LIPOSOME MEDIATED DELIVERY OF 2-MERCAPTOPROPIONYL GLYCINE (MPG) AND CARCINOGENESIS RESPONSE MODULATION

ABSTRACT

By

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DEPARTMENT OF BIOCHEMISTRY

THESIS

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ABSTRACT

The goal of cancer therapy is to eliminate the neoplastic cells without causing any appreciable damage to the normal tissue of the host. Two possible approaches are there in this therapy:

1. Removal of the neoplastic cells/tissues from the host system.
2. Reversal of the process of cellular transformation.

Removal of the neoplastic cells is effected most efficiently by surgical means that has its own obvious fall out. Use of physical, chemical or biological cytotoxic agents are other avenues with their respective limitations. For instance, treatment involving cytotoxic agents is hazardous since they cause serious damage to normal cells. The possibility of reversing the neoplastic cellular transformation may be a better approach but much remains to be done before it can be applicable on population scale. Non-surgically eliminating cancerous growth may be a practical avenue open to us at the moment.

In this non-surgical approach, use of radiations has been one of the best possibilities. The ability of radiations to kill cells by an array of metabolic damages that it causes has been successfully employed to cure cancerous and malignant growth. Radiations, however, act as double edged sword. Since interaction of radiation with matter is random, the radiation interacts with all types of cell - both normal and transformed when a subject is irradiated. Thus, all cells accumulate damages. While the damages transformed cell may not survive and, thereby, cancer may be eliminated, the partially damaged normal cells become a problem. There exists a great probability that these partially damaged normal cell may undergo transformation in due course of time. Several improvisations in the radiotherapeutic protocols, use of different quantities and qualities of radiations and applications of fractionated irradiation schedules have only marginally improved the present clinical efficacy of radiotherapy. It appears that radiotherapy has reached its zenith.

To overcome the limitations of radiotherapy and to improve upon its clinical gains, use of radiomodulatory drugs along with radiation, appropriately named as chemo-radiotherapy, have yielded positive therapeutic advantages in cancer therapy. Two classes of radiomodulatory drugs are in existence in the context of radiotherapy:

1. Radioprotective drugs - This class of drugs essentially should protect normal cells/tissues from the undesirable damaging effects of radiation, thereby, paving way for application of higher doses of radiation for efficient killing of cancerous cells/tissues.

2. Radiosensitizing drugs - The drugs belonging to this class should, in principle, sensitive cancerous cells/tissues so that they are killed even by relatively low doses of

radiations, thereby, reducing the undesirable damages to normal cells.

Chemo-radiotherapy has, indeed, improved the rate of cure of cancer. However, the toxicity of drugs to other tissues, lack of control on the quantity of drug in cancerous tissues, metabolic alteration of drug, non-specific protection/sensitization of tissues, various other side effects have come in way of optimum application of chemo-radiotherapy regimes in clinical practice. In last about two decades, concept of drug delivery system has been tried to overcome the limitations of chemo-radiotherapy regimes to further improve upon it. The concept is based on the fact that certain biologically acceptable carrier may be used to carry the drugs to biological target cells/tissues. Of several possible carriers, liposomes have been found to be promising and convenient.

In the piece of work embodied in this thesis, attempt has been made to test the suitability of liposome as a vehicle for carrying a drug. As it is an exploratory work, 2-mercaptopropionylglycine (MPG), a radioprotective drug, has been used as a model drug. The liposome encapsulated MPG (LEM) is envisaged to be tested for its radioprotective efficacy in normal and transformed system vis-a-vis the free form of MPG. Attempt is also proposed to look into some molecular events triggered by MPG or LEM.

The results embodied in this thesis opens up a possibility of use of liposome as a carrier for radio-modularoty drugs for use in chemo-radiotherapy. The results very emphatically establish that using liposome carrier may offer significant clinical gains in chemo-radiothrapy. The model drug used in this investigation was 2-mercaptopropionylglycine (MPG), a moderate radioprotective drug that has been shown to be a potential radioprotector both in experimental conditions and in clinical trials. The following are the salient findings emerging from this work:

1. The radioprotection affordable by MPG was enhanced when an equivalent amount of drug was administered through liposome vehicle (chapters 2-5).

2. Of several methods for preparation of liposome to encapsulate MPG, the reverse-phase evaporation method was found to be highly convenient, reproducible and effective (chapter 2). The preparation of liposome by this method involves common and simple laboratory equipments and may be performed at any place.

3. The method offers MPG encapsulation into liposome at a rate of over 50 % of the starting concentration of MPG, thus, qualifying to be an efficient method for MPG encapsulation. Five or 10 mM starting concentrations of MPG was found to be optimum for liposome encapsulation (chapter 2).

4. The liposome encapsulated MPG (LEM), as against its free form, afforded significantly higher protection to normal tissues on biological end-points such as, (a) viability of bone marrow and spleen cells and (b) on enzyme acetylcholine esterase (chapter 3).

5. The same was the case in cancer induced or transformed mice. The biological end-points tested for this investigation were cellular glutathione and enzyme γ-glutamyltranspeptidase. On both these limits, the LEM afforded better radioprotection that MPG (chapter 4).

6. It has been shown that MPG was able to influence chromatin organization differently when it was administered in its free form or as LEM (chapter 5).

7. The presence of MPG or LEM was also reflected by the level of cellular poly-ADP-ribosylation, assayed by a new immuno-dot blot assay developed in this investigation (chapter 5).

8. The results point out to the fact that chromatin was poised for better repair in transformed mice when MPG or LEM were administered prior to irradiation (chapter 5).

9. On the parameters of chromatin organization and poly-ADP-ribosylation, MPG and LEM affordable radioprotection was almost similar (chapter 5).

10. Overall, the radioprotection afforded by LEM was higher that free form of MPG (chapters 2-5).

The findings presented in this thesis may have significant impact on the clinical use of radiomodulatory drugs in chemo-radiotherapy as liposome encapsulation could enhance the effectiveness of MPG. A potential use of liposome carrier, however, will be with radiosensitizing drugs. It is known that most of radiosensitizing drugs are highly toxic to normal tissues. Notwithstanding the toxicity problem, use of radiosensitizing drug in chemo-radiotherapy will be more advantageous than that of radioprotective drugs. Liposome as a carrier may be an easy solution to this problem. By liposome encapsulation, the radiosensitizing drug will not be immediately available to other tissues, thus, reducing its toxicity. In addition, it could be visualized that a radiosensitizing drug after liposome encapsulation may be targeted to cancer tissue. Therefore, the cancerous tissue will be sensitized and even a lower dose of radiation may be able to produce enough damage to kill it. Some avenues of specific tissue targeting using immuno-liposome are being investigated.

Even though the work described in this thesis relates to MPG, a radioprotective drug, the findings are of significant clinical value and open up a new line of thinking for improvisation of chemo-radiotherapy.

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Dedicated
to my parents

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To whom it may concern

Certified that the thesis titled **Liposome Mediated Delivery Of 2-Mercaptopropionylglycine (MPG) And Carcinogenesis Response Modulation** submitted by **Sanghamitra Chakraborty** for the award of degree of **Doctor of Philosophy** in **Biochemistry** of the North Eastern Hill University embodies the records in original investigations carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the degree of Ph.D. This work has not been submitted for any degree of any University.

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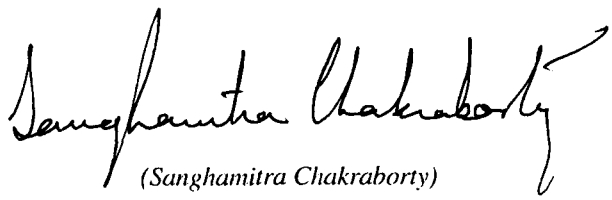

(Sanghamitra Chakraborty)

LIST OF ABBREVIATIONS

%	Percent.
AChE	Acetylcholine esterase.
ADP	Adenosine diphosphate.
ADPR	ADP-ribosylation.
AEBN	Aqueous extract of betel nut.
BMC	Bone marrow cells.
BSA	Bovine serum albumin.
C	Control (untreated).
Chol	Cholesterol.
DCP	Diacetylphosphate.
DD	Detergent dialysis.
DNA	Deoxyribonucleic acid.
DNase I	Deoxyribonuclease I.
DPPC	Dipalmytoylphosphatidylcholine.
DTNB	Dithio nitro benzoic acid.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetraacetic acid.
Fig	Figure.
g	Centrifugal force.
γ	Gamma.
g	Gram.
GGT	Gamma glutamyl transpeptidase.
GSH	Glutathione.
Gy	Gray.
h	Hours.
LEM	MPG encapsulated in liposome.
M	Molar
μ	Micro.
mA	Milliampere.
MADPR	Mono-ADP-ribosylation.
MEM	Minimum essential medium.
mg kg^{-1}	Milligram per kilogram.

µg	Microgram.
mg	Milli gram.
min^{-1}	Per minute.
min	Minute.
ml	Milliliter.
ml^{-1}	per milliliter.
MLV	Multilamellar vesicles.
mM	Millimolar.
MPG	2-Mercaptoprionylglycine.
nm	Nanometer.
p-value	Probability-value (t-test).
PADPR	Poly ADP ribose.
PADPR	Poly-ADP-ribosylation.
PBS	Phosphate buffered saline.
R-buffer	Reaction buffer.
RD	Rehydration dehydration.
RPE	Reverse phase evaporation.
SC	Spleen cells.
SD	Standard deviation.
Sec	Second
SEM	Standard error of the mean.
SF	Survival fraction.
ssb	Single strand break.
TCA	Trichloroacetic acid.
TE	Tris-EDTA (buffer).
Tris	Tris (hydroxy methyl) aminomethane.
ULV	Unilamellar vesicles.
UV	Ultra-violet.
V	Volt.
VMF	Viability modification factor.
°C	Temperature expressed in degree centigrade.

*****

The goal of cancer therapy is to eliminate the neoplastic cells without causing any appreciable damage to the normal tissue of the host. Two possible approaches are there in this therapy:

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Aim and objectives

1. **Development of suitable method for liposome preparation:** Chapter 2 describes results of study designed to develop an convenient method of preparation of liposome to encapsulate MPG.

2. **Biological activity of the liposome encapsulated MPG (LEM) and MPG in normal tissues:** The biological effectivity of MPG and LEM as radioprotector are proposed to be estimated using suitable biological end-points to establish whether LEM offers advantage over MPG or not. This has been describe in chapter 3.

3. **Biological activity of the liposome encapsulated MPG (LEM) and MPG in cancerous tissues:** As normal and cancerous cells differ significantly in their biochemical characteristics, experiments are proposed to be designed to ascertain radioprotective benefits of LEM over MPG in cancerous situation as detailed in chapter 4.

4. **Molecular events associated with radioprotection afforded by LEM and**

MPG: Chapter 5 describes the effects of LEM and MPG on the organization of chromatin and a factor that influence it in order to attempt to understand the radioprotection at molecular level.

*****

2.1 INTRODUCTION

In spite of the enormous amount of efforts on development of new drugs for treatment of diseases, none of the drugs available to this date is free from undesirable biological effects. The latter primarily arises from random toxicity, distribution and nonspecific binding of the drug with undesirable targets in the biophase. In addition, metabolic alterations of drug may also render it ineffective. A logical approach to overcome these problems of chemotherapeutic agents would be to selectively deliver these drugs to the diseased or defective tissue in controlled manner. This may be achieved by using suitable drug delivery systems. Administration of the drug after incorporating them into appropriate delivery system, vehicle, or carrier will make them latent in biophase and would thus protect them from biodegradation or metabolic alterations, inhibit their binding with nonspecific sites and reduce toxicity. The basic requirements of an ideal drug vehicle, thus, are the following:

(a) The carrier should accommodate in its structure sufficient amount of drug either by internalization or by suitable binding of the drug.
(b) The surface properties of the carrier should be such that the release of drugs in biophase can be regulated.
(c) The structure of the carrier should easily accommodate specific molecules (or macromolecules) for transfer to the target sites.
(d) The carrier should be biodegradable.
(e) The chemical constituents of the carrier should not be toxic.

Several possibilities are presently being investigated for their use as drug carriers. Amongst these, microcapsule prepared from polymeric materials, linking of drug with macromolecules (such as, DNA and glycoproteins), cells, sealed erythrocyte, ghosts and liposomes have shown promising results. The biodegradable nature of liposomes along with its ability to associate directly with the target molecules in their structure make them an attractive carrier for use in drug delivery system. Liposomes have been studied as a drug delivery system in the last two decades. Most of its applications have been based on the ability of the liposomes to preferentially migrate to reticuloendothelial systems (RES) or tissues such as, liver and spleen (Vertut-Doi *et al.*, 1996). However, avoiding degradation of liposomes by mononuclear phagocytic system has been a problem (Vertut-Doi *et al.*, 1996). Nonetheless, in principle, liposome as drug carrier for targeted delivery has potentials of applications in chemotherapy of cancer.

2.1.1 Liposome-Composition, Structure And Physical Properties

Liposomes are sphere like structures that possess internal volume and are essentially made up of phospholipids. Their biological properties are to a limited extent controlled by the nature of

their constituents. Most common lipids used for preparation of liposomes are listed in table 2-1.

TABLE 2-I

Chemical Characteristics Of Selected Lipids

NAME	*MOL. WEIGHT (Da)*	*Tc (°C)*	*CHARGE*
Cholesterol (Chol)	387	nk	0
Phosphatidylcholine (PC)	762	- 15	0
Dipalmitoylphosphatidylcholine (DPPC)	734	+ 41	0
Dicetylphosphate (DCP)	547	nk	-
Phosphatidylserine (PS)	760	+ 5	-
Phosphatidic acid (PA)	700	nk	-
Stearylamine (SA)	269	nk	+

(nk = not known)

When phospholipids are dispersed in water they form molecular aggregate due to their amphipathic character. The structure of this aggregate may either consist of bilayers or micelles, depending on the nature of phospholipids and degree of hydration. Among the natural phospholipids, lecithin is known to readily form bilayers (Perez-Soler, 1989; Bachhawat, 1991). Thus, dispersion of lecithin in water form closed structures comprising of a series of concentric bilayers alternate with aqueous compartments. Such structures are called multilamellar vesicles (MLV). Unilamellar vesicle (ULV), comprising a single bilayer, is formed upon brief ultrasonic treatments of multilamellar preparations. The size of MLV varies between 1 and 5 µm whereas that of ULV between 0.05 and 0.2 µm.

MLV forms spontaneously upon addition of an aqueous solution to a preformed lipid film or lipid solution. MLV can passively entrap lyophilic molecules with high efficiency. On the other hand, since the hydrophilic compartment is small (< 10 %), MLV are generally not good carrier for water soluble molecules. Very little information is available on the chemical structure characteristics required for a satisfactory incorporation of drugs into MLV. No studies have yet defined how different substituent groups in a given molecule may effect its interaction with the lipids commonly used for preparation of liposomes (Perez Soler, 1989; Mayhew, *et al.*, 1978; Ryman and Tyrell, 1980). However, available evidence suggests that because of the avidity of the lipids, many lipophilic molecules may be efficiently entrapped within the lipid bilayer of MLV at a lipid:drug ratio (w/w) of 15:1 to 25:1 (Perez Soler, 1989; Yatvin and Lelkes, 1982). This rule has, nonetheless, two important exceptions. Some drugs, though lyophilic, do not

interact with the lipid components of the bilayers or cannot be accommodated in the space between the phospholipid molecules and consequently precipitate outside the vesicles. In other cases, liposomes cannot be prepared because the water insoluble drugs are not soluble in the common organic solvents used for the manufacture of the lyophilized mixture that form MLV upon reconstitutions.

ULV are better suited than MLV to carry hydrophilic molecules because their aqueous space is larger than their lipid space. However, the passive entrapment efficiency of the vesicles is always suboptimal unless a positive gradient type of loading is used. Water dispersions of phospholipids possess unique thermal properties below a certain temperature called "crystalline thermal phase transition temperature" or T_C. Below T_C, the phospholipid structure remains highly ordered whereas at or above T_C the structure becomes greatly disordered. This property of phospholipids is termed as their thermal phase transition behavior. The bilayer possessing phospholipids in disordered form is known as "liquid crystalline phase" (or fluid phase). Liquid crystalline phase of the bilayer is much more permeable to solute as compared to that of the gel crystalline state. However, the permeability is reduced if cholesterol is included in fluid bilayers of lecithin. The cholesterol is known to increase the phospholipid packing in the liquid crystalline state whereas the reverse is true for the gel crystalline phase of the bilayers. Vesicles containing cholesterol have been found more stable and showed a more sustained release of drug.

2.1.2 Liposome As Drug Carriers

Water soluble as well as insoluble substances can be entrapped within the internal space of the liposomes and lipid soluble compounds are accommodated in their membranes. The macromolecules with both hydrophobic and hydrophilic regions occupy accordingly the lipid bilayer and aqueous phases of liposomes. The successful application of liposomes as carriers for drugs and enzymes in therapy is heavily dependent on their stability in circulation, tissue distribution and on their mode of interaction with target cells. These aspects of liposomes have been investigated in great detail (Woodle and Lasic, 1992; Vertut-Doï *et al.*, 1996; Perez Soler, 1989; Kato *et al.*, 1993; Klibanov *et al.*, 1990; Torchilin, 1994).

2.1.2.1 **Mode of administration and distribution:** Liposomes administered intravenously to humans concentrate mainly in the organs with fenestrated capillaries such as liver, spleen and bone marrow. Liposome entrapped agents might, therefore, be particularly effective in treating tumors that infiltrate these organs (e.g., liver micrometases). For treating large, solid, well established metastases, liposome entrapped drugs may be more effective than free drug depending on the ability of the vesicles to cross tumor capillaries and be taken up by the tumor cell. This ability will depend, among other factors on the characteristics of the tumor endothelium and the size, charge and flexibility of the vesicles. Size and surface charge of the

liposomes appear to control the rate of liposome clearance from blood. Large liposome are cleared more rapidly than the smaller one (Juliono and Stamp, 1975). Liposomal preparation of mixed sizes possess biphasic rates of clearance, whereas liposomes of homogeneous size exhibit a simple exponential clearance kinetics (Gregoriadis *et al.*, 1974). Neutral and positively charged liposomes were cleared less rapidly than were unilamellar negatively charged ones (Kirby *et al.*, 1980). Survival of liposomes in circulation is also increase by increased amount of cholesterol in liposomes (Kirby *et al.*, 1980). Liposome consisting of sphingomyelin and cholesterol have longest known survival times (Gregoriadis *et al.*, 1980; Hwang *et al.*, 1980). The stability may also be enhanced by preparing liposomes from phospholipids that possess Tc greater than 37 °C, from the dialkyl analogs of the phosphatidyl cholines or by incorporation of lipids derivatized with hydrophilic polymer polyethylene glycol (PEG) (Kirby *et al.*, 1980; Woodle and Lasic, 1992). The blood clearance and the rate of degradation of liposome can be modified by using different lipid composition. Lipid vesicles that result in a slow drug release can, therefore, be designed and used as carrier of anti tumor agent that are more effective or less toxic when infused continuously (e.g., cytarabine and doxorubicin, respectively).

