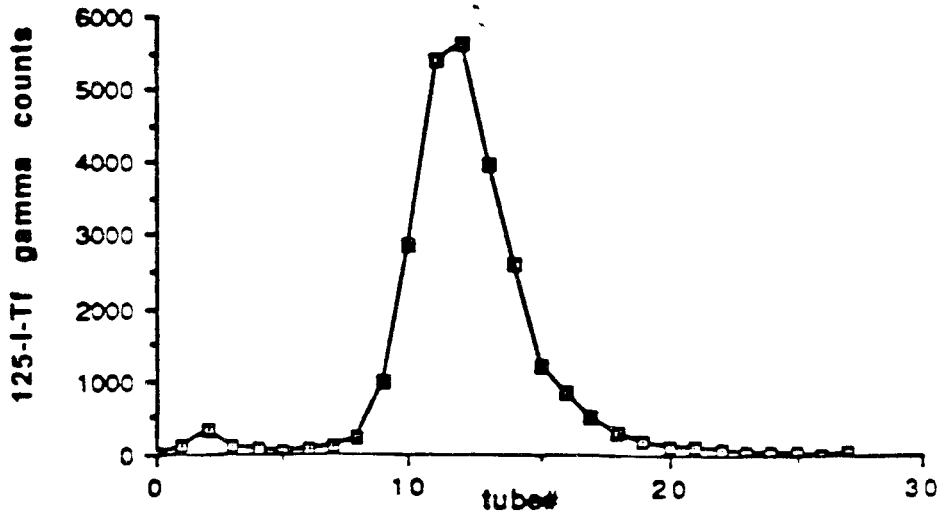


Figure 3. Tf-liposome binding experiment conducted with 200ul liposomes, 20ug ^{125}I-Tf and in the absence of EDCI. The peak represents uncoupled ^{125}I-Tf.

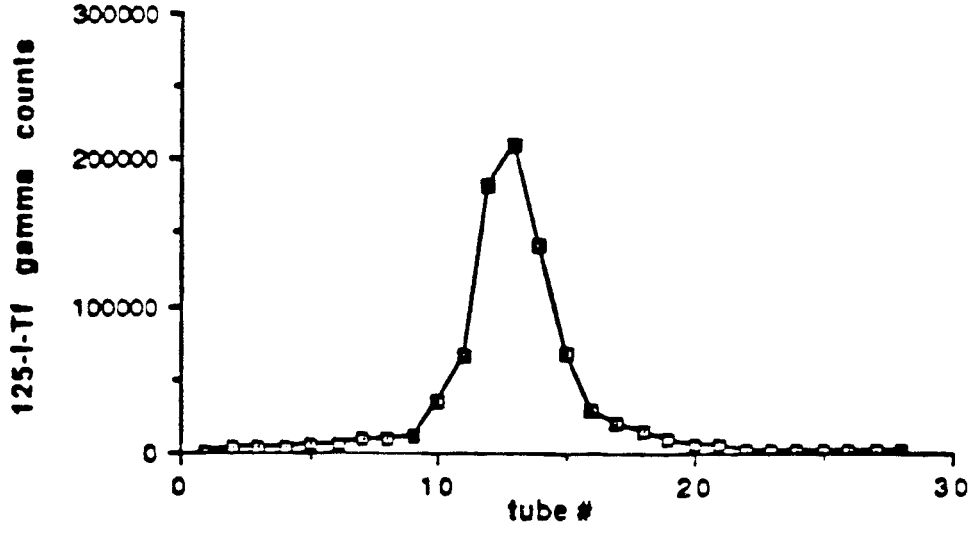
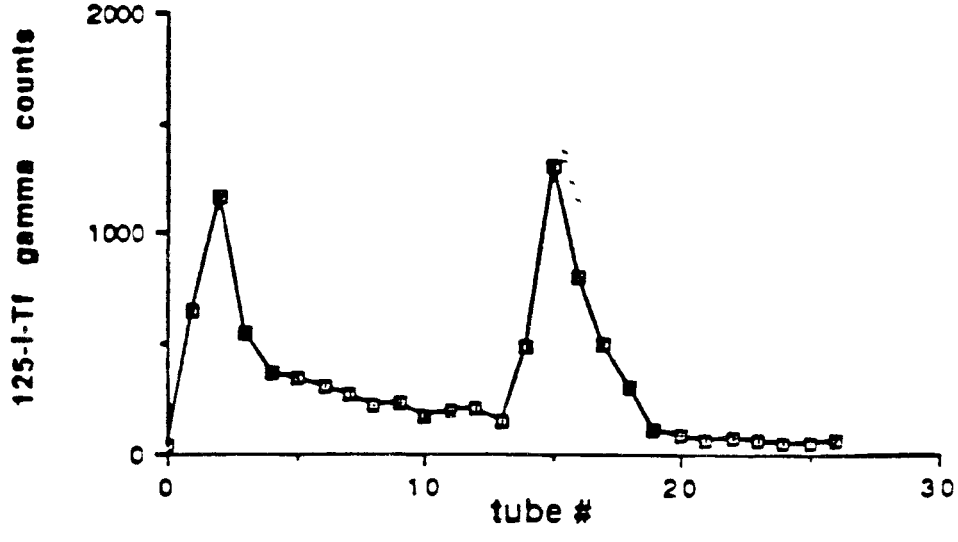
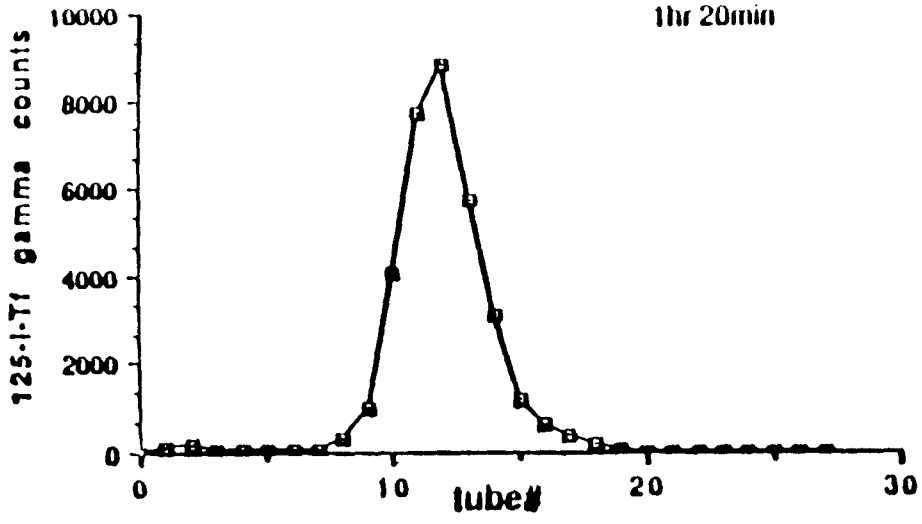