Major proportion of liposomes from blood circulation is captured by liver and spleen (Gregoriadis *et al.*, 1972; Finkelstein and Weissman *et al.*,1978). Other tissues such as lung, kidney, skeletal muscle etc. also participate in the uptake of liposomes. The amounts that are taken up by these organs are rather modest and for multilamellar liposome this amount seldom exceeds 2-5% of the dose per gram of tissue (Gregoriadis *et al* ., 1974; 1977; McDongall *et al.*, 1974). When liposomes are administered subcutaneously, they are drained in part by the lymphatic and concentrate in the regional lymph nodes (Perez-Soler, 1989). Small liposomes tend to be cleared more rapidly from the site of injection than large liposomes, but many of small liposomes are cleared by blood vessels, whereas large liposomes are mostly drained by the lymphatics (Perez-Soler *et al.*, 1985). On the basis of these findings, liposome entrapped drugs may be particularly effective for the treatment or prophylaxis of regional lymph node metastatic diseases e.g., malignant melanoma. Because of their ability to act as a 'depot' system, liposomes may also be particularly useful in altering the pharmacology of drugs administered intraperitoneally or intraplurally and thus marked increase the intracavitary drug levels for a significantly prolonged time. The treatment of peritoneal metastates of ovarian carcinoma might be more effective if such an advantage was exploited.

2.1.2.2 **Release of the liposome encapsulated drugs:** Of the several techniques for the release of the drug includes entrapment of the drug in the polymer matrix, attachment of the drug to the polymeric matrix by covalent bonding and encapsulation of the drug inside the layer of polymer or the liposomes (Williams, 1984).

Release of the drug includes diffusion, i.e., the drug migrates from the initial position to the

outer surface. Diffusion rate can be controlled by two ways: the reservoir and the matrix. In the former case the drug is enclosed in the polymeric film, microcapsules, tubes like hollow fibers and membrane system, liposomes. In the later case, the drug is uniformly distributed throughout the polymer. The matrix system is also known as the monolithic system (Bachhawat, 1991; Bachhawat *et al.*, 1993).

The matrix bound drug is another example of diffusion controlled delivery systems. Drug is dispersed in the polymer matrix. Dispersion may follow three mechanisms. First, it may be that the drug is released at a very slow rate independent of the content of the drug (Gregoriadis and Florence, 1993). In the second case it is possible that the drug is released because of the swelling of the polymer when the environmental fluid is embedded in the system and thus drug diffuses through the swellings. In the third case the polymer that forms the matrix is biodegradable and a slow release of the drug with the surface degradation of the polymer. This process eliminates the unwanted polymer usually left in the body after the drug is consumed. Microcapsules can be undesirable if non biodegradable polymers are used. It is potentially dangerous if the outer layer is burst releasing the incorporated drug at a very high release rate. The main advantages of the matrix system are its low cost of manufacturing and relative safety in case of leakage (Bahadur, 1983). It can also release the leakage of larger molecular weight molecules like insulin, enzymes, antibodies, etc. This method has certain disadvantages as a constant release rate is not attainable as it slows down with the gradual consumption of the drug. The drug and the polymer are linked through covalent bond that undergoes fission releasing the drug at a desired rate. The drug is attached to the polymer by a spacer group. The drug is released when water reacts to break the bond thereby freeing the drug. Release rate in each case changes by changing the hydrophobic character of the polymer backbone (Zaffaroni, 1980). Liposomes can be designed that the enzymes can also break the bond between the drug polymer bond.

Benefits of controlled release system:

(1) Maintenance of drug at its controlled therapeutic level.

(2) Localized drug administration.

(3) Preserving volatile medication.

(4) Decreased expense and wastes.

2.1.2.3 **Scope of improvisation:** To reduce the uptake of liposomes in liver and spleen and to stop random distribution of liposomal material in biophase much progress has been made in the last few years. Methods have been developed that have decreased uptake by reticuloendothelial system (RES) and increased blood circulation half life (Allen and Chonn, 1987; Allen *et al.*, 1989; Kibanov *et al.*, 1990). Such developments appear to have brought liposome research into a new era. Many reports have appeared in the literature demonstrating

the increased therapeutic efficacy by using such long circulating liposomes over those with conventional compositions (Allen, 1994; Papahadjopoulos *et. al.*, 1991; Gabizon, 1992). The conventional term is used to signify liposomes composed of various phospholipids, cholesterol and possibly other lipids, without additional components that might confer the property of long circulation in blood and diminished recognition by the liver and spleen macrophages. The rapid clearance of conventional liposomes from the circulation has limited their prospects as an *in vivo* delivery system for transporting drugs to the site of disease beyond the RES. Recent reports have described new liposomes formulations that exhibit a prolonged circulation time in blood following intravenous administration in mice and increased accumulation in implanted tumors (Allen and Chonn, 1987; Gabizon, 1990). Because of their ability to evade normally rapid uptake by the resident macrophage cells of the liver and spleen, these sterically stabilized liposomes (Woodle and Lasic, 1992) have been referred to as "stealth". The term stealth is used to signify liposomes containing specific molecules such as GM1 ganglioside, phosphatidylinositol, PEG derivatized phospholipids etc. Their properties have the potential for significantly expanding the therapeutic utility of liposomes both by a more sustained release of various pharmaceutically active molecules within the circulation and by increasing effective targeting to specific cells and tissue within vasculature. It has been postulated (Gabizon and Papahadjopoulos, 1988) that steric "shielding" of negative charges on the liposome surface may contribute to the long circulation time of "stealth" liposomes, by inhibiting interaction with plasma proteins (Woodle and Lasic, 1992). These plasma proteins, opsonins, are thought to be responsible for the removal of conventional liposomes by the RES (Gregoriadis, 1988). It is also possible that similar steric hindrances may inhibit recognition of surface groups on the liposome by cell surface proteins responsible for their binding (Allen *et al.*, 1991). Unlike conventional liposomes that show dose dependent blood clearance kinetics, the new formulations show clearance kinetics that is completely independent of dosage over a wide range (Papahadjopoulos *et al.*, 1991). Microscopic evidence indicates that accumulation of liposomes in tumors involves extravasation, presumably due to increased permeability of the capillary endothelia (Jain, 1987). The new liposome formulations produce a marked enhancement of the anti tumor activity of encapsulated doxorubicin and epirubicin in mice against intraperitonial lymphoma and subcutaneous colon carcinoma, with a concomitant decrease in toxicity (Gabizon, 1992). Thus the therapeutic studies with "stealth" liposome mentioned above showed a significant increase in therapeutic index of anti tumor drugs in mice. The evidence obtained indicates the presence of intact extra cellular liposomes within the tumor area is likely to provide a local depot for sustained drug release at a relatively high concentration (Jain and Gerlowski, 1986).

2.1.3 **Preparation Of Liposomes**

Presently, several methods are available to make liposome of different size and charge. A short review of the various methods available for preparation of liposomes follows:

2.1.3.1 **Sonication method**: This simplest method was originally described by Bangham (1965). The phospholipids are dissolved in a solution of chloroform : methanol (v/v, 2:1). Solvent is evaporated to make a lipid film. The lipid film is then dispersed in water at a temperature above the T_c of the lipid. Encapsulation efficiency of drugs is about 4 % - 7 %. A gentle shaking increases the encapsulation efficiency. Usually MLV are formed. The size of the vesicles may be reduced by disrupting the lamellar structure by sonication.

2.1.3.2 **Ethanol injection method**: This method uses injection of a solution of lipid in ethanol in the buffer. This method was described by Batzri and Korn (1973). Nearly all vesicles formed by this method is unilameller. The major drawback in this method is that the lipid dispersion is diluted and the encapsulation is poor.

2.1.3.3 **French pressure method:** The lipids are mixed in chloroform:methanol mixture (v/v, 1:1) and dried on the wall of a glass bottle by evaporating the liquid in nitrogen and then evaporated under vacuum in a desiccater. Drug to be entrapped is then added to the film as aqueous solution and the lipid is completely dispersed by vigorous vortexing. In this method large MLV are formed (Barenholz *et al.*, 1979). The white MLV are transferred to a French pressure cell that is held upside down after the shaft is tightly closed and mantained at 4 °C. The pressure cell is inserted in the hydraulic press and the pressure is applied. If the closure of the cell is performed properly the cell will hold the set pressure. Very slow extrusion is a crucial step in the preparation procedure. The size of the resulting liposome depends on the pressure applied. The higher the pressure the smaller the liposome size provided the operations are performed above the T_c of the phospholipids.

2.1.3.4 **Ether infusion method**: This method was first described by Deamer and Bangham (1976). The original procedure used diethyl ether as the solvent and was termed as the "Ether injection method". The phospholipids are dissolved in diethyl ether or diethyl ether:methanol mixture and an aqueous solution of the material to be entrapped is added either at 55-65 °C or under pressure at 0 °C. The solvent is then removed by evaporation and large ULV of mean size of 1500-2500 Å are formed. This method is suitable for macromolecules. The drawback of this method is the unnecessary exposure of the material to high temperature and organic solvent. Materials like proteins are likely to loose their original structures and will lose its properties. This method has low encapsulation efficiency and produces heterogenous population of liposomes.

2.1.3.5 **Detergent dialysis (DD) method**: This method involves stabilization of a lipid dispersion with a detergent such as, sodium deoxycholate or sodium cholate followed by its removal with gel filtration or by dialysis (Kagawa and Racker, 1971). Advantage of this

method is that this method produces are homogenous of small ULV. Lipid to detergent ratio plays an important role in the size of small ULV. Complete removal of detergents may not be possible making this method less practicable.

2.1.3.6 **Reverse phase evaporation (RPE) method**: To a mixture of lipids in an organic solvent an aqueous solution is added. The organic solvent is removed under pressure. Large ULV and oligolamellar liposomes are formed (Szoka and Papahadjopoulos, 1978). The entrapment efficiency is as high as about 65 %. This method cannot be used for the entrapment of the proteins as they get denatured on exposure to organic solvent.

2.1.3.7 **Rehydration-dehydration (RD) method**: This method is used for the industrial preparation of liposomes. The phospholipids are dissolved in organic solvent and the solvent is removed under reduced pressure and then dispersed into aqueous phase. This procedure is mild requiring the presence of neither solvents nor detergents. The entrapped efficiency ranges from 27-70 %. Optimal entrapment efficiency is achieved when the lipid is fully dehydrated. The method produces small ULV. This method was first developed by Kirby and Gregoriadis (1984). The major advantage of this method is that the liposome can be freeze dried and can be stored for more than a year without any major leakage of the entrapped material. Whenever needed the dried lipid can be regenerated into liposome by adding water. The lipid powder could be stored for a long time. The mildness of this condition is particularly important for the encapsulation of labile materials such as enzymes, DNA, cofactor and other biologically active substances.

2.1.3.8 **Freeze and thaw method:** Solutes are added to a concentrated liposome suspension (about 40 mg lipid ml^{-1}) prepared by dry film of lipids suspended in aqueous phase. The mixture is rapidly frozen in dry ice/acetone bath and thawed slowly at room temperature. The cycle is repeated at least three times to ensure efficient trapping. The untrapped solutes are removed by the ficoll flotation procedure. About 5 % to 6 % of the total solute can be encapsulated (Pick, 1981). Although this method is more rapid and equally efficient in solute entrapment, the resulting liposomes are heterogeneous in size. Also many oligolamellar liposomes are produced.

2.1.4 2-Mercaptopropionylglycine (MPG)

Sulfhydryl compounds, widely distributed in animals, plants and microorganisms, are long known for their efficiency in restoring the activity of protein bound sulphydryl groups (Udupi and Rice-Evans, 1992). Consequently they are able to restore radiation-induced decativation of proteins. The sulphydryl protectors are can be categorized in two types. One is of natural origin

with low toxicity such as, glutathione while the other type of sulphydryl protectors are synthetic with high toxicity such as, cystamine. A synthetic aminothiol, 2-mercaptopropionylglycine (MPG), has been one of several such thiol compounds which has generated considerable interest as a radioprotector. The molecular weight of MPG is 163.20 Da and is water soluble white powdery substance. The chemical structure of MPG is shown in Fig.2-1

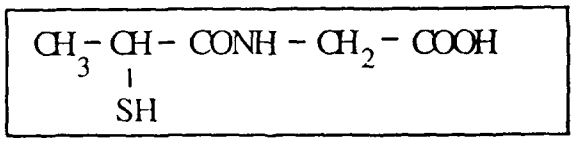

FIGURE 2-1: Chemical structure of MPG.

MPG is an exceptional synthetic radioprotector *in vivo* and *in vitro*. whose effective dose (20 mg kg^{-1} body weight) is sufficiently below its toxic dose of (2100 mg kg^{-1} body weight) (Sugahara & Srivastava, 1976; Ayene & Srivastava, 1985; Garner *et al.*, 1986). The nontoxic nature of MPG at its optimum dose for radioprotection has been the main cause of the interest for using it in chemical radioprotection. In addition, it has some pharmacological properties such as, antiallergic action and detoxificating action against heavy metals and various drugs protecting the liver (Chiba *et al.*, 1973). Two derivative of MPG, namely, 3-MPG, and MPG-amide has been synthesized. Contrary to expectations, the derivatives partially lost the radioprotective ability of MPG (Sugahara and Srivastava, 1976). Consequently, MPG is reported to be one potent radioprotectors among its related compounds (Sugahara and Srivastava, 1976).

MPG is known to chemically "heal" the primary lesion by a donation of an H-atom to a site where it was initially abstracted and thus facilitate DNA single strand break (ssb) rejoining in areobic system (Revesz *et al.*, 1984). Patt *et al.* (1949) observed the effect of cystamine in animals for their ability to protect against the ionizing radiations. Many sulphydryl compounds have been reported to protect the animals though the protection is not universal. MPG and a related compound, WR 2721, show radioprotective effect at subtoxic doses (Nagata *et al.*, 1972). MPG is reported to be an antitoxic agent and an enzyme activator due to the presence of easily available -SH group (Bhanumathy *et al.*, 1986). Therefore, the MPG affordable radioprotection is believed to be mediated by several mechanisms. MPG is reported to mediate release endogenous protectors (Revesz *et al.*, 1972), act as OH and free radical scavanger (Mishra and Srivastava, 1981) or cause elevation of redox potential (Sugahara, 1972) to offer radioprotection to cells. A possibility of MPG induced delay in glutathione metabolism has been suggested (Uma Devi and Prasanna, 1990).

2.1.5 Encapsulation Of MPG In Liposome

MPG is a suitable radioprotective drug which has shown promising radioprotective effects in experiemental systems against radiation induced damages (Sugahara and Srivastava, 1976). The radioprotective effect of MPG was reported *in vitro* for gamma induced radiolysis of catalase (Wary and Sharan, 1988) as well as *in vivo* (Wary *et al.*, 1989, Sharan, 1990). However, it offorded radiosensitization in cases of γ-induced microsomal lipid peroxidation (Ayene & Srivastava, 1985), catalase radiolysis (Wary & Sharan, 1988) and DNA strand break in human lymphocytes *in vitro* (Wary *et al.*, 1989; Sharan, 1990). The causes for this undesirable reversal of MPG affordable radioprotection was attributed to (a) the lack of optimal concentration of MPG in tissue of interest for radioprotection and (b) metabolic alterations of MPG to its oxide, disulphide and other derivatives after its administration (Sharan *et al.*, 1995). Since liposomes can, in principle, encapsulate MPG it is possible to overcome the two, essentially undesirable, conditions which reverse the MPG affordable radioprotection. No such report is available in leterature where such attempts were made.

2.1.6 Aims And Objectives

Therefore, in this piece of work it was desired to encapsulate MPG in a suitable liposome vesicle. As several methods for encapsulation of the drugs in liposomes are available, it has been attempted to use mild and relatively convenient methods of liposome preparation. The objective of this work was to:

(a) Select and standerdise a mild, convenient and reproducible method of liposome preparation.
(b) Encapsulate MPG in such liposomal preparations.
(c) Compare the entrapment efficiency to recommend the best method for MPG encapsulation.
(d) Establish the influence of concentration of MPG on the efficiency of MPG entrapment into liposome by such methods.

2.2 Methods And Materials

2.2.1 Chemicals

2-mercaptopropionylglycine (MPG) was a gift from Prof. T. Sugahara and Santen pharmaceuticals Co., Japan. Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), cholesterol (Chol), dithio-bis-nitrobenzoic acid (DTNB), deoxycholic acid, sodium azide, Triton-X-100, phosphate buffer saline (pH-7.9) and tris base (Trizma) were purchased from Sigma Chemicals Co., USA and Sepharose CL-4B, Sephadex 200 from Pharmacia Fine

Chemicals, Sweden. The other chemicals like ethylenediaminetetraacetic acid (EDTA), chloroform, ethanol, sodium chloride, etc. were of highest purity grades chemicals available from various indigenouce sources. Glass double distilled water was used for preparation of all reagents.

2.2.2 **Other Materials**

The work involved use of ordinary laboratory facilities like, liquid nitrogen, water bath, rotary evaporator, vortex, spectrophotometer, etc.

2.2.3 **Preparation Of Liposomes**

The following methods were used to prepare liposomes and to encapsulate MPG into it:

2.2.3.1. **Preparation of required buffers/solutions**: The following reagents were prepared and stored refiregerated for use:

(A) Tris-acetate buffer - It consisted of:

Tris base	20 mM
NaCl	100 mM
EDTA	5 mM

The pH of the buffer was adjusted to 8.1 with 1 M acetic acid.

(B) PBS (pH 7.9) - The constituents of this buffer was:

Na_2HPO_4	0.1 M
NaH_2PO_4	0.1 M
NaCl	0.9 M

Appropriate amouts of Na_2HPO_4 and NaH_2PO_4 were separetely dissolved in water to prepare their respective 0.1 M solutions. The two solutions were mixed until pH of 7.9 was obtained. To this calculated amount of NaCl was added to get its molarity.

(C) MPG solution - Separate solutions of 2.5 mM, 5 mM and 10 mM MPG were prepared by dissolving 0.408 mg, 0.816 mg and 1.632 mg, respectively, of MPG in 1 ml of PBS (pH 7.9). Similarly, another set of MPG solutions were prepared by dissolving MPG in tris-acetate buffer (pH 8.1).

2.2.3.2 **Rehydration dehydration (RD) method**: The method is essentially based on the method of Kirby and Gregoriadis (1984). DPPC (5.0 mg), DCP (1.0 mg) and cholesterol (2.5 mg) were added in 1 ml of chloroform:ethanol mixture (1:1) in a glass test tube. After thorough vortexing, it was incubated at 37 °C for about 5 min for proper solubilization. The solution was then dried to a thin film on the wall of the tube by flushing the tube with N_2 while rotating the tube immersed in a water bath mainained at 37 °C. Typically it took a few min to achieve this.

The dried film was then dispersed in 1 ml of either 2.5 mM, 5 mM or 10 mM MPG solution. For this MPG solution was added drop-wise into the tube with continuous vortexing until the entire lipid film was completely dispersed in the solution. The liposomes thus formed had encapsulated MPG into it. For preparing blank liposomes, PBS was used in place of MPG solution.

2.2.3.3 **Reverse phase evaporation (RPE) method**: The method is based on the method of Szoka and Papahadjopoulos (1978) which was used with minor modifications. A mixture of DPPC (5 mg), DCP (1 mg) and cholesterol (2.5 mg) was dissolved in 1 ml of chloroform:ethanol mixture (1:1) in a round bottom flask at 40 °C. To the organic solution, either 5 mM or 10 mM aqueous solution of MPG was added in small aliquots while the flask was being vortexed continuously. After complete dispersion, the flask was fitted in a rotary evaporator maintained at 40 °C to remove the organic solvents. It usually took about 45 min to comlete the process. Blank liposomes were prepared by using PBS instead of MPG solution.

2.2.3.4 **Detergent dialysis (DD) method**: The method is modified from that described earlier by Kagawa and Racker (1971). DPPC (22.0 mg) and cholesterol (5.5 mg) were completely dissolved in 1 ml of chloroform:ethanol mixture (1:1) in a test tube. The solution was then dried at 37 °C by passing a steam of N_2 on the walls of the tube which was being continuously rotated. The resulting thin film was dispersed by addition, in small aliquots, of either 5 mM or 10 mM aqueous solution of MPG in 20 mM tris-acetate buffer (pH 8.1). Finally, 1.0 ml of deoxycholic acid (dissolved in 20 mM tris-acetate buffer, pH 8.6) was added into the tube. The tube was continuously vortexed.during the entire operation lasting about 5 min. The content of the tube was dialysed for 48 h in tris-acetate buffer with several changes. Blank liposomes were prepared by using tris-acetate buffer in place of MPG solution.

2.2.4 **Separation of free MPG from liposome entrapped MPG (LEM)**

MPG encapsulated in liposome was separated from the free or non-trapped MPG by the following methods:

2.2.4.1 **Dialysis:** A dialysis bag was boiled in water containing 10 mM EDTA and 1mM $NaHCO_3$ for 10 min. After cooling, the bag was washed extensively in water before using it. A known volume of solution containing LEM and free MPG was packaged into the dialysis bag which was dialysed in 250 ml of 20 mM tris-acetate buffer (pH 8.1) with several changes. After 48 h, the liposome preparation was recovered from the bag and its volume measured.

2.2.4.2 **Gel filtration on sepharose**: LEM and free MPG were separated by passing the mixture through sepharose CL 4B column.

Packing of column and chromatography - A column (30 x 15 cm) was packed with sepharose containing 0.02% sodium azide. Sepharose was equilibrated with 10 mM PBS, pH 7.4. The column was developed with the same buffer. Gel filtration used to remove trace of chloroform remaining in the liposome.and free MPG from the encapsulated MPG. Fractions were collected and read at 412 nm.

2.2.4.3 **Centrifugation**: To separate free MPG from LEM, the mixture was centrifuged at 46,000 x *g* in a Beckman centrifuge for 30 min. The supernatant was decanted. The pellet was resuspended in the same buffer. The suspension was recentrifuged and the whole process was repeated thrice. After the final centrifugation, the pellet was recovered and suspensed in volume equal to the initial volume.

2.2.5 Assay Of MPG

Since MPG contains a -SH group (Fig. 2-1) the qunatification of MPG can be conveniently done by the assay of the -SH group using Ellmans (1959) method using aqueous solution of MPG as a standard. This spectrophotometric method is very convenient and highly *reproducible.*

2.2.5.1 **Preparation of assay reagent:** The assay reagent consisted of:

DTNB	10 mM
PBS (pH 7.9)	100 mM
EDTA	0.1 mM

The reagent was stored refrigerated.

2.2.5.2 **Methodology**: The liposomes encapsulating MPG was first disrupted to ensure complete availability of MPG for the quantification. For this, 0.1 ml of 1 % solution of triton X-100 was added to 0.1 ml of liposome preparation and vortexed. This solution was subjected to the assay.

The assay reagent was first flushed with N_2 for 1 min. To 2.9 ml of N_2 flushed assay reagent, 0.1 ml of MPG sample was added and vortexed. The absorbance of the solution was read immediately at 412 nm at intervals of 30 sec up to 3 min in a spectrophotometer. The blank contained PBS with 1% solution of triton X-100 in place of MPG sample.

2.2.6 Calculation Of Percent Entrapment Of MPG In Liposomal Preparation

The percent encapsulation of MPG into liposome was calculated by the following equation (Hiroaki Jizomoto *et al.*, 1989):

$$\frac{\text{Total amount of the MPG estimated in the liposome prepararion x 100}}{\text{Total amount of the MPG used for the prepation of liposome}}$$

2.3 RESULTS

2.3.1 **Liposome**

Liposomes were prepared by three standard methods, namely, rehydration dehydration (RD) method, reverse phase evaporation (RPE) and detergent dialysis (DD) method, after suitable modifications. All these methods are known to produce ULV. Fig. 2-2 shows that whiile RD

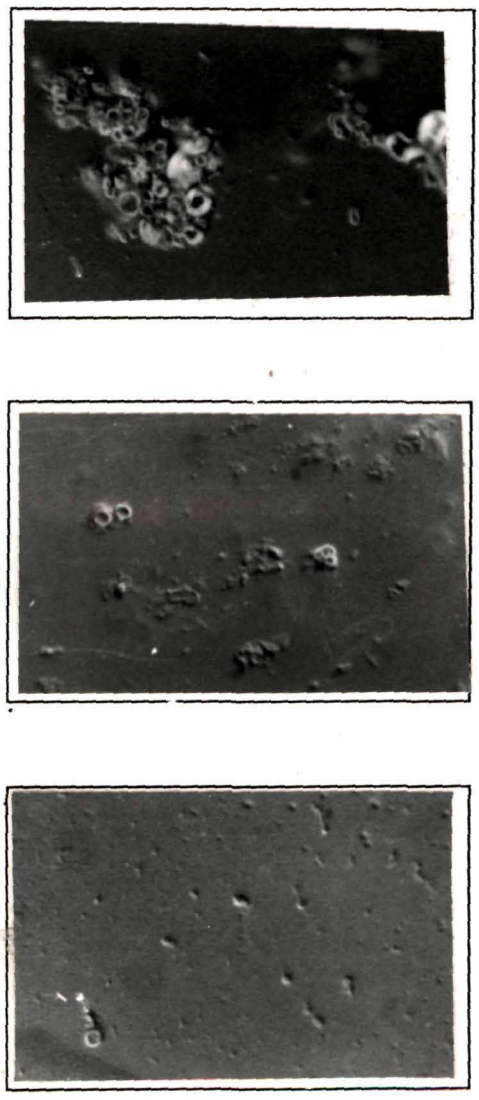

FIGURE 2-2 - Photomicropgraph of liposomes prepared by rehydration dehydration method (A). reverse phase evaporation method (B) and detergent dialysis method (C) three methods (252 X).

(A) and RPE (B) methods produced small sized ULV liposomes, the liposomes produced by DD method were relatively large (C). The size variability was minimum in liposomes produced by RPE method (Fig. 2-2 C).

2.3.2 Composition Of Liposome

The lipid composition of liposome prepared by the RPE and RD methods and lipid to MPG ratio were calculated and are are shown in table 2-II:

TABLE 2-II

LIPID COMPOSITION	QUANTITY OF LIPIDS (mg)	MOLAR RATIO	LIPID TO MPG RATIO
DPPC, Chol & DCP	5, 2.5 & 1	1 : 0.9 : 25	5.21

The lipid composition of liposome prepared by the DD method and the lipid to MPG ratio came out to be as shown in table 2-III:

TABLE 2-III

LIPID COMPOSITION	QUANTITY OF LIPIDS (mg)	MOLAR RATIO	LIPID TO MPG RATIO
DPPC & Chol	22 & 5.5	1 : 0.48	16.85

2.3.3 Effect Of MPG Concentration On Entrapment Efficiency

The effect of starting concentration of MPG on percent of its encapsulation into liposome prepared by the three methods are shown in Figs. 2-3 to 2-5. The RD method yielded very low percent entrapment (19.47±5.49 %) when 2.5 mM MPG was used. The percent of entrapment (69.970±10.650 %) was highest when starting concentration of MPG was 5 mM (Figure 2-3). The entrapment efficiency (37.770±8.740 %) was low when 10 mM MPG was used.

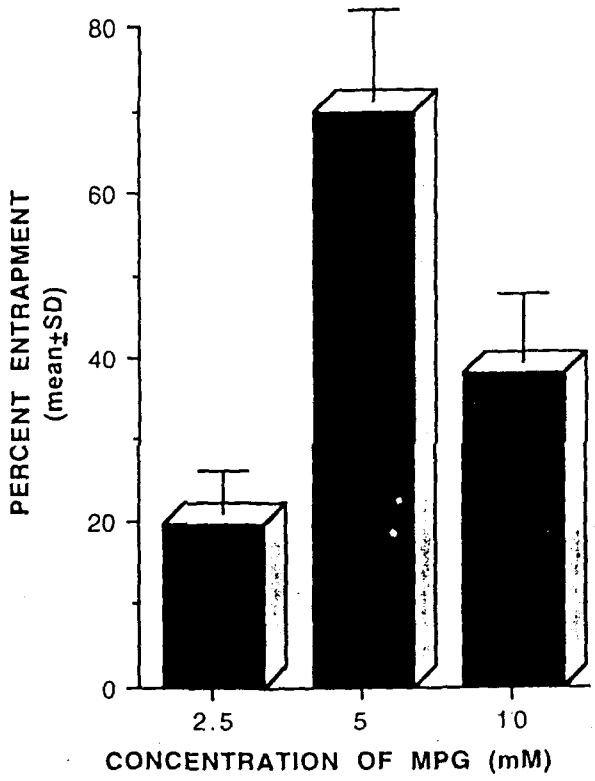

FIGURE 2-3: Percent entrapment of MPG into liposome prepared by RD method.

Similarly, the detergent dialysis method did not show (Fig. 2-4) enhancement of percent of MPG entrapment when the starting concentration of MPG was raised from 5 mM (39.990±3.330 %) to 10 mM (31.910±4.09 %). Since percent entrapment was very low (19.47±5.49 %) when 2.5 mM MPG was used (Fig. 2-3), this concentration of MPG was not used in the DD or RPE methods.

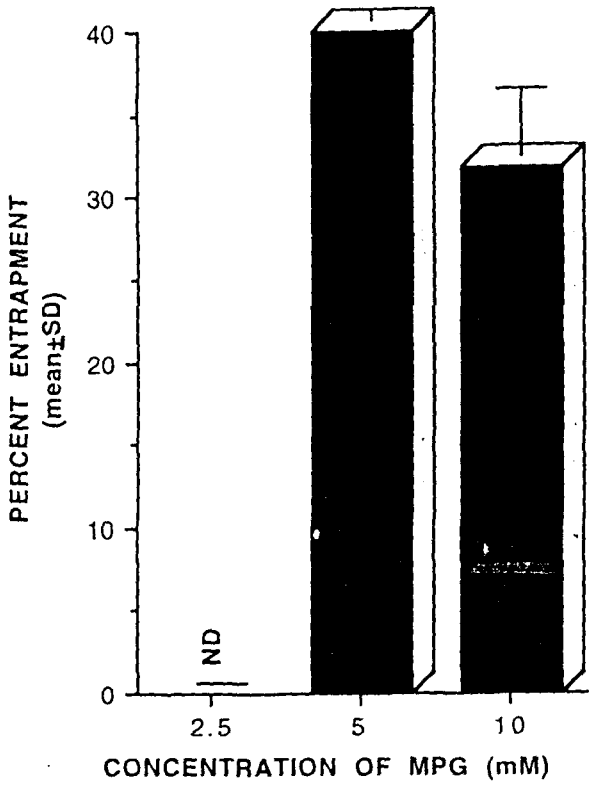

FIGURE 2-4: Percent entrapment of MPG into liposome prepared by DD method.

On the other hand, RPE method showed progressively increasing efficiency of MPG entrapment (54.54 ±4.95 % to 68.33±6.49 %) when the starting concentration of MPG was raised from 5 mM to10 mM (Fig. 2-5).

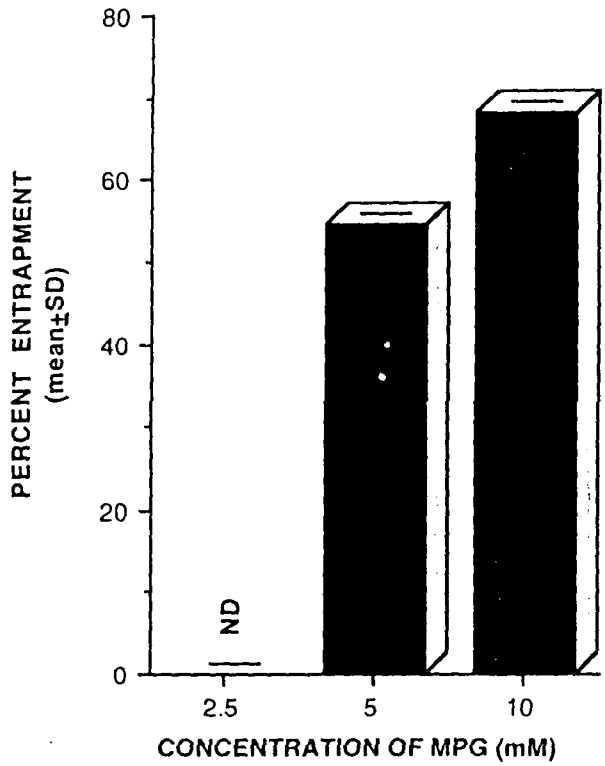

FIGURE 2-5: Percent entrapment of MPG into liposome prepared by RPE method.

2.3.4 **Effect of Method of Liposome Preparation On Entrapment Efficiency**

Fraction of MPG entrapment into liposome prepared by different methods is shown in Fig. 2-6. In the RPE method the fraction of MPG entrapped was found to progressively and significantly increased from 0.545±0.050 for 5 mM MPG to 0.683±0.065 for 10 mM MPG (curve ii). The DD method showed relatively poor entrapment. The fraction of MPG entrapped was 0.400±0.033 for 5 mM MPG which showed a marginal decrease to 0.320±0.040 for 10 mM MPG (curve iii). In RD method (curve i) the increase in the fraction of MPG entrapment was from 0.194±0.055 (for 2.5 mM MPG) to 0.699±0.106 (for 5 mM MPG) which was significantly reduced to 0.378±0.87 (for 10 mM MPG).

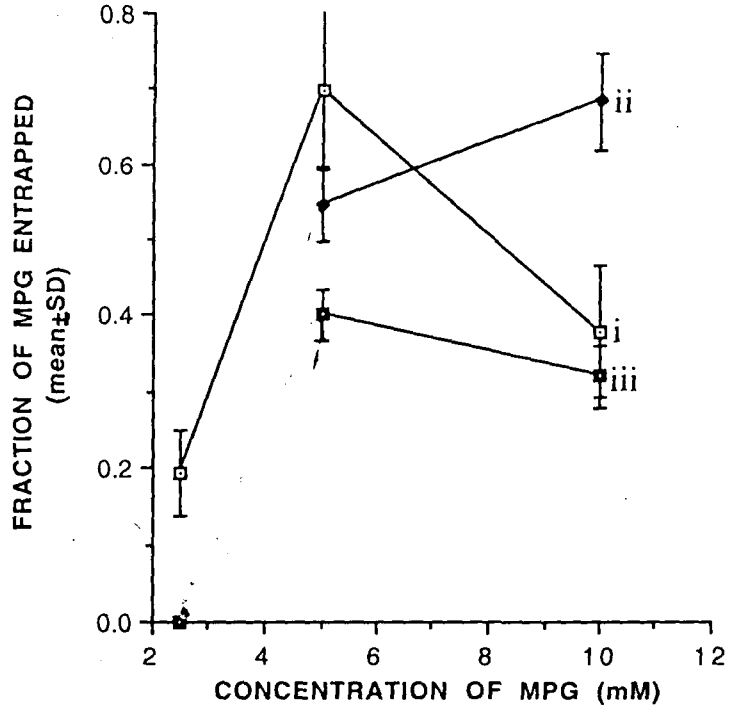

FIGURE 2-6 - Fraction of MPG entrapped into liposomes prepared by rehydration-dehydration (curve i), reverse-phase evaporation (curve ii) and detergent dialysis (curve iii) methods.

2.4 DISCUSSION

Ever since the discovery of liposome in 1965, persistent efforts have been made to use them in studies other than the structure and function of biological membranes (Gregoriadis, 1986). During the last two decades, it was discovered that this model cell system may serve a duel role as a valuable experimental tool for membrane research (Bangham *et al.*, 1974; Papahadjopoulos *et al.*, 1973 and Jost *et al.*, 1982) and in addition, as an *in vivo* delivery system for enhancing the efficacy of various biologically active molecules (Gregoriadis, 1988).