Figure 4. Tf-liposome binding experiment conducted with 6.4ug ^{125}I-Tf, 21%EDCI and in the absence of liposomes. The first peak (between collected aliquot tubes 0 and 4) represents ^{125}I-Tf-Tf homocoupled complexes. The second peak (between collected aliquot tubes 13 and 19) represent uncoupled ^{125}I-Tf.





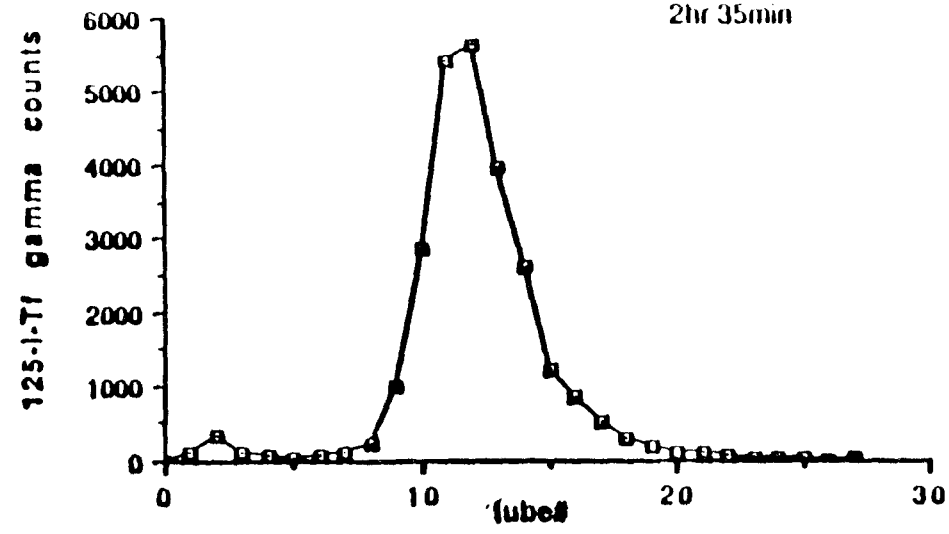
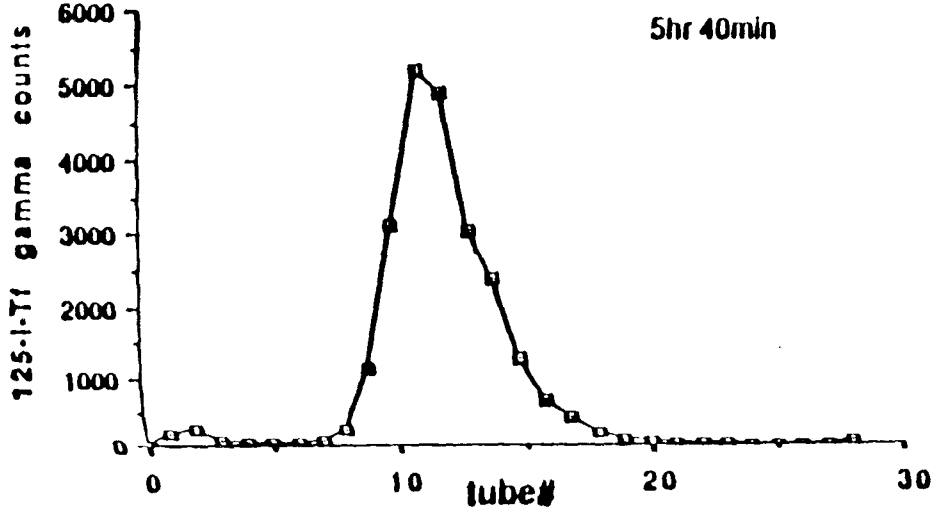
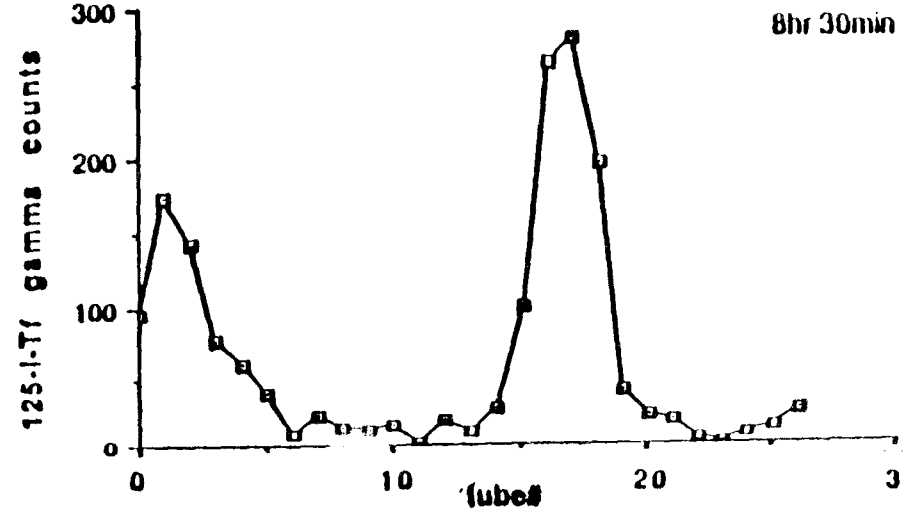