Animal studies have shown that liposome can decrease the toxicity of several antitumor and antifungal drugs (Papahadjopoulos *et. al.*, 1991; Gabizon, 1992; Jones, and Hudson, 1993; Conley, 1993; Thierry *et. al.*, 1994, Allen *et al.*, 1995; Nagayasu *et al.*, 1996). The use of liposome as a carrier of antitumor agents has only recently become a clinical reality. Preclinical studies showed that these particulate carriers may improve the delivery of antitumor agents to certain tumors or organs, may decrease drug levels in certain organs particularly sensitize to the drugs toxic effects and may be used as a vehicle of the new lyophilic agents. On the basis of the information available, it is evident that liposome entrapped antitumor agents may offer a clear therapeutic advantage for several specific diseases. Among clinical situations in which liposome

entrapped antitumor agents may result in a higher antitumor activity, the prophylactics of liver metastases of colon carcinoma (treatment of microscopic diseases at the time of reaction of primary tumor) is well supported by the pre clinical data (Perez-Solar, 1989). Besides, liposomes have also been found to be efficient carriers of macrophage activators for increasing tumoricidal activity in models of metastasis (Koff *et al.*, 1985; Vertut-Doi *et al.*, 1996; Killon and Fidler, 1994; Fidler, 1994; 1988). No such study has been conducted to test the efficasy of radioprotective drugs, MPG.

MPG is evidently a moderate radioprotective drug *in vivo* and *in vitro* experimental animals (Sugahara and Srivastava, 1976; Ayene and Srivastava, 1985; Garner *et al.*, 1986; Wary and Sharan, 1988). In limited chemical studies on humans recieving radiation for cancer treatment, administration of MPG 15 to 30 minutes prior to irradiation afforded significant radioprotection (Sugahara and Srivastava, 1976). This was shown by the studies several biological end points. However several studies have shown the radioprotection afforded by MPG could be reversed (Ayene and Srivastava, 1985; Wary and Sharan, 1988). The main cause of this undesirable reversal was attributed to, suboptimal concentration of MPG in the tissue of interest, interaction of MPG with some metabolites or chemical alterations of MPG after its administration to animals. Encapsulation of MPG into liposome may offer several advantages and help mantain the affordable radioprotection.

The major consideration for selecting suitable methods for liposome preparation is to achieve highest efficiency of MPG entrapment as well as to contain the encapsulated MPG into the liposome for a long period. Further, all methods require exposure of the drug to organic solvents and detergent which might alter chemical characteristics of the drug or inactivate it. The third main consideration, therefore, should be that the MPG comes in contact with organic solvents for the shortest period of time so that the probability of its chemical alteration is minimum. The choice of the three methods used in this investigation was based on these considerations. All the three methods, rehydration dehydration method, reverse phase evaporation and detergent dialysis used for encapsulation of MPG meets the criteria atleast to a certain extent.

Figs. 2-3 to 2-5 shown that MPG may be encapsulated into liposomes by all the three methods used in this investigation. Depending on the concentration of MPG, the percent entrapment of MPG showed a dose dependence only in the case of RPE method (Fig. 2-5). The percent entrapment did not increase by increasing the concentration of MPG when DD method was employed for liposome preparation (Fig. 2-4). When RD method was used, higher concentration of MPG was inhibitory to its encapsulation efficiency into MPG (Fig. 2-3). These observations are very clearly summerized in Fig 2-6. Since only RPE method of preparation of liposme for MPG encapsulation showed progressively increasing efficiency of

MPG encapsulation, this method seems to be the best of the three methods tried in this work. The entrapment efficiency of MPG into liposome by RPE method was also very reproducible. The method has also been reported to be simple and reproducible for encapsulation of other macromolecules (Alam *et al.*, 1992).

Fig. 2-6 also shows the effect of the starting concentration of MPG on its fractional entrapment into liposome. It is evident that RPE method offers a MPG dose dependent increase in the entrapment of MPG into liposome. This method of preparation of liposome encapsulating MPG did not show any influence of starting concentration of MPG in the range used in this investigation. The other two method that have been used in this investigation were influenced by the concnetration of MPG used for encapsulation. It was reported that entrapment of MPG in the liposome is dependent on the pH of the buffer and a major determinant in this is the chemical composition of the buffer (Gregoriadis,1993). It is possible that the observed inhibition in efficiency of entrapment (RD method) or no increase in efficiency of entrapment (DD method) was due to this.

The major pharmacological problem is the use of cytotoxic agents for treating tumor while reducing or eliminating exposure of susceptible normal tissues. Of many possible unique solutions to this problem, explored over the last few decades, drug encapsulation in synthetic phospholipids vesicles (liposomes) has been found to be promising. The liposome offers infinite possibilities of alteration of its size, charge and chemical structure. This flexibility is a distinct advantage for formulation of tailored liposome for specific pharmacological goals and present unique clinical and experimental opportunities. Drugs that have serious and dose limiting toxic effects on normal organs can be packaged in liposomes to avoid exposure of those organs. High peak drug concentration can be reduced by a liposome formulation resulting in slow drug release. Thus liposomal drug delivery system has great potential for many purposes. Future clinical use is likely provided the preparation of liposome can be reproduced in hospital and pharmacies.

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3.1 INTRODUCTION

MPG has been shown to afford radioprotection in mice *in vivo* and in several *in vitro* studies as well as in limited clinical trials (Nawalkha *et al.*, 1984). Of several radioprotective drugs that has been used in radiotherapy of cancers to diminish the damaging effects of radiation to healthy tissues, MPG has come out to be a promising radioprotector and detoxifying agent (Sugahara and Srivastava, 1976; Nawalakha *et al.*, 1984). Several clinical trials have been conducted and MPG has been accepted as a moderate radioprotector at an effective dose of 20 mg kg^{-1} of body weight significantly below it reported toxic dose of 2100 mg kg^{-1} body weight (Ayene and Srivastava, 1985; Uma Devi and Saharan, 1978; Saini and Uma Devi, 1979; 1980; Sugahara and Srivastava, 1976; Kumar *et al.*, 1985; Sharan *et al.*, 1992; 1995). Of the two related drugs, MPG and WR-2721, the later has been reported to be more effective radioprotector than MPG for bone marrow (Gupta and Uma Devi, 1985) but its toxicity is too high to use it in clinical radiotherapy (Meistrich *et al.*, 1984).

In conventional protocols, 20 mg kg^{-1} dose of MPG is administered, either through oral or intraperitoneal route, 30 min prior to irradiation because its effectiveness depends not only on the dose of MPG but also on the interval between drug administration and irradiation (Bisth *et al.*, 1990). As discussed in chapter 2, the main reason for the effectivity of MPG is its nontoxic nature at its optimum effective dose for radioprotection (20 mg kg^{-1} of body weight) approximately 30 min before irradiation.(Sugahara *et al.*, 1970; Nagata *et al.*, 1972; Tanaka, 1972; Nawalkha *et al.*, 1984). Perhaps this time interval between administration of MPG and irradiation is required to permit metabolic accumulation of MPG in its optimum concentration in the tissues that are envisaged to be protected. However, it must be noted that once administered, the distribution and metabolic fate of MPG is totally physiology dependent. Therefore, accumulation of MPG in different tissues and metabolic alterations of MPG cannot be controlled. This fact suggests that depending on the physiological state of the subject, radioprotective effect of MPG may show variations. This is a serious matter which ought to be considered when MPG is to be administered to humans in chemo-radiotherapy protocols.

There have been reports where the radioprotective effect of MPG was reversed and it behaved as a radiosensitizer. MPG was found to behave like a radiosensitizer against spontaneous and chemically induced microsomal lipid peroxidation *in vitro* (Cheeseman *et al.*, 1981; Ayene and Srivastava, 1985). It has been proposed that MPG may be at the level of secondary radical scavenger or chain breaking antioxidant to influence lipid peroxidation. It was, nonetheless, shown that MPG affordable radioprotection was restored in the presence of EDTA (Cheeseman *et al.*, 1981). Similarly, inactivation of enzyme catalase *in vitro* in the presence of MPG showed both radioprotective and radiosensitizing effects of MPG under different situations. The MPG affordable radioprotection was reversed to radiosensitization when Fe^{++}/Fe^{+++} ions

were allowed to interact with MPG resulting in formation of an unstable catalase·Fe^{++}/Fe^{+++}-MPG complex (Wary and Sharan, 1988). MPG also induced radiosensitization at higher doses of gamma rays in human lymphocytes *in vitro* when assayed for radiation induced DNA strand break (Wary *et al.*, 1989; Sharan, 1990). The possible reasons for reversal of MPG affordable radioprotection are:

(a) Alteration of the chemical structure or characteristics of MPG after its administration losing its radioprotective form.
(b) Sub-optimal concentration of MPG in the tissue envisaged to be protected.
(c) Circumstantial interaction of MPG with other macromolecules.

Further, due to physiologically dependent tissue distribution of MPG, it is possible that undesirable tissues (like tumors) show radioprotection thereby the desired effect of radiotherapy. All these have seriously limited use of MPG in clinical chemo-radiotherapy.

In order to maintain MPG in its radioprotective form it is, therefore, important that the chemical structure and characteristics are not metabolically altered in the 30 min prior to irradiation period to keep MPG in its radioprotective form. Secondly, the effective concentration of MPG in the tissues envisaged to be protected should be optimum for radioprotection and its concentration be minimum or totally nil in tissues (such as, tumors) that are not envisaged to be protected. Thirdly, circumstantial interactions of MPG with cofactors or ions, known to effect affordable radioprotection (Ayene and Srivastava, 1985; Wary and Sharan, 1988; Wary et al., 1989; Sharan, 1990) should be avoided as much as practicable. In principle, MPG after liposome encapsulation gets endowed with characteristics that may satisfy all the above three points. Due to liposome encapsulation, the MPG molecules are not immediately accessible to metabolic enzymes and co-factors that may alter the chemical structure and characteristics of MPG. Therefore, alterations of the chemical characteristics of MPG after administration is slowed down. For the same reasons, circumstantial interaction of MPG with other macromolecules is seriously hindered. Finally, as liposomes may be potentially targeted to tissues of interest, it will be possible to ensure optimum MPG concentration for radioprotection in tissues of choice and limited or no MPG in other tissues that are not envisaged to be protected.

As it has been reported in chapter 2, encapsulation of MPG by reverse-phase evaporation method was very satisfactory, this chapter deals with testing the biological effectiveness of MPG as a radioprotector after liposome encapsulation. To quantify the MPG affordable radioprotection in biological system two parameters are envisaged to be used:

(a) Protection of γ-irradiation induced cell killing by assaying viability of cells.
(b) Protection of γ-irradiation induced release of membrane bound acetylcholine esterase

of liver by assaying the amount of the enzyme in the soluble fraction.

Over all viability of cells after radiation assault is of utmost importance for assessment of impact of radiation. This strategy has been employed in several studies as the most important parameter of assessment of radiation induced damage as well as for monitoring the impact of factors which can rescue cells from radiation induced damage. Since membrane constitutes the major component of a cell, dye exclusion technique to monitor viability of cell has been very widely used (Wary and Sharan, 1988; Sharan *et al.*, 1995).

Acetylcholine esterase (acetylcholine acetylhydrolase, AChE), an oligomeric enzyme, is predominantly membrane anchored (Massoulié and Toutant, 1988) mediating cholinergic neurotransmission by rapid hydrolysis of transmitter acetylcholine. Radiations are known to disrupt membrane structure, thereby, releasing the membrane bound or anchored enzyme (Bacq and Alexander, 1966). Thus, accumulation of AChE in the soluble fraction of liver has been used as a parameter to monitor radiation induced damage (Sharan *et al.*, 1995). The cellular substrate of AChE is acetylcholine that is hydrolyzed by the enzyme to choline (Jürss and Maclicke, 1981; Bazelyansky *et al.*, 1986).

3.1.1 Aim And Objectives

In order to achieve the objective, the investigation aimed to do the following:

1. To assess the effect of increasing dose of γ irradiation on the survival of bone marrow and spleen cells of mice.

2. To monitor the effect of increasing dose of γ irradiation on the release of membrane bound acetylcholine esterase (AChE) enzyme of liver of mice.

3. To monitor the two parameters mentioned above when the mice had been administered bioequivalent dose of either free MPG or LEM prior to irradiation in order to establish the biological effectiveness of MPG after liposome encapsulation.

3.2 METHODS AND MATERIALS

3.2.1 Chemicals

2-mercaptopropionylglycine (MPG) was obtained from Santen Pharmaceuticals Co., Japan. Dipalmytoyl phosphatidyl choline (DPPC); Dicetylphosphate (DCP); Cholesterol (Chol), Triton X-100, Trypan blue and Acetylcholine were purchased from Sigma Chemical Co. USA;

Dithionitrobenzoic acid (DTNB) from SRL, India and Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden. Minimum essential medium (MEM) was product of from HiMedia, India. Other chemicals of highest purity grade were purchase from local suppliers. Glass double distilled water was used for all preparations.

3.2.2 **Animal**

Female Swiss albino mice (6-8 weeks), inbred colony maintained in animal room (22±2 °C) on standard dry pellet feed and water *ad libitum*, were used in this study. The average weight of the mice was 20±3 g.

3.2.3 **Experimental Groups**

The animals were divided into the following groups for this investigation:

1. Control group - This consisted of normal mice without the injection of MPG or LEM. The animals were not irradiated. 15 mice was used for each group.

2. Positive control group - A group of animals were irradiated to different dose γ rays (1 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy).

3. MPG group - The mice belonging to this group received intraperitoneal injection of aqueous solution of MPG 30 min prior to irradiation at different doses (1 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy). Three different doses of MPG used were: 10 mg kg^{-1} body weight, 20 mg kg^{-1} body weight and 40 mg kg^{-1} body weight.

4. LEM group - The mice belonging to this group received intraperitoneal injection of liposome encapsulated MPG (LEM) 30 min prior to irradiation at different doses (1 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy). Three different doses of MPG used were: 10, 20 and 40 mg kg^{-1} body weight.

3.2.4 **Preparation Of Liposomes**

Liposomes were prepared by reverse phase evaporation method. The details of the methodology has been described in chapter 2 (section 2.2.3). Briefly, using 5 mg of DPPC, 1 mg of DCP and 2.5 mg of cholesterol which were dissolved in chloroform:ethanol (v/v, 1:1). To the solution, an aqueous solution of MPG was slowly added while vortexing. Chloroform and ethanol were removed by rotary evaporator at 40 °C for about 45 min. LEM was separated from the free MPG by centrifugation or by gel filtration on Sepharose CL-4B. The concentration of MPG in LEM form was calculated by the assay of -SH group as described in chapter 2 (section 2.2.5).

3.2.5 Dose And Mode Of Administration Of MPG

Separate solutions of 2.5 mM, 5 mM and 10 mM MPG were prepared as described in chapter 2 (section 2.2.3.1). The same MPG solutions were used for entrapment into liposome. Half ml of the MPG solutions (free MPG) or 1 ml of LEM were intraperitoneally injected onto mice to deliver 0.204 mg, 0.408 mg and 0.816 mg, respectively, of MPG per mouse. The dose of MPG in these three cases was respectively equivalent to 10, 20 and 40 mg MPG kg^{-1} body weight of mouse. MPG was administered by a single intraperitoneal injection about 30 min prior to irradiation.

3.2.6 Dose And Mode Of γ–Irradiation

Mice were whole body irradiated to doses of 1 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy of γ rays in a Gamma chamber 900 (Bhabha Atomic Research Center, Bombay). The source of radiation was a ^{60}Co delivering radiation at a dose rate of 23.65 Gy min^{-1}.

3.2.7 Assay Of Cell Viability

The method was based on monitoring the number of cells which are able to exclude trypan blue dye indicating that they are metabolically active and live (Wary and Sharan, 1988). Therefore, non-blue cells were taken as live while cells which accumulated dye and became blue were taken as dead.

3.2.7.1 **Preparation of required buffers/solutions**: The following reagents were prepared:

(A) The reagent for dye exclusion assay was made by boiling the following in 90 ml of water.

Trypan blue	400 mg
Sodium chloride	800 mg
Dipotassium hydrogen phosphate	60 mg
Methyl p-hydroxy benzoate	50 mg

The pH of the solution was adjusted to 7.2 with 0.1 N sodium hydroxide, the volume made up to 100 ml and the solution was stored at room temperature (Phillips, 1973).

(B) Minimum essential medium (MEM): MEM (11.7 g) was dissolved in 1000 ml of double distilled water. After autoclaving, it was stored refrigerated.

3.2.7.2 **Methodology:**

Immediately after irradiation, the mice were killed by cervical dislocation. The cells were flushed out of the spleen and the bone marrow and care was taken to minimize cell damage. The

spleen and the bone marrow cell suspensions were prepared in MEM. To 0.9 ml of the cell suspension 0.1 ml of the assay reagent were mixed and incubated at 37 °C for 5 min. Viable (non-blue) and dead (blue) cells were counted on a Burker chamber under a Zena phase contrast microscope. The cell number was calculated using the following formula:

$$\#\ \text{of cells ml}^{-1} = \#\ \text{of cells square}^{-1} \times 25000 \times \text{dilution factor}$$

3.2.8 Calculation Of Viability Modification Factor (VMF)

To quantify the radioprotective effect of MPG or LEM the VMF was calculated. It is a factor derived by dividing the % viability of the cells after irradiation in the presence of either free MPG or LEM by that of radiation alone.

$$\frac{\%\ \text{viability of cells in the presence of either free MPG or LEM after X Gy } \gamma\text{-irradiation}}{\%\ \text{viability of cells after X Gy } \gamma\text{-irradiation}}$$

3.2.9 Assay Of Acetylcholinesterase (AChE)

The enzyme assay methodology was based on Ott *et al.* (1975) with minor modifications.

3.2.9.1 **Preparation of required buffers/solutions**: The following reagents were prepared and stored refrigerated for use:

(A) PBS (pH 7.9) - The constituents of this buffer was:

Na_2HPO_4	0.1 M
NaH_2PO_4	0.1 M
NaCl	0.9 M

Appropriate amounts of Na_2HPO_4 and NaH_2PO_4 were separately dissolved in water to prepare their respective 0.1 M solutions. The two solutions were mixed until pH of 7.9 was obtained. To this calculated amount of NaCl was added to get the desired molarity.

(B) Sucrose solutions: To prepare a 0.2 M solution 68.460 g of sucrose was dissolved in 1000 ml of double distilled water.

(C) The AChE assay mixture in 3 ml contained the following

Acetylcholine	1 mM
DTNB	0.125 mM
Triton-X-100	0.05 %
Enzyme preparation	0.1 ml.

3.2.9.2 **Methodology**: Immediately after irradiation the animals killed by the cervical dislocation and livers removed in 0.2 M sucrose solution. The livers homogenized in PBS (pH 7.9) using a motorized tissue homogenizer. The homogenate centrifuged at 2000 x *g* for 30 min at 4 °C using a Heraeus RS 20 centrifuge. The resulting supernatant (enzyme preparation) subjected to the assay of AChE.

The assay mixture containing the enzyme preparation in a cuvette placed in a Shimadzu UV 150-02 spectrophotometer. The reaction followed by observing an increase in absorption (412 nm) at intervals of 30 sec for 3 min and the increase in absorption min^{-1} calculated. One unit activity of enzyme is defined as the amount of enzyme which consumes 1 M of the substrate at room temperature min^{-1}.

3.2.10 Data And Statistical Treatment

The data presented in this thesis are mean±SD or SEM representing minimum of six to nine independent sets each consisting of at least three mice. In a few experiments, only one mouse constituted an independent set. Such experiments were independently repeated nine times. The controls were age-matched mice, sham-injected with PBS 30 min before irradiation. Student's t-test was applied to calculate the significance of differences. All values with $p \geq 0.01$ have been taken as significant.

3.3 RESULTS

Administration of 2.5 mM, 5 mM or 10 mM MPG is equivalent to 10 mg, 20 mg or 40 mg kg^{-1} body weight, respectively, to each mouse. The doses of whole body γ–irradiation to mice were 1, 2, 4, 6 and 8 Gy. In all the cases, the MPG, either as free MPG or in liposome encapsulated MPG (LEM), was intraperitoneally administered to mice approximately 30 min before irradiation.

3.3.1 Effect Of γ–Irradiation On Survival Of Spleen Cells

Fig. 3-1 shows that the decrease of survival of the spleen cells was radiation dose dependent in the absence of MPG (curve i). The presence of free MPG significantly enhanced the percentage of surviving cells in all the cases (curve ii). The LEM further enhanced the survival of the cells at all doses (curve iii) and exhibited reversal of the trend for the dose of 20 mg Kg^{-1} body weight (panel B). For this dose of LEM, the relative protection afforded by MPG was highest at 8 Gy of γ irradiation.

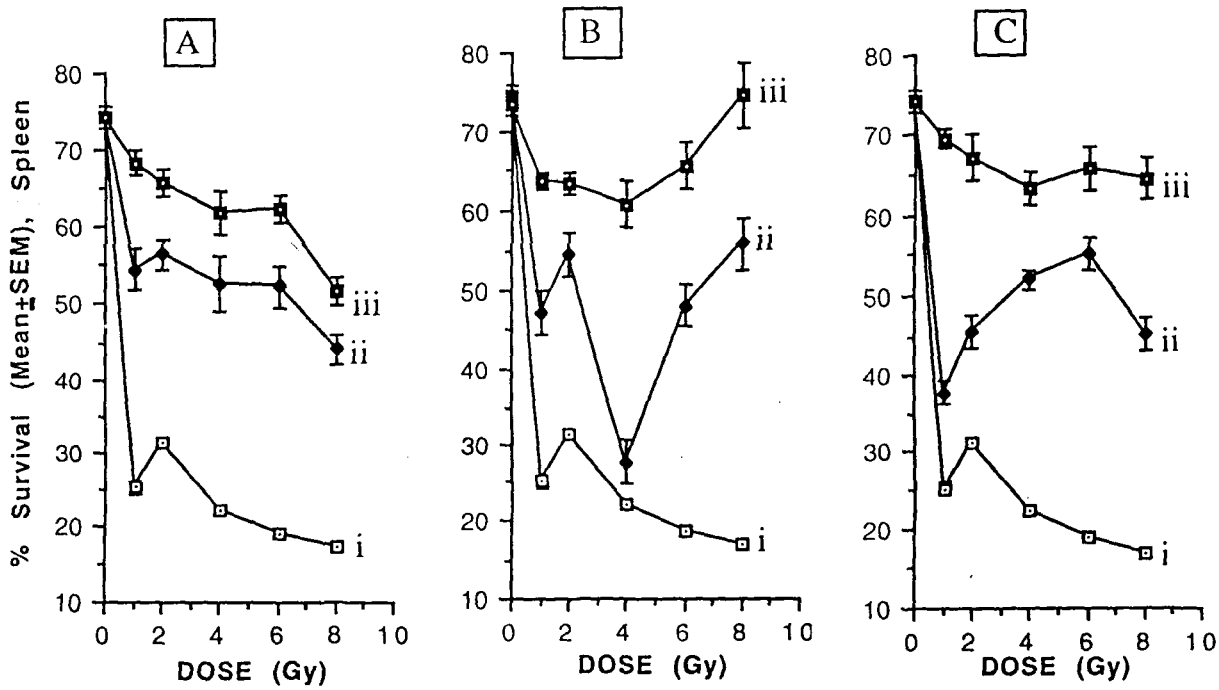

FIGURE 3-1: Percentage survival of spleen cells following γ-irradiation at different doses (curve i) and that after administration of different concentrations of either free MPG (curve ii) or LEM (curve iii) 30 min prior to irradiation. Dose of MPG = 10 (panel A), 20 (panel B), or 40 (panel C) mg kg^{-1} body weight. No bar means the SEM was smaller than the thickness of the point.

3.3.2 **Effect Of γ–Irradiation On Survival Of Bone Marrow Cells**

Fig. 3-2 shows that the decrease of survival of the bone marrow cells was radiation dose dependent (curve i). The presence of free MPG significantly enhanced the percentage of surviving cells in all the cases (curve ii). Like spleen cells, the LEM protected BMC from radiation induced killing at all doses (curve iii). It again exhibited reversal of radiation dose dependent cell killing at the dose of 20 mg kg^{-1} body weight of MPG (panel B).These results also show that the relative protection afforded by LEM at the dose of 20 mg kg^{-1} body weight was optimum at 8 Gy.

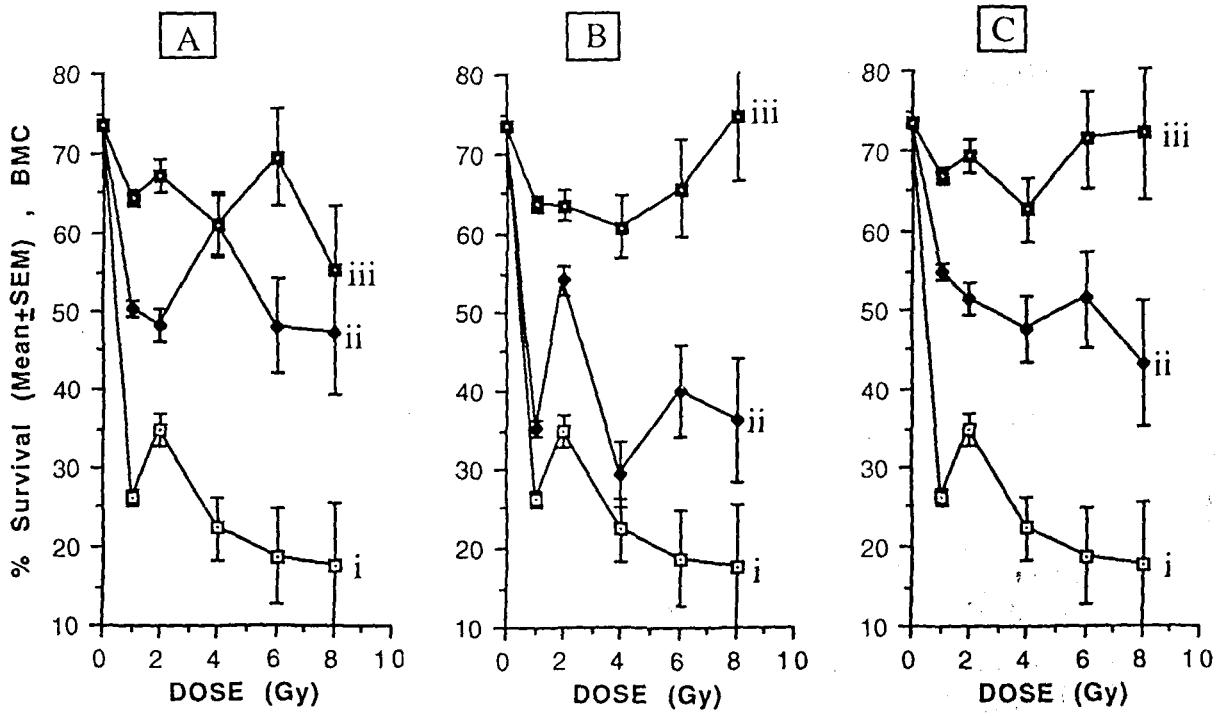

FIGURE 3-2: Percentage survival of bone marrow cells following γ-irradiation at different doses (curve i) and that after administration of MPG (curve ii) and LEM (curve iii) 30 min prior to irradiation. Dose of MPG = 10 (panel A), 20 (panel B) and 40 mg kg^{-1} body weight. No bar means the SEM was smaller than the thickness of the point.

3.3.3 **Viability Modification Factor Of Free MPG or LEM**

Fig 3-3 shows the viability modification factor (VMF) of MPG, either as free MPG or LEM, for spleen cells after 1 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy of γ–irradiation.

The VMF afforded by free MPG (curve i) administered at the dose of 10 mg kg^{-1} body weight (panel A) was essentially invariant for all doses of radiation. When the same dose was administered as LEM (curve ii), the initial high protection at 2 Gy leveled off at higher doses (curve ii, panel A). The situation was not very different when either free MPG (curve i) or LEM (curve ii) was administered at the dose of 40 mg kg^{-1} body weight (panel C). However, free MPG at the dose of 20 mg kg^{-1} body weight exhibited a clear and statistically significant radiation dose dependent increase in VMF (panel B). The VMF was highest for LEM at this dose (panel B).

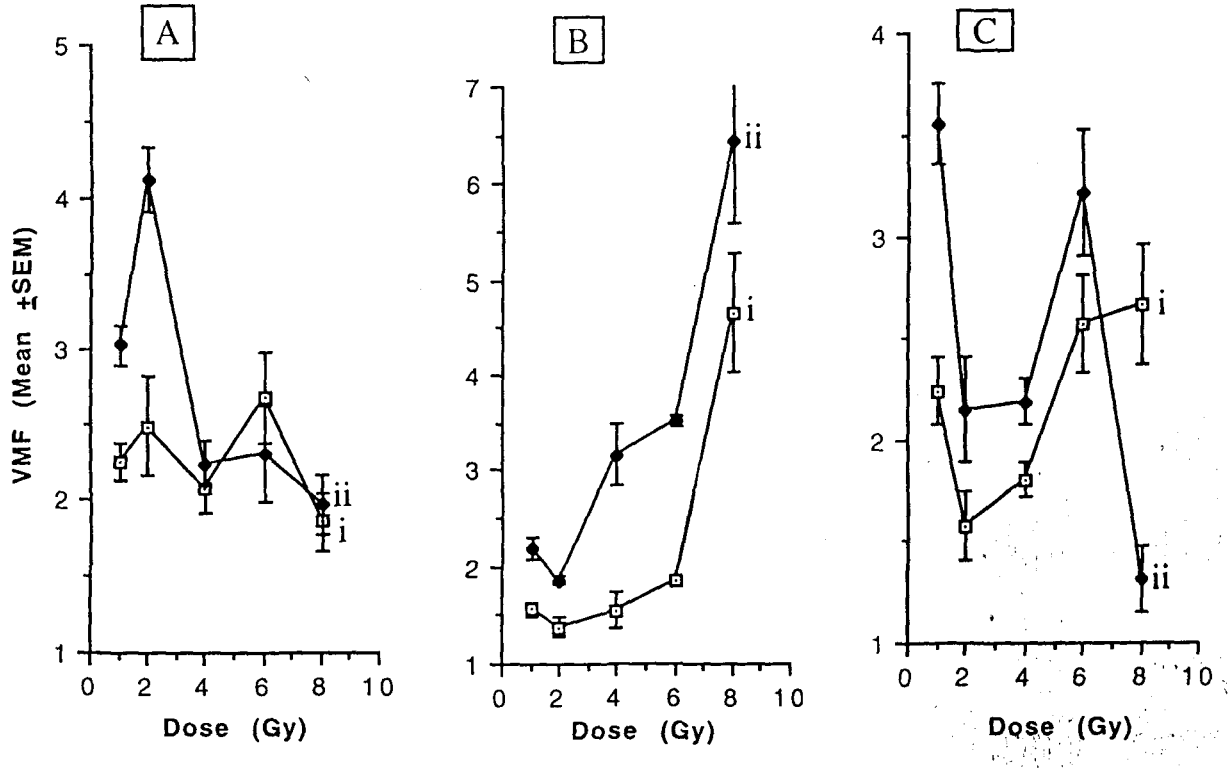

FIGURE 3-3: VMF calculated for spleen cells following different doses of γ-irradiation to mice after administration of free MPG (curve i) or LEM (curve ii) 30 min prior to irradiation. Doses of MPG = 10 (panel A), 20 (panel B) and 40 (panel C) mg kg^{-1} body weight. No bar means the SEM was smaller than the thickness of the point.

Fig 3-4 depicts the VMF afforded by free MPG (curve i) or LEM (curve ii) for BMC at the dose of 10 (panel A), 20 (panel B) and 40 mg kg^{-1} body weight. (panel C) of MPG or LEM 30 min prior to irradiation. While the dose of 10 mg kg^{-1} body weight did not show clear radioprotective gains for the range of radiation dose used (panel A), both 20 mg kg^{-1} body weight (panel B) and 40 mg kg^{-1} body weight (panel C) groups of mice exhibited significant increases in VMF after administration of MPG by either way. The affordable radioprotection, especially for the LEM treated group was very clear and statistically highly significant for its dose of 20 mg kg^{-1} body weight (panel B).

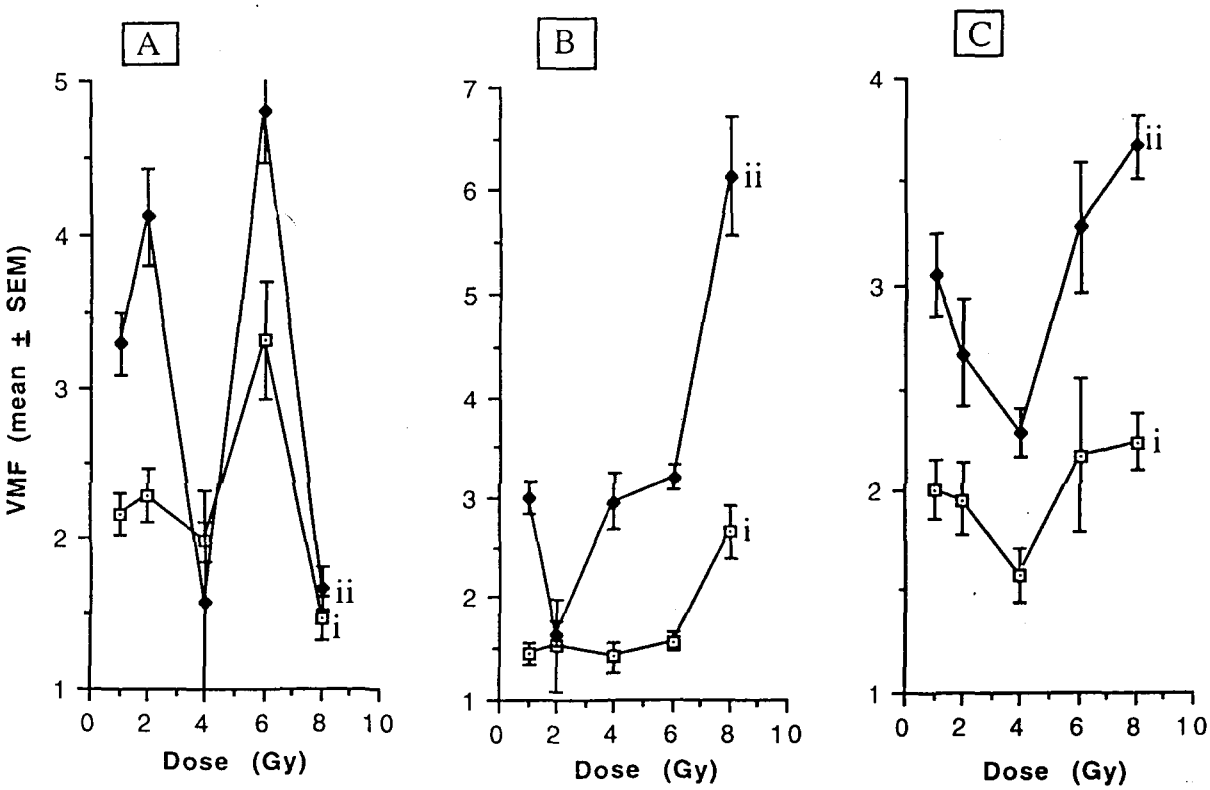

FIGURE 3-4: VMF calculated for bone marrow cells following whole body γ-irradiation to mice at different doses after administration of free MPG (curve i) or LEM (curve ii) 30 min prior to irradiation. Doses of MPG = 10 (panel A), 20 (panel B) and 40 (panel C) mg kg^{-1} body weight. No bar means the SEM was smaller than the thickness of the point.

3.3.4 Effect Of γ– Irradiation Or Release Of Liver AChE Into Supernatant

The radiation induced damage to liver was monitored by observing the activity of membrane bound acetylcholine activity in the supernatant fraction at different doses γ radiation either in the presence or absence free MPG or LEM at the dose of either 10, 20 or 40 mg kg^{-1} body weight. Fig. 3-5 shows the results obtained from mice treated with free MPG or LEM at 10 mg kg^{-1} body weight 30 min prior to irradiation. Except for the radiation dose of 2 Gy, all other doses showed that LEM prevented the release of AChE into supernatant. When 20 mg kg^{-1} body weight dose of MPG or LEM was administered prior to irradiation (Fig. 3-6), both MPG and LEM affordable radioprotection was statistically similar to the possible exception of that at 1 Gy. As depicted in Fig. 3-7, the dose of 40 mg kg^{-1} body weight of MPG, either in free form or as LEM, offered no radioprotection as more AChE was assayed in the supernatant fraction.