Figure 5. Duplicate runs of Tf-liposome binding experiments done in 1.77% EDCI and in the absence of liposomes. An increase in ^{125}I-Tf-Tf homocoupling (represented by the first peak which begins at tube# 0) can be seen occurring as a function of time. The second peak which begins after tube# 8 represents uncoupled ^{125}I-Tf. 2ug ^{125}I-Tf and 200ul liposome were used for each sample. Note: there is no significant crosslinking after 2 hrs.





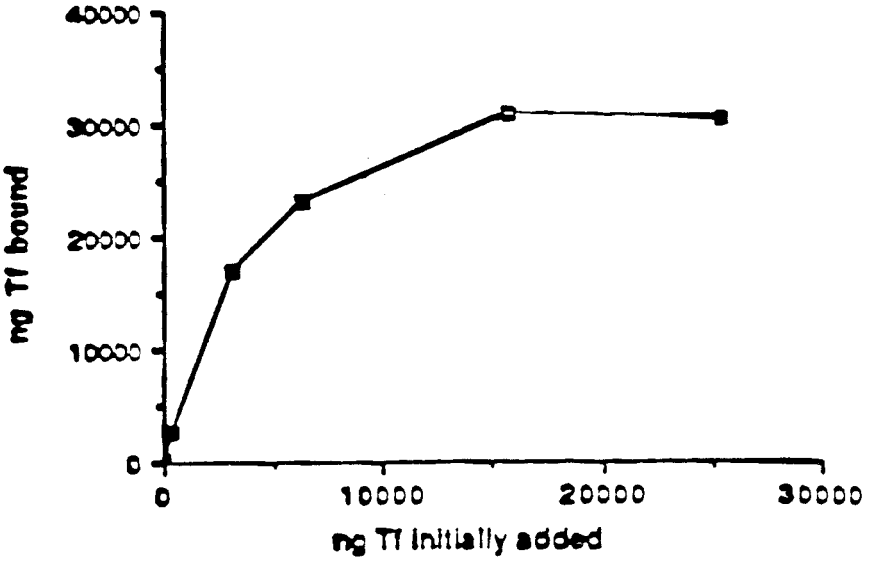
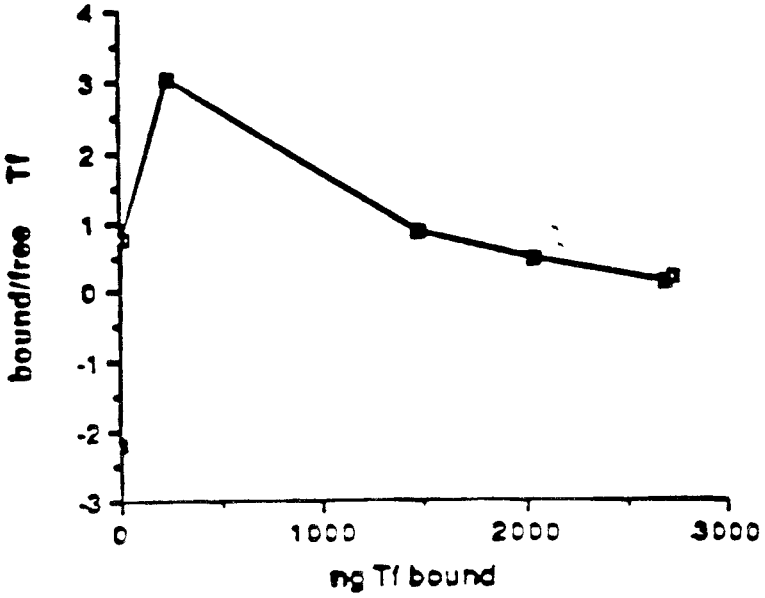


Figure 6. Graphed data from a cell binding experiment with H-9 cells and ^{125}I-Tf.

ABOVE: Various amounts of ^{125}I-Tf were incubated with 2 x 10^6 cells at 0°C for 90 min. After cells were washed with cold PBS, the radioactivity associated with the cell membrane was counted.

BELOW: The above data has been transposed to a Scatchard plot.



PROPOSITION 1

Studies Concerning the Cellular Uptake

of Indium bound Transferrin

Abstract

Proliferating cells require iron (Fe). To take up Fe, transferrin receptors (TfR) on the cell surface bind transferrin (Tf) which is capable of binding 2 Fe molecules. The TfR--Tf-Fe complex is then endocytosed into the cell. The TfR and Tf are recycled back outside of the cell while the Fe remains inside. Besides Fe, Tf binds many other transition metals[1-6]. These various metals have different affinities for Tf[1-6]. These differrent Tf-metal affinities as well as the differences in the Tf-metal configurations result in each Tf-metal complex having its own cell binding characteristics and therefore its own cell uptake characteristics as well[1-4]. Since TfR appears to be constantly endocytosed and recycled by cells whether or not Tf is attached[7], it would seem that any Tf-M complexes having similar cell binding characteristics might also have similar cell uptake characteristics.

Although Fe and indium (In) seem to bind in the same manner and with similar affinity and although Tf-Fe and Tf-In have similar binding affinities for cells, the uptake of Fe and In by cells is vastly different[3]. Previous experiments show that after incubating Tf-In with reticulocyte-rich erythrocytes, very little In is found inside of the cells[3]. An important question then is whether Tf-In somehow blocks TfR--Tf-In

uptake or whether Tf-In is taken up but the In - like Tf and TfR - is recycled back outside of the cell. Two aspects of this question will be addressed. Since the lowering of pH is considered a major reason for the Tf-Fe dissociation inside the cell[1,8-10], pH studies will be done. The pH dependent dissociation of Tf-In and Tf-Fe will be compared. Studies will also be done to determine if Tf-In is actually taken up by reticulocytes and, if so, to what degree compared to the uptake of Tf-Fe.

I. Introduction

The presence of iron within a cell is vital for the growth of the cell[1]. Tf binds iron and transports it into cells. Besides iron, transferrin will bind many other metals as well, such as indium, hafnium, chromium, cobalt, copper, gallium, manganese and zinc[1-6]. All of these metals are in the form of positively charged ions. The binding characteristics of Tf for different metals and the structures of the resulting Tf-metal (Tf-M) complexes will be different[2-4]. As a result of these differences, the binding characteristics of Tf-M to TfR will also differ.

Cell growth requires the presence of Tf for incorporation of Fe. Hence, Tf is vital for many types of cells *in vivo* and *in vitro*. Proliferating cells, whether normal or malignant, will have comparatively large amounts of TfR on their surface[1]. Reticulocytes are pre-erythrocyte cells and require Tf for their maturation into erythrocytes, which do not need Tf and have no TfRs on their surface. The best studies of Tf-In--cell interactions used a fresh suspension of reticulocyte-rich erythrocytes[3]. In order to be consistent with these previous studies, populations of reticulocyte-rich erythrocytes will also be used for the experiments proposed here. Henceforth, "cells" and "reticulocytes" will refer to this reticulocyte-rich erythrocyte suspension.

An interesting anomoly has been found involving Tf when it is assosciated with In. By competitive binding experiments, In and Fe appear to bind to primarily the same Tf sites[3]. Tf-In and Tf-Fe bind with equal strength to cells with TfR. Since the TfR is continually recycled in and out of cells independent of its binding to Tf[7], it would seem likely that Tf-Fe and Tf-In would be taken into the cell at similar rates. This, however, has yet to be shown.

Very little In is found inside cells after incubation with Tf-In[3]. This does not necessarily indicate the amount of Tf-In which has entered the cell. Once inside, In may be transported back outside of the cell along with Tf or in some other manner. A decrease in pH can be directly correlated with the dissociation of Fe from Tf within the cell[10]. Under the intracellular conditions to which Tf-In is exposed, dissociation may not occur. Another possibility is that Tf-In, although binding to TfR in an apparently similar manner as Tf-Fe, either changes the TfR conformation or induces some signal which inhibits the uptake of the TfR--Tf-In complex. For the above reasons, two important questions will be addressed here. One, does pH affect the association of Tf and In in a similar manner as that of Tf and Fe? Two, does the Tf-In complex actually enter the cell?