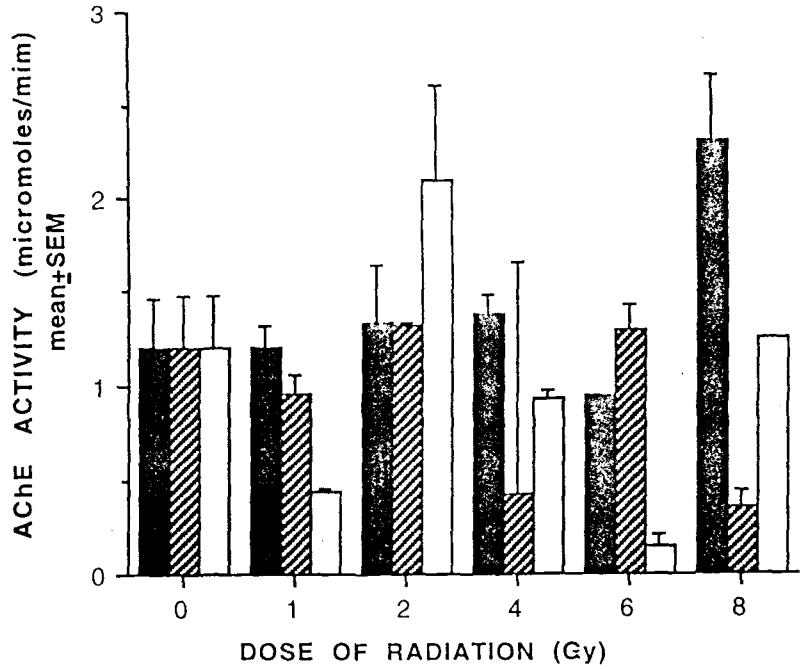

FIGURE 3-5: Liver AChE activity in the supernatant fraction following whole body γ-irradiation of mice at different doses (filled) and that after administration of 10 mg kg^{-1} body weight of MPG (slashed) or LEM (dotted) 30 min prior to irradiation. No bar means the SEM was too small to be visible on this scale.

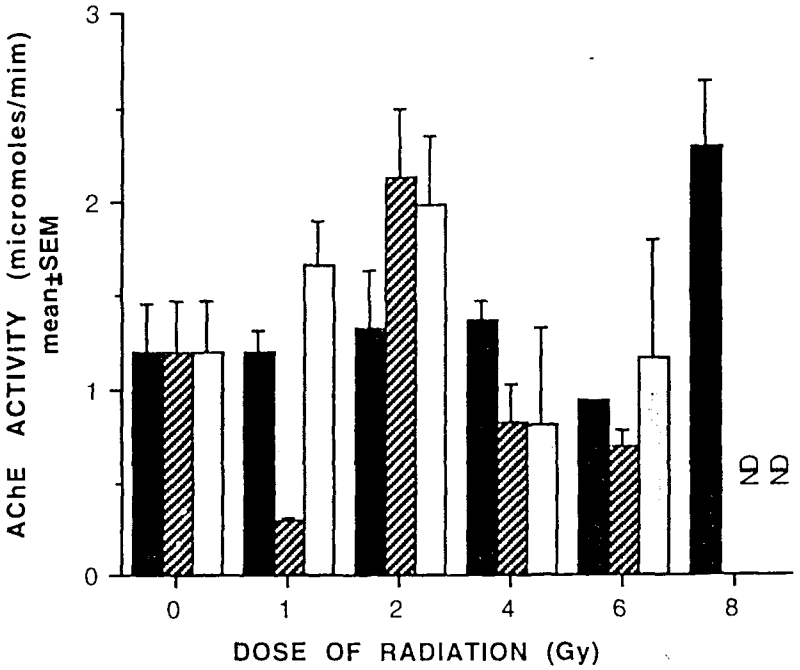

FIGURE 3-6: Liver AChE activity in the supernatant fraction following whole body γ-irradiation of mice at different doses (filled) and that after administration of 20 mg kg^{-1} body weight of MPG (slashed) or LEM (dotted) 30 min prior to irradiation. No bar means the SEM was too small to be visible on this scale.

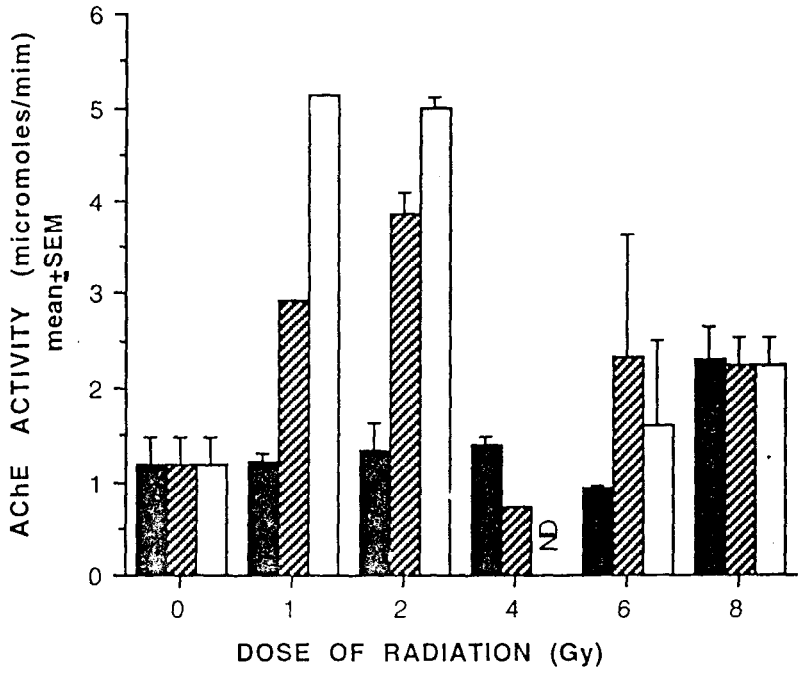

FIGURE 3-7 C: Liver AChE activity in the supernatant fraction following whole body γ-irradiation of mice at different doses (filled) and that after administration of 40 mg kg^{-1} body weight of MPG (slashed) or LEM (dotted) 30 min prior to irradiation. No bar means the SEM was too small to be visible on this scale.

3.4 DISCUSSION

The biodegradable nature of liposome and its capability of encapsulating a target molecule (Gregoriadis, 1988; Allen and Cleland, 1980) are the main reasons for the selection of liposome as a drug delivery system. Reverse phase evaporation method was selected for the encapsulation of MPG into liposomes as the method is simple and reproducible with high entrapment efficiency (Sharan *et al.*, 1992). This method produces large unilamellar vesicles that are suitable as a carrier for MPG. The liposome preparation had the molar ratio of DPPC:Chol:DCP as 1:0.25:0.9 and the lipid/MPG ratio as 5.21. The percentage entrapment of MPG was found to be 54.54±4.95 when 5 mM MPG solution was used (chapter 2). Administration of 0.5 ml of 5 mM MPG aqueous solution or 1 ml of LEM, therefore, delivered 0.408 mg MPG equivalent to each mouse. This is quantity of MPG per mouse shall deliver a dose of 20 mg MPG kg^{-1} body weight that is the effective radioprotective dose of MPG (Ayene and Srivastava, 1985; Saini and Uma Devi, 1979; 1980; Sugahara and Srivastava, 1976;

Sharan *et al.*, 1992; 1995).

Upon administration free MPG migrates to different tissues including the reticuloendothelial system i.e. the spleen cells, bone marrow cells and the liver (Chiba, 1973; Toshioka *et al.*, 1970; Carlsson *et al.*, 1990). MPG molecules are subjected to metabolic alterations depending on the physiological state of the subject. The present finding on BMC shows the protection of pronormoblasts and normoblasts by MPG (Saini and Uma Devi, 1980). The behavior of LEM differs from that of MPG as liposome preferentially migrates to the liver, spleen cells, and bone marrow cells that is rich in reticuloendothelial cells and fenstrated capillaries (Gregoriadis, 1988). At 5 mM there is an increase in protection with the increase in radiation up to 8 Gy. At 10 mM the protection by MPG and LEM remains to almost the same whereas for spleen at 8 Gy the protection by LEM drops below that of MPG. In each case LEM shows a higher viability over the respective free MPG. The encapsulation of the drug in the liposomes delays the metabolic alteration of the entrapped drug (Papahadjopoulos *et al.*, 1991) and so LEM offers a significantly higher radioprotection than that of free MPG to spleen cells and bone marrow cells.

Acetylcholine esterase activity on liver shows a supporting trend with LEM. Spleen cells and bone marrow cells show a higher viability with increasing doses of radiation in LEM treated groups but not so in the cases of free MPG. Since free MPG and LEM were injected in the mice 30 min before radiation, a part of free MPG could have been metabolically altered (Chiba, 1973; Toshioka *et al.*, 1970; Carlsson *et al.*, 1990) making it a less effective radioprotector. The release of the enzyme acetylcholine into supernatant signifying the radiation induced membrane damage was significantly reduced by MPG and LEM shows a higher tendency of protection. The dose of MPG was far below the toxic dose and so it is assumed that the drug did not involve in the metabolism or the survival of the treated animals. LEM as compared to free MPG afforded greater protection for all doses of radiation statistically highly significant ($p \leq 0.001$) in the case of BMC and at lower levels of statistically probability ($0.1 \geq p \geq 0.0001$) in the case of spleen cells (Sharan *et al.*, 1995).

On the other hand metabolic alteration of MPG is reduced to the minimum level in the case of LEM and so it remains in its protective form. The encapsulation of MPG also prevents the circumstantial interaction of MPG with Fe^{+2} which causes radiosensitization effects (Ayene and Srivastava, 1985; Wary and Sharan, 1988). It is possible to assume that MPG has leaked out at higher doses of radiosensitization into the cell membranes and thus a higher protection. Undesirable radiosensitization by liposome encapsulation is prevented and a higher radioprotective effect of MPG in the experimental conditions. In an attempt to reasons for it Gabizon have reported that liposome encapsulated DOX, an anti tumor drug, to be more effective in compared to free form. On liposome encapsulation efficiency of radioprotection is

enhanced of the drug. Liposome can successfully be a vehicle for other drugs in radiotherapy whose use is limited to high toxicity and metabolism problems.

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4.1 INTRODUCTION

In the preceding chapter, it has been conclusively shown that as compared to the free form of MPG, the liposome encapsulated MPG (LEM) afforded statistically significant enhancement of the affordable radioprotection to normal tissues of mice. The dose of MPG used, either as free MPG or as LEM, was 20 mg kg^{-1} body weight of mice and the drug was administered 30 min prior to whole body γ-irradiation. The result may be viewed to suggest that the use of LEM in human radiotherapy instead of free MPG offers clinical gain in terms of radioprotection at the equivalent dose of MPG. In other words. an equivalent dose of free MPG when administered as LEM offers enhanced radioprotection indicating positive gain in clinical efficacy of MPG. Whether the very mild toxic effects of MPG was also reduced when MPG was administered as LEM has not been ascertained. However, it may be logical to think that it would be the case since the same degree of radioprotection would be achieved when lower dose of MPG is administered, albeit in LEM form. Nonetheless, the results of previous chapter relates to MPG affordable radioprotection to normal tissues. Since there are significant metabolic and physiological differences between normal tissues and cancerous tissues (Pitot, 1986), it would be desirable to monitor the radioprotective activity of free MPG vis-a-vis LEM in cancerous tissues. Such an approach will be practical since normal tissue radioprotection does not directly apply to actual situation of radiotherapy where the subject of radiotherapy is a patient bearing cancer. Study of this aspect will further strengthen the attempt to enhance radiotherapeutic index of MPG for clinical application. Therefore, in this chapter attempts have been made to evaluate the MPG affordable radioprotection in a cancer situation when MPG is administered as free MPG or LEM. For the evaluation, cell viability assay, assay of glutathione (GSH) and level of enzyme γ-glutamyltranspeptidase (GGT) have been used as the biological end-points.

4.1.1 The Carcinogen

Keeping this in mind, the first task was to induce cancer in mice. Though it is possible to induce cancer using commercially available chemical carcinogens, aqueous extract of betel nut (AEBN) has been chosen as inducer of cancer in mice due to its relevance to a large section of human population all over the world (IARC, 1987), especially in this part of India. It is well documented that ingredients of betel nut, a customary masticating natural plant product, is a suspected cause of a significant number of human cancer at different sites (IARC, 1984; 1987; Sharan, 1996). The major site of betel nut cancer is the oral cavity that accounts for about 30 % of the total cancers in males in India though other sites have also been reported (WHO, 1984; Sharan, 1996). Notwithstanding subtle differences between different varieties of betel nut from different parts of the globe, the average betel nut has been reported to contain the following major groups of chemicals (Raghavan and Baruah, 1958; Sharan, 1996):

TABLE 4-I

Major constituents of betel nut

CONSTITUENT	QUANTITY
ALKALOIDS	0.25 to 0.38 %
Arecaidine	*0.1 to 0.2 %*
Arecoline	*0.15 to 0.24 %*
others	*trace amount*
CARBOHYDRATES	25 to 30 %
FATS	2.5 to 12 %
POLYPHENOLS	≥ 15 %
PROTEINS	7.5 to 18 %
TANNINS	≥ 18 %

Several analysis of the constituents of betel nut indicate that the suspected carcinogen of betel nut is the alkaloid group of chemicals. Alkaloids are reduced pyridine compound capable of producing various adducts and alkylation products. Therefore, after metabolic activation, alkaloids may produce an array of nitrosation products, the betel nut specific nitrosamines, which may interact with DNA, proteins of other targets in cells to cause transformation (Sharan, 1996). Of different alkaloids, arecoline has been of serious concern since it the most abundant alkaloid and was found to induces molecular alterations conducive to cellular transformation within three to four weeks (Saikia, 1997; Pariat, 1997). Arecoline shows carcinogenic and mutagenic potential (IARC, 1985; Balachandran and Sharan, 1995). However, it has been reported that equivalent amount of arecoline alone and that in combination with other constituents of betel nut shows different potentials of carcinogenesis; the later being more potent carcinogen (Wary and Sharan, 1988). It is obvious that the mastication of betel nut by humans causes extraction of constituents of betel nut in water based saliva and exposes the chewer to arecoline in the presence of entire saliva extractable components of betel nut (Sanghavi, 1981; IARC, 1985). To mimic this situation, the water extractable components of betel nut has been used in this investigation . This extract has been called as aqueous extract of betel nut (AEBN). Mice has been exposed to AEBN in drinking water for 3 months creating a chronic exposure condition. After this, the mice were subjected to whole body exposure, either in the absence or presence of free MPG or LEM. Earlier, it has been reported that intraperitoneal injection of an aqueous extract of betel nuts (1.5 mg arecoline) increased glutathione content and decreased protein -SH groups in liver, kidney and muscle of Swiss albino mice (Shivapurkar and Bhide, 1978). Similarly, the AEBN induced DNA strand breaks in the primary kidney cells *in vitro*

and appreciably enhanced rate of cell proliferation that was inferred as a preneoplastic change (Wary and Sharan, 1988). Chewing betel quid with or without tobacco increased the frequency of micronucleation in the buccal mucosa of the chewers (Jeng *et al.*, 1994). It increased the frequency of sister chromatid exchange in the peripheral blood lymphocytes of the chewers (IARC, 1985).

4.1.2 **Glutathione**

γ-glutamylcysteinylglycine or glutathione is a low molecular weight natural tripeptide and the major free thiol in the living cells (Meister *et al.*, 1981). It is ubiquitously present in variable concentrations in both normal and preneoplastic tissues (Tate and Meister, 1974). Its chemical structure is shown in fig. 4-1.

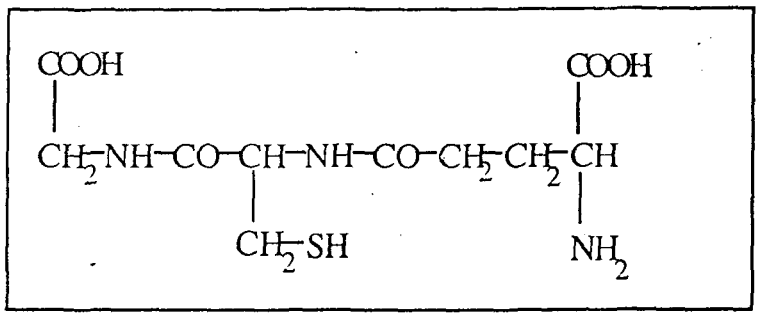

FIGURE 4-1. Chemical structure of glutathione.

In animals it is reported to be present in the cytosol in soluble form though it may also be found in the form of mixed disulfide with the -SH groups of proteins (Revesz and Modig, 1965). A small amount may also be found intercellularly and in the body fluids. It has been shown that enzymatic oxidation of the reduced glutathione (GSH) produces glutathione disulfide (GSSG) and glutathione mixed disulfide (GSSR) (Theodorus *et al.*, 1981). In the living cells, however, the GSH predominates over its oxidized forms. This endogenous GSH has been implicated in such diverse biological processes as in protection against radiation induced damages (Bhattathiri, 1995), detoxification of xenobiotics (Andrews *et al.*, 1989; Pendyala *et al.*, 1990), removal of hydroperoxides, maintenance of sulphydryl status of proteins and maintenance of enzyme activity to name the most important ones. Therefore, the glutathione, which is present extra- and intracellularly in many tissues and are capable of binding to heavy metals, increased cellular glutathione levels may protect against oxidative damage, toxic compounds and radiation (Meister and Anderson, 1983; Dunn, *et al.*, 1987; Meister, 1988; Singhal *et al.*, 1987). GSH content is reported to decreases on starvation which returns to the normal level on refeeding (Meister, 1981).

Glutathione is a versatile protector against the radiation damage. GSH has been shown to act as a free radical scavenger (Simone *et al.*, 1983). Glutathione and the enzymes of glutathione

metabolism are reported to protect the plasma enzyme against radiation injury (Ryskulova, 1983). The depletion of GSH was found to increase the sensitivity of the cells to radiation (Dethmers and Meister, 1981). It has been shown that the radioresistance may be related to enhanced level of cellular GSH rather than intracellular GSH levels *per se* (Moore *et al.*, 1989). In adult rats, the cerebellum and the cerebrum contain high level of GSH as compared to the brain stem and the spinal cord. This has been reported to reflect in the relative radiosensitivities of these tissues (Kudo *et al.*, 1990).

The GSH has two interesting structural features: the -SH groups and the γ–glutamyl linkage. The -SH group is thought of importance in maintaining the sulphydryl group of other molecules like proteins affording radioprotection. On the other hand, the γ–glutamyl linkage serves as a catalyst for disulfide exchange reactions and in the exchange of foreign compounds, hydrogen peroxide and free radicals. Glutathione, therefore, becomes a major endogenous defense against exogenous toxins and antioxidants in the cells (Pendyala, 1995). It has been reported that GSH may non-enzymatically conjugate and inactivate many electrophiles including some drugs used in cancer chemotherapy (DeleveI and Kaplowitz, 1991).

Therefore, monitoring the level of GSH before and after radiation may serve as an excellent indicator of effects of radiation and the extent of radioprotection to tissues afforded by MPG either in its free form or in LEM form.

4.1.4 γ-Glutamyl Transpeptidase

γ–glutamyl transpeptidase (GGT), a lymphoid cell surface marker enzyme, plays an important role in S-substituted glutathione compound metabolism and in the transport of amino acids and peptides across the membrane. GGT is localized in the basal portion of the epithelial cell of the ciliary body and at sites extensively involved in transport (Meister, 1981). It is a predominantly membrane bound enzyme in certain epithelial cells in the normal and preneoplastic tissues (Novogrodsky *et al.*, 1976). The livers of adult mice, rats or hamsters, in contrast to that of guinea pigs or human liver, is almost totally devoid of GGT. Unlike that of the adult animals the livers of the fetal or the neonatal animals show a very high activity of GGT that drops to the low adult level a few days after birth (Boelsterli, 1979).

GGT is a glycoprotein the saccharide moiety of which exhibits high heterogeneity and organ specificity. It catalyses three types of reactions: hydrolysis, transpeptidation, and autotranspeptidation (Dvorakova *et al.*, 1996) which leads to the transfer of γ–glutamyl moiety of a γ–glutamyl donor to a variety of acceptors like amino acids and peptides including glutathione (Tate and Meister, 1974) as shown in fig. 4-2:

2-Glutathione ⇔ γ–Glu-glutathione + Cys-SH-Glu

FIGURE 4-2: Autotranspeptidation of glutathione.

This enzyme can serve as a marker of injury in the liver, pancreas, brain and kidney (Dvoráková *et al.*, 1996). GGT in liver has been recognized as a positive marker in hepatocytes which have undergone malignant transformations (Boelsterli, 1979). Therefore, GGT is a widely used indicator of the preneoplastic transformations of several tissues of various animal species (Richards, 1983). GGT activity have been found to increase in many growing tissues, aging cells and differentiating tissues (Richards, 1983). P-nitroaniline is released during transpeptidation and hydrolysis. It may be that GGT is a donor and also acts as an acceptor and so it may result in autotranspeptidation. The effectiveness of the drug may be limited by the emergence of the drug resistant tumor cells (Kessel, 1989). The enzyme responsible for the cleavage in Gamma Glutamyl linkage was thought to be restricted to kidney and intestine while the one responsible for the hydrolysis of cystine glycine has a broad tissue distribution. Nonetheless, the K_m values of the fetal or transformed cells and that of the adult tissues are identical (Köttgen *et al.*, 1976).

4.1.5 Aims And Objectives

The aims of this piece of study was to monitor the relative effects of free form of MPG vis-a-vis LEM on the radiation induced regression of carcinogenesis. To achieve this mice were chronically exposed to AEBN for 3 months. The following biological end-points were observed in the cancer initiated mice:

(1) Effect of radiation on viability of spleen cells in the presence of free form of MPG or LEM.
(2) Effect of radiation on the liver GSH content in the presence of free form of MPG or LEM.
(3) Effect of radiation on the liver GGT level in the presence of free form of MPG or LEM

4.2 METHODS AND MATERIALS

4.2.1 Chemicals

2-mercaptopropionylglycine (MPG) was obtained from Santen pharmaceuticals Co., Japan. Trypan blue, ethylene diamine tetra acetic acid disodium salt (EDTA), 5,5'dithio-bis(2-nitrobenzoic acid) (DTNB), tris base, glycylglycine and L-γ–glutamyl-p-nitroanilide were procured from Sigma Chemicals Co., USA. Sodium chloride, dipotassium hydrogen phosphate, methyl p-hydroxy benzoate, sodium bicarbonate, potassium chloride, magnesium

chloride, sodium dihydrogen phosphate, sodium sulphate, calcium chloride, trichloroacetic acid (TCA), disodium hydrogen phosphate, glucose and oxygen were bought from indigenous sources. Glass double distilled water was used for preparation of all reagents.

4.2.2 Animals

Four weeks old female Swiss albino mice bred by a random breeding method from an inbred colony maintained in our animal house were used for this investigation. They were housed at 23±2 °C with standard mouse pellet and drinking water *ad libitum*.

4.2.3. Experimental Groups

The animals were divided into the following groups for this investigation:

(1) Control group - This consisted of age matched normal mice without any exposure to AEBN, MPG or LEM. The animals were not irradiated.

(2) AEBN control group - This group consisted of the mice chronically exposed to AEBN for 3 months. The animals were not irradiated.

(3) Positive control group - A group of animals chronically exposed to AEBN for 3 months were irradiated at a dose of 8 Gy of γ-rays.

(4) MPG control group - The mice belonging to this group were chronically exposed to AEBN for 3 months. They received intraperitoneal injection of free form of MPG at the dose of 20 mg kg^{-1} body weight.

(5) MPG group - The AEBN treated mice were intraperitoneally injected with free form of MPG at the dose of 20 mg kg^{-1} body weight 30 min prior to 8 Gy γ–irradiation.

(6) LEM control group - The mice exposed to AEBN for 3 months received intraperitoneal injection of LEM at a dose of 20 mg kg^{-1} body weight.

(7) LEM group - The AEBN exposed mice received intraperitoneal injection of LEM at a dose of 20 mg kg^{-1} body weight 30 min prior to 8 Gy γ–irradiation.

4.2.4 Preparation Of Aqueous Extract Of Betel Nuts (AEBN)

Raw betel nuts (*Kwai*), grown locally in the North-East part of India, were procured from the local markets. The betel nut (100 g) was crushed and left in 250 ml water for 24 hours for extraction. It was filtered through Whatman #1 filter paper and the filtrate was dried by lyophilization to a powder form. This has been called aqueous extract of betel nuts (AEBN).

The extract was stored in cold until use.

4.2.5.Dose And Mode Of AEBN Treatment

A group of 4-week old mice were chronically exposed to AEBN in drinking water for 3 months. The dose of AEBN was 1 mg ml^{-1}. For this appropriate amount of AEBN was dissolved in a known volume of drinking water which was supplied to the mice in the place of normal water.

4.2.6 Dose And Mode Of Irradiation

The mice of the experimental groups were whole body irradiated to 8 Gy of ^{60}Co γ-rays at a dose rate of 20.56 Gy min^{-1}.

4.2.7 Dose And Mode Of Administration Of MPG

MPG, either as free MPG or liposome encapsulated MPG (LEM), was administered by a single intraperitoneal injection at a dose of 20 mg kg^{-1} body weight 30 min prior to irradiation.

4.2.8 Assay Of Cell Viability

The methodology has been described in chapter 3 (section 3.2.7.2) in details. It was followed without any change. Briefly, the spleen cells were flushed out in MEM and was mixed with equal volume of trypan blue dye. The mix was incubated at 37 °C for 5 min. Viable (non-blue) and dead (blue) cells were counted under a phase contrast microscope on a Burkers chamber.

4.2.9 Perfusion Of Liver

The standard method for perfusion (French *et al.*, 1981) was adapted for use on a mouse system .

4.2.9.1 **Preparation of required buffers/solutions**: The following solutions were prepared and stored refrigerated:

(a) Solution 1 contained

NaCl	115 mM

(b) Solution 2 contained

$NaHCO_3$	25 mM

(c) Solution 3 consisted of

KCl	5.9 mM
$MgCl_2$	1.18 mM
NaH_2PO_4	1.23 mM
Na_2SO_4	1.20 mM

(d) Solution 4 contained

$CaCl_2, 2H_2O$	1.25 mM

(e) Glucose 5 mM

Equal volumes of solutions 1, 2, 3 and 4 were mixed together. To this appropriate amount of glucose was added to get 5 mM concentration of glucose in the mixture and the pH was adjusted to 7.4. This has been referred to as perfusion medium that was prepared immediately before use.

4.2.9.2 **Methodology:** A mouse was anaesthetized in chloroform for a minute. A midline incision was made in the mouse to expose the liver. The liver was freed from the surrounding tissues and a loose thread was tied on the common bile duct and the portal vein together. Another thread was used to tie the vena ceva. With a fine pair of scissors an incision was made in the ventral surface. A polyethylene tube with a fine tip was introduced into the first branch of the portal vein and the thread was then tightened. The swelling of the liver was avoided by severing the abdominal aorta. The perfusion was carried out for seven hours at the end of which the perfused liver was used for experiments. The mouse died during this operation.

The perfusion medium was oxygenated by bubbling O_2 gas and was maintained at to 38 °C. The perfusion was carried out in a non-circulating manner (French *et al.*, 1981; Saha *et al.*, 1995) at a flow rate of 7-8 ml g^{-1} of liver min^{-1}.

4.2.10 **Assay Of Glutathione**

The assay is based on Ellmans (1959) method with minor modification.

4.2.10.1 **Preparation of required buffers/solutions:** The following reagents were prepared and stored refrigerated:

(a) Trisodium citrate solution - 2.0 g dissolved in 200 ml of double distilled water to prepare a 28.04 mM solution.

(b) DTNB - 4 mg of DTNB was dissolved in 10 ml of trisodium citrate solution to get 1.0081 mM concentration.

(c) Na_2HPO_4 - 4.2588 g of Na_2HPO_4 was dissolved in 100 ml of double distilled water to get a solution of 0.3 M.

(d) NaCl - 0.84 g of NaCl was dissolved in 100 ml of double distilled water.

(e) EDTA - 0.3664 g of EDTA was dissolved in 50 ml of NaCl solution giving 24.96 mM solution of EDTA.

(f) TCA - 20 g of TCA was dissolved in 100 ml of trisodium citrate solution.

4.2.10.2 **Methodology:**

One gram of perfused liver was homogenized in saline EDTA (solution e). Equal volume of 20 % TCA (solution f) was added, vortexed and centrifuged at 5000 x *g* for 10 min at 4 °C. To the supernatant, 6 x volume of solution c was added. Finally 1 ml of solution b was added, thoroughly mixed and the absorbance of the coloured solution was read at 412 nm for 2 min at an interval of 1 min against a blank containing 1 ml of saline EDTA, 1 ml of 20% TCA, 6 ml of Na_2HPO_4 and 1 ml of DTNB.

4.2.11 **Assay Of γ–Glutamyl Transpeptidase (GGT)**

The methodology followed for this assay is based on Meister *et al.* (1981) with minor modification.

4.2.11.1 **Preparation Of Required Buffers/Solutions** - The following were prepared:

(a) 0.1 M Tris -HCl buffer, pH 8.0 at 25 °C

12.14 g of tris was dissolved in 980 ml of water and pH was adjusted with 1 M HCl. The volume was made up to 1 liter.

(b) 1 M Glycylglycine buffer pH 8.0

1.32 g of glycylglycine was dissolved in 80 ml of water and the pH was adjusted by using 2 M NaOH. The final volume was made up to 100 ml.

(c) 5 mM L-γ–Glutamyl-p-nitroanilide, pH 8.0

0.080 g was dissolved in 60 ml of water and the pH adjusted with tris buffer.

4.2.11.2 **Methodology** : Liver was removed from the mice killed by cervical dislocation. After rinsing it in 0.1 M tris HCl it was homogenized in 1 ml of the Tris HCl buffer using a motorized tissue homogenizer. The whole homogenate was centrifuged at 2000 x *g* at 4 °C for 30 min in a Heraeus RS 20 centrifuge. The supernatant fraction was decanted and stored. The pellet was then resuspended in equal volume of 0.1 M tris HCl buffer. Both were subjected to the assay of GGT.

To 0.2 ml of L γ-glutamyl-p-nitroanilide in a cuvette, 0.2 ml of glycylglycine and 0.5 ml of Tris-HCl buffer were added. The reaction was initiated by adding 0.1 ml of the enzyme preparation. The change in absorbance was read at 410 nm at 37 °C in a spectrophotometer. The final concentrations of the constituents of the assay were:

γ - glutamyl-p -nitroanilide	1 mM
Glycylglycine	20 mM
Tris HCl	20 mM

4.3. RESULTS

The mice chronically exposed to AEBN in drinking water for 3 months looked and behaved normal. There were no visible cancers on the mice. As compared to the age-matched normal mice, the body weight of the AEBN exposed mice were on the higher side.

4.3.1 Viability Of Spleen Cells

The viable and dead cell counts were taken. From this data, the survival fraction (SF) of the

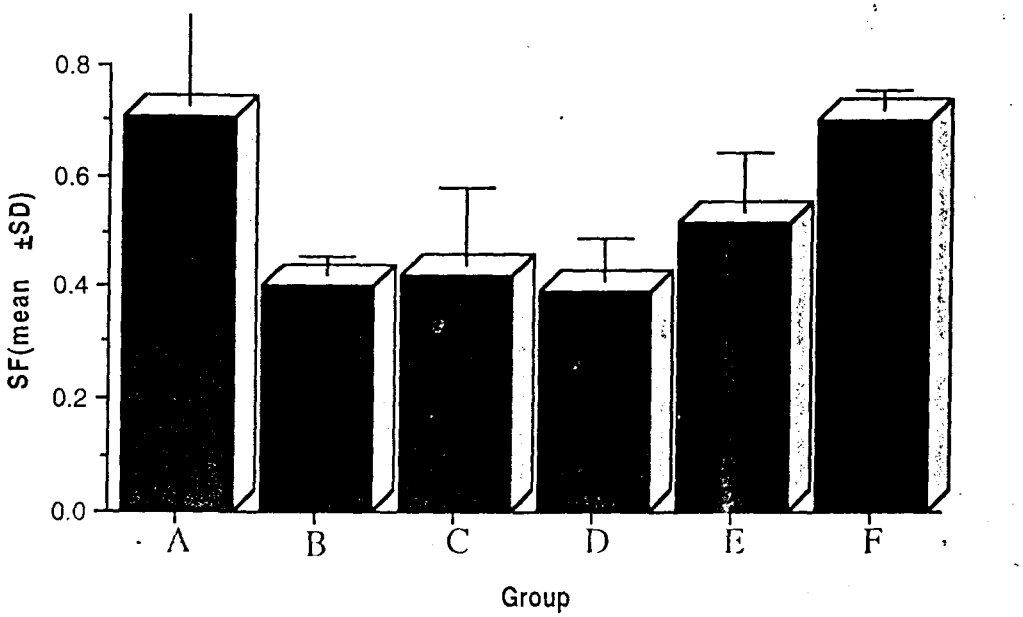

FIGURE 4-3 The survival fraction of normal and AEBN treated mice with and without irradiation in the absence or presence of either free MPG or LEM. A = Normal, B = AEBN, C = AEBN + MPG, D = AEBN + 8 Gy, E = AEBN + MPG + 8 Gy, F = AEBN + LEM + 8 Gy.

spleen cells (SC) was calculated (Fig. 4-3). The SF of SC was significantly reduced to about half in mice treated with AEBN for 3 months (B) as compared to the untreated control (A).

Treatment of AEBN exposed mice to either 20 mg kg^{-1} body weight of free from of MPG for 30 min (C) or 8 Gy of γ-rays (D) did not statistically change the status of SF of SC. However, the SF showed recovery towards the control value when MPG and γ-rays were applied in combination. The recovery was partial when free form of MPG was used in combination with 8 Gy of γ-rays (E). It was nearly normal when LEM in combination with 8 Gy of γ-rays was applied (F).

4.3.2 **Liver Glutathione (GSH)**

Fig. 4-4 shows the level of liver GSH in mice belonging to different experimental groups. The normal (A) and AEBN treated (B) mice showed almost similar levels of GSH. When free MPG (C) or LEM (D) at an equivalent dose of 20 mg kg^{-1} body weight was injected to AEBN treated mice, the level of GSH shot up about 3 to 6 folds after 30 min of injection. Even 8 Gy γ-ray exposure enhanced the level of GSH by about 4 folds (E). However, combination of free form of MPG with 8 Gy γ-rays (F) or LEM with 8 Gy γ-rays (G) brought down the exaggerated level of GSH towards normal. The liver GSH level in the AEBN treated mice after being treated with LEM+radiation (G) was statistically same as that in the normal control.

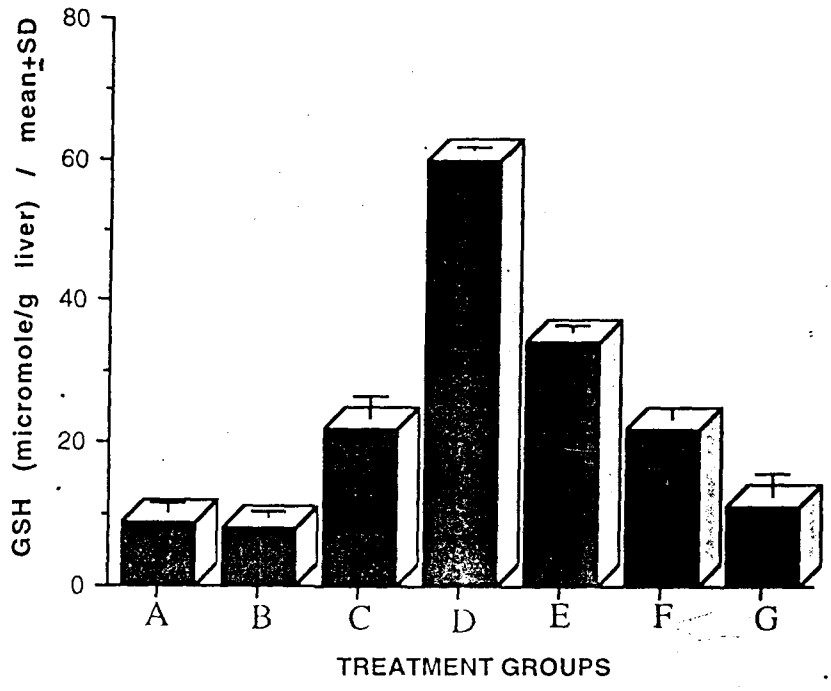

Figure 4-4 The level of GSH in liver of normal mice and that treated with AEBN with and without irradiation in the absence or presence of either free MPG or LEM. A = Normal, B = AEBN, C = AEBN+MPG, D = AEBN+LEM, E = AEBN+8 Gy, F = AEBN+MPG+8 Gy, G = AEBN+LEM+8 Gy.