II. Experiments

Determination of the wavelength of the absorbance maximum for saturated Tf-In

Saturation procedures are done as explained below. Once the originally acidic Tf-In solution has reached a pH of 7.4, the Tf-In solution will be scanned from wavelength 280 - 700nm. This scan should reveal the wavelength giving a maximum absorbance for In saturated Tf. To confirm the validity of this wavelength, a concentrated In solution will be added to the Tf-In solution until a final 6M In solution is obtained. Absorbance will be measured as the concentration of In increases. If the absorbance of the 6M solution is within 10% of the absorbance from the 3M solution, the wavelength is valid for determining In saturation of Tf. If more than a 10% change is observed, new scans can be done, followed by confirmation tests as above.

pH binding study of Fe and In to Tf

Apo-transferrin will be saturated with either Fe or In by the methods of Chitamber.[4] Tf will be dissolved in 20mM acetic acid, 150mM NaCl, pH3.5. $FeCl_3$ or $InCl_3$ will be added to achieve a molar ratio

of 3 Fe or In to 1 apo-Tf. Small amounts of 1M $NaHCO_3$ will be added to the solution in order to raise the pH gradually. The degrees of Tf saturation at the increasing levels of pH will be noted by reading the absorbance at 465 for Tf-Fe and at the wavelength discerned to give the maximum absorbance for the Tf-In saturated complex. Saturation should be complete at pH 7.4.

Tf-Fe and Tf-In solutions should be of equal Tf and metal concentrations. For each Tf-M solution, the pH of a 2ml aliquot will be gradually lowered the by the addion of HCl. As the pH is lowered, the absorbance will be measured at the appropriate wavelengths and the dissociation of Tf-In compared with that of Tf-Fe.

Radioactivity pH binding study of Fe and In to Tf

If more precise binding data of Tf to metal is required, the radioisotopes ^{59}Fe and ^{111}In can be used. Saturation experiments would be conducted as explained above, and the final saturated solutions concentrated and washed several times using Amicon concentration membranes. The amount of bound metal can then be determined by counting the radioactivity. HCl can then be used to lower the pH. Following several washes, the radioactivity can be counted at that desired pH level in order to determine the amount of metal bound to Tf.

Cell system

Reticulocyte-rich erythrocytes will be obtained in the same manner as the previous Tf-In studies to which this paper earlier referred[3]. Blood will be obtained from male Sprague-Dawley rats after continued venesection. Once obtained, cells will be washed three times in cold pH 7.4 buffered saline solution (0.9% NaCl and 0.01M $NaHCO_3$). The supravital stain, brilliant cresyl blue, will be used to count the percentage of reticulocytes in the reticulocyte-rich erythrocyte solution. A percentage between 14 and 30 should be obtained.

Subcellular study - equilibrium density gradient

Tf-Fe and Tf-In will be radiolabeled with ^{125}I. Absorbance readings are taken to insure that Tf remains saturated with either Fe or In. If necessary, additional metal will be added in order to saturate Tf. Several aliquots of $1x10^5$ - $2x10^6$ cells will be incubated with 0.1mg of either Tf-Fe or Tf-In at 4^oC for 1 hour. This results in the Tf-M complex binding to cells but not in its uptake by the cells. Cells will then be washed well with cold media. To begin uptake, cells will be warmed to 37^oC. With various cell aliquots, uptake will be allowed to continue for 0, 5, 10, 15, 25 and 40 minutes. Cells will then undergo homogenization, followed by

fractionation on a 20% Percoll colloidal silica gradient[1,11,12]. Density markers will be used to determine gradient density and hence the locations of subcellular particles such as the plasma membrane, Golgi, and lysosomes. The fractions are counted to estimate and compare the cell locations of the ^{125}I-Tf, resulting from Tf-In and Tf-Fe endocytosis. This experiment will determine the level of Tf-In taken up by the cells, as well as allow this level to be compared with the Tf-Fe level of uptake.

Further uptake studies - Trypsinization

Samples will be prepared as explained above. After cells are incubated at 37^{o}C for the desired lengths of time, trypsin will be added to the cell suspensions in order to cleave off any proteins bound to the exterior of the cell. Cells will be spun down, washed, and counted to determine the amounts of ^{125}I-Tf in the cells. These experiments will also be done using ^{59}Fe and ^{111}In.

III. Conclusions

pH experiments

The first question to be answered from this study is whether In does release from Tf at low pH. If so, the next question to address is whether Fe and In release from Tf in a similar manner. Following uptake by clathrin coated pits, the TfR--Tf-M complex is found in vesicles called endosomes or receptosomes[1]. These receptosomes have been found to have a pH of ~4.5[13]. If In and Tf are found to be appreciably dissociated at pH 4.5, In should be released from Tf during endocytosis. Comparing the dissociation of the Tf-In complex to that of Tf-Fe may also lead to further insights on the intracellular requirements needed to dissociate Fe from Tf as well as the nature of this process. For example, if pH does not affect the dissociation of Tf-In in the same way as it does Tf-Fe and if Tf-In is not dissociated at pH 4.5, Tf may never release In during its intracellular cycle. This would be a further confirmation of the importance of pH for the intracellular dissociation of Tf-Fe. The above scenario is one possible explanation for only small amounts of In being found inside of cells after incubation with Tf-In.

Subcellular - equilibrium density experiments

The first question which must be answered from this study is whether Tf-In is endocytosed. This is the central question of this proposal. If Tf-In is endocytosed, the uptake of Tf-Fe and Tf-In by cells will be compared. The experimental results obtained will indicate whether similar amounts of Tf-Fe and Tf-In are being taken up by cells and whether the two intracellular pathways are similar.

Future studies

If it does appear that Tf-In is endocytosed, In must also be exported. Further studies examining the intracellular pathway of Tf and In are in order. Some methods which may be employed in these future studies are fluorescence studies with fluorescently labeled Tf as well as fluorescently labeled Abs to Tf and TfR and perhaps even to Tf-In. Light microscopy studies using colloidal gold or hydrogen peroxidase as well as electron microscopy studies may provide more detailed information as to the Tf or Tf-In intercellular locations. One other very valuable technique is that of cell fractionation. Some fractionation studies have been proposed here, but if endocytosis of Tf-In is confirmed, more detailed studies are in order. Cells can be fractionated to a further extent than has been proposed by applying successively higher g forces and lengths of time

for centrifugation. The different intracellular components can then be analyzed by Abs, or for more precision, analyzed by radioactivity using ^{125}I-Tf and ^{111}In.

If the experiments described in this study show that Tf-In is not taken up by cells, many experiments can be done to begin understanding why the uptake of the TfR--Tf-In does not occur. Structural studies of complexes of Tf with many metals (i.e. Fe, In, Hf, Cr, Cu, Zn, ...) should be done. These should be followed by structural studies of these Tf-M complexes with TfR. The best structural studies employ crystallography. Other less time consuming and less complicated methods should be tried initially. Computer simulation may be of use. The Tf-M structures should be compared, looking for differences which might result in the reduction or inhibition of its binding to and /or uptake by cells. The hypotheses should then be checked with the known binding and uptake tendencies of the Tf-M by cells.

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PROPOSITION 2

The Effect of Heat Shock Proteins on Untreated Cells

Abstract

Heat shock proteins (hsps) represent a relatively new and unexplored scientific field. They are induced in cells exposed to any of a wide variety of stresses. Hsps are not confined to a specific cell or a specific species but are conserved throughout many life forms. There are many families of hsps ranging from the small ubiquitin protein at 8kd to a family of hsps around 110kd. Cells produce hsps of different families and often different hsps within a family. Though they are induced after exposure to stress, such as a 5^{o}C increase in temperature for 30min, the processes involved in this induction and the subsequent down-regulation are still not known. Also unknown is whether the presence of one hsp might affect the regulation of another. It seems very possible that one hsp might intiate the induction or repression of itself or another hsp. This research proposes to determine if hsps participate in their own regulation.

In order to investigate the possible self regulatory role of hsps, Chinese hamster ovary (CHO) fibroblast cells will be used. The cells will be incubated at elevated temperatures in order to induce hsp production. Hsps will be isolated and added to cells which have not been exposed to elevated temperatures or other hsp inducing conditions. Protein synthesis

in these cells will be monitored by the use of ^{35}S-methionine labeling and 2-dimensional gel electrophoresis. By incubating CHO cells with varying amounts of one or more hsps and by comparing the amount of proteins synthesized, in particularly hsps, regulatory relationships between hsps may be revealed and insights may be provided into the mechanisms of their regulation and their possible role in regulating the synthesis of other proteins.

I. Heat Shock Proteins - Background

The first evidence of heat shock proteins can be traced back to 1962 when Ritossa observed chromosomes of drosophila to be puffed following exposure to mild heat[1,2]. This observation was not thought to be of great importance by most of the scientific community. The importance was greatly magnified by Tissieres and Mitchell in 1973 when they showed that these "puffs" were associated with the synthesis of new proteins[1,2]. This phenomenon was thought to be confined to Drosophila until 1978 when similar responses were observed in E. coli, Tetrahymena, and both avian and mammalian tissue culture cells[1].

Hsps are named according to their molecular weight. There are three major families of hsps: hsp83-90, hsp70, small hsps (20-30kd)[1]. The

hsp70 family is the most universally prominent[1-3]. It seems to be important for protein synthesis and is essential to some cells even under normal growth conditions[1,4]. Hsp83 has a transient association with retroviral transforming proteins[1]. It has been hypothesized that hsp83 serves as a shuttle for intercellular proteins[1]. At least one, but usually more than one, small hsp is produced by all organisms[1,4]. Plant cells in particular produce many small hsps[1]. There are also other hsps outside of these 3 families. Ubiquitin (8kd) has been found to be induced by heat and other cellular stresses[5-9]. Some cells, particularly mammalian cells have been found to produce hsps in the range of 100 - 115kd[1,10-13].

The genes for these hsps are highly conserved, whether derived from animals, plants, or bacteria[1-4]. The temperature increase needed to induce these proteins varies between 3 and 15^{o}C depending on the species, the type of cell, and the degree of heat shock desired (for example, at lower temperatures the process is reversible)[1,14-17]. Chemicals such as certain drugs and metals also induce the synthesis of hsps[11-13,17-20](Figure 1). Hsps appear to be a defense mechanism of cells responding to stress. Therefore, as might be expected, hsps are associated with inflammation[2] and viral infection[1,4]. They might also be involved during embryonic development and have been found to have protective properties against cancerous tumors. However, the natures of

all of these associations are still unknown.

Understanding the functional roles of these hsps could be of great importance. If they truly protect cells from harmful stress, perhaps their mechanisms or simply their induction could be used in treating diseases such as cancer or viral infections, or developmental defects. Understanding the mechanisms involved in hsp action might increase our understanding of inflammation or so many other processes where the detailed biochemical and molecular biological mechanisms are not fully known. Understanding these protective processes of a cell might bring new possibilities for medical treatment of many diseases.

The functional roles of the hsps and the regulatory processes responsible for their induction and repression are still very unclear. For the most part, studies of hsps have resulted in an abundance of disjointed information. The degree of synthesis of the various hsps depends on the specific type of cell as well as the amount of inducer[4,16]. Different cells may react differently to the same stress, whether they are different cells from the same species or analogous cells from different species[1,4,16]. Different cells may also react in similar ways. No correlating factor has been found for predicting hsp production. In fact, some cells have small amounts of one or more hsps - especially hsp70 - even without stress inducement[22]. For at least some cells, hsps are vital for life even under

normal growth conditions[22]. Obviously, there remains much to be learned about hsps.

One big area in question concerns the regulation of hsps. What chemical processes cause their induction and their repression? Are hsps involved in their own regulation? It seems possible that a hsp could be a part of a cascade, regulating the induction or repression of itself or another hsp or other proteins. To study the regulatory ability of these proteins, hsps will be isolated under gentle conditions in order to minimize denaturation. Cells will be incubated with one or more hsps. The incorporation of hsps by the cell and the production of newly synthesized proteins, in particularly hsps, will then be analyzed. Hsps may not be taken up by cells. If they are, the efficiency of uptake may be poor. Furthermore, once inside the cell, the hsps may not reach the cytosol or a necessary organelle. For these reasons, it will be important to examine the effects of hsp when they are placed inside the cytosol, by a method such as electroporation, rather than being taken up by the cell.

II. Experimentation

Cell Line

Chinese Hamster Ovary (CHO) cells were chosen as the experimental

cell line for these experiments for three primary reasons. First, considering various cell lines, CHO is one of the most widely used mammalian cell lines for studying hsps. Second, there appears to be good, representative levels of hsps in the cell cytoplasm. Third, CHO cells have undergone initial electroporation studies which have succeeded in transfection of DNA. CHO cells will be grown in Eagles Media, supplemented with 10% fetal calf serum, pennicilin, streptomycin, and 50ug/L gentamycin and kept in a humidifier incubator at 37^{o}C with 95% air, 5% CO_2[20].

Isolating intact hsps

Antibodies have been made to all classes of hsps[1]. Since the focus of this proposition is not on the production of Ab's, it will be assumed - and not unreasonably so - that Ab's can be obtained. Depending on the stage of their induction, hsps are primarily found in the cytoplasm or the nucleus[1,20]. Recently, however, there is evidence of high levels of hsps associated with other cellular compartments[10-23]. To isolate hsps in a simple and gentle manner, cells which have been heat shocked will be fractionated in order to separate out the cytosol with its soluble components from the remaining cellular material. To begin, cells are disrupted by lysis by resuspension in media suplemented with 1% Triton X.

Cells are then homogenized, followed by centrifugation for 120 minutes at 100,000g. Abs to hsps are attached to cyanogen bromide activated Sepharose 4B beads. The Ab-Sepharose is mixed with the cytosol solution and then poured into a Buchner funnel and washed with buffered saline (pH7.4). Buffers at pH2-5 will be used to elute and wash the hsps into a pH8 buffer. By using a funnel rather than a column and by eluting into a neutralizing buffer, hsps will be quickly isolated with a minimum of exposure to the harsh acidic conditions. The buffered solution with the collected hsps will then be concentrated via Amicon membrane ultra filtration. Aliquots of the cytosol suspension before elution from Ab-Sepharose and after elution at different acidic pHs will be subject to 2-dimensional electrophoresis in order to determine and compare the recovery of the various hsps and to detect possible adverse affects of low pH. Cells will also undergo more detailed fractionation by applying different g forces and times of centrifugation in order to isolate subcellular components of the noncytosolic material. 2-dimensional electrophoresis will follow in order to determine the hsp composition in the various subcellular components and to determine if the cytosol isolated hsps give a good representation of the complete set of hsps in the cell.

The hsps families will be separated from one another by gel

filtration with a Sephadex G-150 column. Initially, ^{35}S-methionine labeled hsps will be used so that the protein concentration in the collected, elution aliquots can be determined. The appropriate collections of eluate will then be concentrated via Amicon membranes. After sufficient concentration, the final protein concentrations will be approximated using the Lowry technique.

Endocytosis and incubation studies of hsps with CHO cells

An all important question which must now be addressed is whether the hsps will be endocytosed by the CHO cells. Perhaps the simpliest and least biologically altering way to label hsps for endocytic studies is to initially grow the hsp producing cells in the presence of ^{35}S-methionine which in turn will be taken up into newly synthesized proteins i.e. hsps. These ^{35}S labeled, isolated hsps are then incubated with cells in ^{35}S-methionine free culture. The incubated cells will be fractionated and the various fractions analyzed with 2-dimensional electrophoresis and autoradiography in order to determine the amount of endocytosis of each hsp as well as the general location of the endocytosed hsps.

After the level of endocytosis of each of the hsps has been established, the next question to be studied is whether incubation of cells with hsps (unlabeled) affect the synthesis of proteins at either normal or

hsp inducing temperatures. The most prominent hsp, especially for synthesis, seems to be hsp70. So far, every hsp producing cell known produces hsp70. Some cells require hsp70 for normal growth even at normal temperature. Therefore, the most interesting result may come from incubation of cells with this hsp alone. For example, hsp70 might be an initial component of a cascade inducing hsps. Small hsp's as well as hsp87 and hsp110 will also be incubated separately with cells. Just prior to aliquoting the cells, a total of 30uCi (specific activity ~1200uCi/mmol) of ^{35}S-methionine per cell aliquot sample will be added to the 'methionine-free' MEM cell suspension . For each sample, 10^5 to $2x10^6$ cells will be incubated with 0.01, 1, 25 or 200 ug of the appropriate hsp. The same experiment will also be done by incubating the cells with all the hsp families together. Incubation times will be 15, 30, 60, 120 and 180 minutes. For a control, the same number of cells will be incubated under the exact same conditions, but in the absence of hsps. Both controls and samples will be subject to 2-dimensional electrophoresis followed by autoradiography in order to compare the synthesis of hsps as well as any other profound changes in protein synthesis.

hsps added to the cytoplasm - Electroporation

Experiments which add hsps to the cytoplasm are also in order. Previous studies have used electroporation to transfect CHO cells with DNA[24]. Therefore, it would seem likely that hsps could be introduced into the cells by this method. For other reasons, electroporation may be the best choice. For example, the recovery time for the cells is on the order of seconds as opposed to that of other methods such as osmotic perforation which requires minutes to hours for recovery[24,25]. Time is very important since the induction and down-regulation of hsps can be a rapid process (<30min). In the previous study mentioned above, the cell density used was 1:20 packed cells:solution(media). The most homogeneous results - as far as each cell being subjected to the same field strength - were found when applying a pulse of 4000V/cm with a pulse length of 2-5ms. This gave a transfection frequency of ~$2x10^{-4}$. Decreasing the pulse or the pulse length will broaden the range of field strengths acting on the cells but will increase the transfection frequency to ~$1x10^{-3}$. Before applying electroporation to the task of incorporating hsps into the cells, various sizes of control proteins corresponding to hsp weights will be used to establish the best pulse to use with a pulse length of 3ms. Cell vitality will be monitored with trypan blue.

As in the incubation studies, electroporation will be conducted with

each family of hsps individually as well as all hsps together. ^{35}S-Methionine will also be introduced to the cell media just prior to electroporation. Since electroporation itself may induce the synthesis of hsps, cells in solution free of hsps will undergo electroporation as well. The protein synthesis of these control cells and the above described sample cells can then be compared using 2-dimensional electrophoresis and autoradiography.

III. Discussion

From the incubation of hsps with CHO cells much may be learned about the regulatory roles of hsps, both individually and collectively. At normal temperatures, either an increase or a decrease in hsp synthesis would be of great interest. An increase in protein synthesis at normal temperatures would indicate that hsps are involved in upregulation. A decrease in hsp production might imply that hsps are involved in down-regulation. It might also imply that hsps are important for normal cell functioning. This would be the case if the excess hsps shut down normal production before these added hsp(s) have been incorporated into the necessary intracellular sites to continue protein synthesis. More extensive time dependent studies may be required in order to see this type

of relationship.

The results of experiments conducted at temperatures which normally induce hsps will also be of great interest. The results will probably be larger simply because of the increase of hsps produced at the higher temperature. A decrease in hsp synthesis would indicate that hsps down-regulate their own production. Similarly, an increase in hsp synthesis would imply up-regulation.

Down-regulation is a common biological mechanism. Whether a hsp is down regulated by itself or another hsp would be of great regulatory interest. Inter-relation between hsps would also be of great interest. Since all experiments, both at normal and elevated temperatures will be conducted with individual hsp families as well as all hsps together, inter-regulation should be observed if it is present. An important aspect of these studies is to observe if there is any significant effect of hsps on the synthesis of non-hsp proteins. If the latter are down regulated it would seem that hsps are somehow involved in the down-regulation of normal cellular proteins. Another factor which might be important is the amount of hsp(s). Small amounts may induce hsp synthesis whereas large amounts may reduce or stop hsp synthesis.

If only large amounts of hsp are found to reduce or stop hsp synthesis, a logical question to ask is how much hsp is required to effect

synthesis. Is the effect gradual or is it sudden? Is the amount of hsp needed to effect hsp synthesis related to the degree of stress the cell has undergone? For example, cells incubated at 4°C above normal may require less hsps to initiate down-regulation than cells incubated at 8°C above normal. These latter questions are a few which may arise from the studies proposed. The study of hsps is new, with many questions yet to be answered and many more questions yet to be asked.

Figure1: A partial list of components which have been shown to induce or not to induce hsp synthesis.

Heat Shock Protein Inducers[1,11-13,17-20]

Heat	Metals	Chelating Drugs
3-15 C	Cu^{2+}	Kethoxal bis(thiosemi-
	Zn^{2+}	carbazone
	Cd^{2+}	Disulfirm
	Hg^{2+}	

Vicinal Sulfhydryl reagents	Amino Acid Analogous
Sodium Arsinate	Canavanine
Iodoacetamide	
p-chloromercuribenzoate	
p-chloromercuribenzoate sulfonate	

NONINDUCERS

Co^{2+}
Ni^{2+}
Fe 3+ & 2+
Mn^{2+}
Pt^{4+}
Pb^{2+}

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