4.3.4 Liver GGT Activity

The activities of GGT in the liver of mice of different experimental groups were separately monitored in the supernatant and the pellet fractions (Fig. 4-5). The results indicate that major portion of GGT was membrane bound and there was no significant release of the enzyme to supernatant fraction. The membrane bound low activity of GGT in normal liver (pellet, A) was significantly hyperactivated when AEBN treated mice were irradiated to 8 Gy of γ-rays (pellet, B). The combination of free form of MPG with 8 Gy γ-rays (pellet, C) or LEM with 8 Gy γ-rays (pellet, D) sequentially brought down the level of GGT to normalcy. The supernatant residing GGT activity remained subdued in all cases.

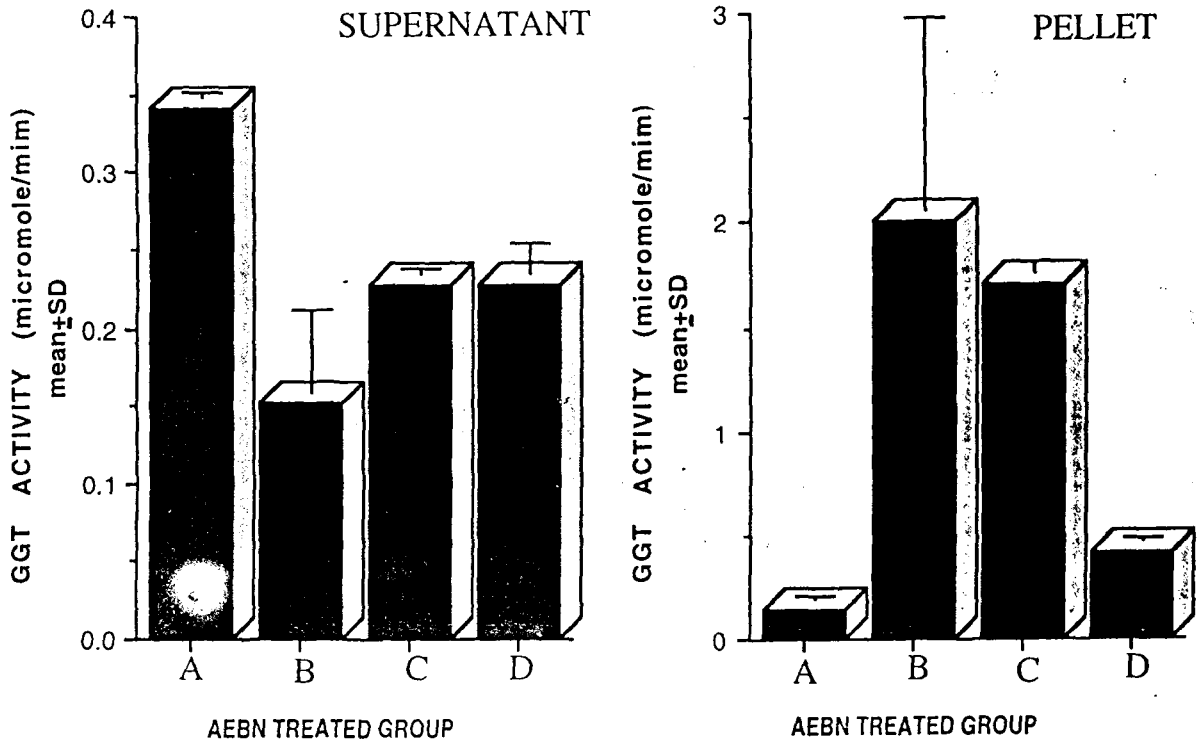

Figure: 4-5 Liver GGT activity in the supernatant and in the pellet fractions of normal mice and that treated with AEBN with irradiation in the presence of either free MPG or LEM. A = Normal, B = AEBN + 8 Gy, C = AEBN + MPG + 8 Gy, D = AEBN + LEM + 8 Gy.

4.4 DISCUSSION

Aqueous extract of betel nut (AEBN) has been taken for the experiment to initiate cancer in mice. Since equivalent dose of arecoline has been shown to initiate carcinogenesis in mice in about 3 weeks (Saikia, 1997), it is assumed that a 3-month long chronic exposure to AEBN, as

was the case in this investigation, would have certainly initiated the process of cancer. No full blown cancer in mice was observed physically after the 3-month period of exposure to AEBN. It is not unlikely since the progression phase and establishment of cancer usually occur after a relatively long gestation period following initiation phase. Several genetic and epigenetic factors influence this gestation period (Pitot, 1986). Nonetheless, the mice used in this work were metabolically different from the normal in the sense that they were initiated for carcinogenesis and were at some stage in the progression phase. As has been reported in the literature, the major suspected carcinogen of betel nut, arecoline, possesses chromosome damaging ability (Panigrahi and Rao, 1982; 1983) which was enhanced in the absence of exogenous metabolic system (Stich *et al.*, 1981). Ashby *et. al.* (1979) have reported that arecoline and arecaidine from betel nuts can induce cell transformation. The arecoline has been shown to be potentiated in combination with other constituents of betel nut present in AEBN (Wary and Sharan, 1988). Therefore, the chronic exposure protocol for AEBN used in this investigation is likely to introduce cancer like situation in the exposed mice. Such mice may be taken to represent a cancer bearing patient undergoing radiotherapy. Therefore, the influence of free form of MPG as against LEM on radiation induced changes in the biological parameters is likely to represent the responses expected in actual radiotherapy.

The function of the liver can be studied with a spectrum of liver ranging from intact organ, liver slices, isolated hepatocytes, homogenate membrane fraction, isolated enzymes and perfused liver (Dirk *et al.*, 1981). The use of isolated and perfused liver is attractive as the intact architecture of the liver and the possibility of controlling hepatic blood supply as well as neuronal and hormonal influences is possible. A large number of perfusate samples can be collected and compared with intact animal organ (Dirk *et al.*, 1981). The perfusion system does not require the damaging treatment with Ca^{+2} free solution and digesting enzymes and the normal polarity of the cells and their localization on the liver lobule is maintained. With the perfused liver only short term experiments are possible up to a duration of 6-8 hours. Many modifications of the original perfusion technique have been described (Staib and Scholzm, 1968; Bartosek *et al.*, 1973).

Glutathione and the related enzyme forms a part of the system which protect the plasma membrane against radiation injury (Ryskulova *et al.*, 1983). GSH can spontaneously and non-enzymatically conjugate and inactivate many electrophiles including the drugs used in chemotherapy (Deleve, *et al.*, 1995). Increased cellular glutathione levels may protect against oxidative damage (Meister and Anderson, 1983; Dunn, *et al.*, 1987; Meister, 1988; Singhal *et al.*, 1987). GSH protects the cell against ionizing radiation, maintaining the -SH status of the protein and modulation of the enzyme activity by disulfide exchange. (Theodorus *et al*, 1981). In this investigation the circulating GSH was completely flushed out by perfusion so as to assess the cellular level of GSH in liver (Fig. 4-4). It is interesting to note that the cellular GSH

content did not change following chronic AEBN treatment for 3 months. The level of cellular GSH, however, was elevated significantly 30 min after intraperitoneal injection of free form of MPG which was further boosted when instead LEM was injected (Fig. 4-4). This is likely because the assay of GSH involves -SH group, the amount of which is expected to increase when a -SH containing compound such as MPG is also present. It is known that liver is the major site for the detoxification of the foreign compounds. Thus, after intraperitoneal injection, MPG or LEM shall migrates to liver. Since liposomes are known to have preferential migration to liver (Allen and Cleland, 1980; Greorgdias,1980; Kirby *et al.*, 1980), in case of LEM administration, the contribution of -SH groups from MPG encapsulated in liposome will be higher. This may be the reason for several folds higher GSH content in liver after administration of LEM. Nonetheless, 8 Gy γ-ray exposure also enhanced the cellular GSH level (Fig. 4-4) which was progressively brought down to the normal level when irradiation followed MPG or LEM administration, the later being most effective. Radiation induced enhancement of cellular GSH level may indicate radioresistance. Progressive lowering of the GSH level when MPG or LEM was present should normally be interpreted as radiosensitivity. However, if one considers that sufficient amount of exogenous -SH groups (made available by MPG or LEM due to their administration 30 min prior to irradiation) was available in the liver, it is logical to think that cellular GSH content need not increase. In other words, since sufficient amount of -SH groups from MPG or LEM is present, there is no biological need to generate endogenous GSH resource. It may be a case of biological economy. Such observation on biological economy has been made earlier in context with influence of post-translational modification of chromosomal proteins to cause structural changes in chromatin. It was shown that level of poly-ADP-ribosylation increases in order to relax chromatin. However, prior hyperacetylation leads to lowering of poly-ADP-ribosylation because both processes are known to lead to relaxation of chromatin superstructure (Bohm *et al.*, 1997).

The above interpretation is supported by the observation made on the activity of liver GGT enzyme (Fig. 4-5). It is apparent from Fig. 4-5 that major portion of GGT was membrane bound and only a minor portion was found in the supernatant fraction (Fig. 4-5). It may be noted that the membrane bound GGT (pellet fraction) was hyperactivated by 8 Gy γ-irradiation indicating damage to the enzyme. Hyperactivation of enzymes is a known phenomenon following irradiation (Sharan and Srivastava, 1984). The presence of free form of MPG 30 min prior to irradiation was able to partially restore normalcy of the enzyme which reached the control level when LEM was administered 30 min prior to irradiation. The regaining of control level when LEM was administered before irradiation indicates highest level of radioprotection. It was only partial when free form of MPG was administered.

Therefore, the results show that even in cancerous condition, LEM affords better radioprotection than the free form of MPG.

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5.1 INTRODUCTION

Carcinogenesis is a multistep process involving multiple alterations in the normal gene expression patterns as well as mutations during cellular transformation. The mutation includes rearrangements, loss or amplification of genes (Pitot, 1986). In the process some neogenes are known to be expressed while others are shut off (Ames and Gold, 1991). All these processes are almost entirely dependent on the structural organization of the chromatin (Boulikas, 1991). For instance, a highly condensed chromatin segment will have low or no transcriptional activity due to low or no possibility of interaction of chromosomal proteins/enzymes/factors with the gene. A relaxed superstructure of chromatin provides accessibility of proteins/enzymes/factors to genes and, thereby, favors transcriptional activity. On the other hand, radiation induced damages to the genetic material, DNA, and its repair are also under a great influence of the status of the organization of the chromatin superstructure. Similar to transcription, a condensed chromatin structure prevents accessibility of DNA segment to radiation induced free radicals and other activated species. This also prevents DNA damaging effects of radiation. For repair systems to correct damaged DNA, likewise, proper accessibility of damaged sites of DNA to repair systems is essential. It is obvious again that the state of organization of chromatin, therefore, has serious bearing on the damage and repair processes associated with radiation.

For radiotherapy, the basic operating principle is that radiation should cause enough damage to the genetic material and other components of a cell or tissue so that it does not survive. Among the two broad category of cellular targets, the genetic material and the other component, damage to DNA, the genetic material, is of utmost importance. It is obvious that the cell or the tissue envisaged to be killed by radiation is cancerous. From radioprotection view point, it is envisaged that the undesirable radiation induced damages to normal cell are kept to the minimum. It logical, therefore, to think that if the chromatin is in condensed state both radiation induced damage and the repair of damages will be slow. A relaxed status of chromatin will favour induction of damages to DNA. In this context, it may be worthwhile to look into the status of chromatin organization during radiotherapy and the influence of the presence of MPG, either in its free form or as LEM on it during radiotherapy.

Of several factors that influence chromatin organization, poly-ADP-ribosylation, a post translational modification of chromosomal proteins, has been shown to greatly influence the level of condensation/relaxation of chromatin (Weisbrod, 1982). Therefore, monitoring the level of poly-ADP-ribosylation would give an insight into state of chromatin. Further, it is possible to directly monitor the extent of chromatin condensation/relaxation by its enzymatic fragmentation using DNase I (Modrich, 1994; Tarnaletti and Pfeifer, 1994, Vidaldi *et al.*, 1977). This investigation has been designed to probe into this aspect.

5.1.1 Chromatin

The DNA carrying genetic information is combination with a large array of chromosomal proteins to constitute chromatin (Lewin, 1994; Weisbrod, 1982). The structural basis of chromatin organization revolves around five types of histones proteins (Kornberg and Klug, 1981). Four types of histones (H2A, H2B, H3 and H4) constitute the core histone around which the DNA coils to create a nucleosomes. Since DNA is a continuous piece, a series of nucleosomes, spaced by a small piece of DNA where the fifth histone (H1) is proposed to interact, is created. This polynucleosome undergoes further folding to make the chromatin. Therefore, a chromatin can be present in a condensed state or in a dispersed state. The process of transcription necessitates relaxation and unwinding of chromatin to make DNA accessible to enzymes/factors responsible for transcription.

The organization of chromatin is essentially achieved by the electrostatic and weak force interactions between the chromosomal proteins, mainly histone proteins, which are basic and the DNA strand which is acidic (Weisbrod, 1982). The strength of interaction between these two components of chromatin decided the condensed or relaxed status of the chromatin. The change in the organization of chromatin, therefore, may be affected when the interaction between the chromosomal proteins and the DNA change under specific conditions. This has been shown to have significant bearing on the level as well as the pattern of gene expression (Arwood and Spikes, 1990; Paranjape *et al.*, 1994).

In order to monitor the structural changes in chromatin, several approaches are possible. A convenient way of monitoring the status of the organization of chromatin is chromatin activity assay using DNase I that fragments DNA of the chromatin randomly (Weisbrod *et al.*, 1980; Goodwin and Mathew, 1982). The fragmentation efficiency of DNA by DNase I depends on the availability of DNase I cutting sites on the chromatin (Schneeweiss *et al.*, 1997). If the chromatin is condensed, fewer sites for DNase I fragmentation will be available and the reverse will be true when the chromatin is in a relaxed state. Therefore, DNase I mediated fragmentation is a convenient way of monitoring the state of organization of chromatin (Weisbrod *et al.*, 1980; Schneeweiss *et al.*, 1997)

5.1.2 Poly-ADP-Ribosylation

Poly-ADP-ribosylation (PADPR) is an enzyme catalyzed post-translational modification of essentially chromosomal proteins wherein ADP-ribose moieties of endogenous nicotinamide adenosine dinucleotide (NAD+) is covalently attached to an acceptor amino acid residue of a target protein (Saikia, 1997; Althaus, 1992; Poirier and Moreau, 1992). A branched or unbranched homopolymer of repeating ADP-ribose units may be created on the target proteins. This is referred to as poly-ADP-ribosylation (Althaus and Richter, 1987). This is predominately a nuclear reaction accounting for over 95 % of all such reaction (Ueda, 1989).

The main target proteins for PADPR are chromosomal proteins include histones, endonucleases, topoisomerase I, DNA polymerase etc. besides poly-ADP ribose polymerase enzyme which catalyses the PADPR reaction (Ferro and Olivera, 1984; Cressien and Shall, 1982; Boulikas, 1989; Schneeweiss *et al.*, 1995 Sharan *et al.*, 1996).

The metabolism of PADPR reaction involves enzymes such as, poly-ADP-ribose polymerase, poly-ADP-ribose glycohydrolase and ADP-ribose protein lyase. The former enzyme is responsible for anabolism, that is, biosynthesis of poly-ADP-ribosyl moieties on a target protein while the latter two are involved with catabolism, that is, degradation of poly-ADP-ribose moieties from the target proteins (Althaus and Richter, 1987). This post-translational modification of chromosomal proteins has been suggested as one of the most important post-translational processes known to affect interaction of chromosomal protein with DNA in chromatin (Poirier *et al.*, 1982; Niedergang *et al.*, 1985; de Murcia *et al.*, 1988). Therefore, the PADPR reaction has significant bearing on the state of organization of chromatin (Poirier *et al.*, 1982).

The PADPR reaction has been shown to be implicated in such diverse biological processes such as, repair of radiation induced DNA damages (Durkacz *et al.*, 1980; Sharan *et al.*, 1996), regulation of cell cycle (Surowy and Berger, 1983; Althaus, 1987), cell differentiation and gene expression (Miyakawa *et al.*, 1972; Althaus, 1987), carcinogenesis (Miwa and Sugimura, 1990) and in several cellular processes (Althaus, 1987; Schneeweiss *et al.*, 1995). The enhancement of the level of PADPR of chromosomal proteins has been reported after exposing chromatin to DNA damaging agents as well as during DNA excision repair (Shall, 1995). Modrich (1994) and Tarnaletti and Pfeiffer (1994) have shown the crucial role of chromatin superstructure in excision repair and have indicated that the PADPR reaction may be a cellular regulator of this process. It has been proposed that enhancement of PADPR of nuclear proteins, especially of histone proteins, causes relaxation of chromatin and lowering of PADPR causes condensation of chromatin (Althaus, 1978; de Murcia *et al.*, 1988) and, therefore, may regulate the structural organization of chromatin. This alters the accessibility of genes (DNA) to various enzymes/factors (Althaus *et al.*, 1980; Althaus, 1992; Modrich, 1994; Tarnaletti and Pfeiffer, 1994)) which may be an avenue for accomplishing the biological functions enlisted above. However, there are reports were enhancement of PADPR was shown to result in inhibition of several chromosomal enzymes (Haiashi and Ueda, 1982). While the involvement of the process of PADPR in a variety of cellular process is known, the exact mechanisms of its varied functions remains far from being clear.

The process of carcinogenesis has been directly shown to be influenced by the level of PADPR or to the chemical inhibitors of PADPR (Miwa and Sugimura, 1990; Boulikas, 1993; Saikia, 1997). It is known that deregulation of initiation of DNA replication, entry of cells into mitosis

and malfunction of DNA repair machinery may push forward cellular transformation (Boulikas, 1991). Since all these processes are potentially regulated by PADPR, it has been suggested that PADPR has a profound regulatory function in the process of carcinogenesis (Boulikas, 1992; 1993).

The assay of PADPR uses ^{32}P-NAD$^+$ as the substrate. When the radiolabelled NAD$^+$ mixes with the cellular pool of cold NAD$^+$, which is the substrate for PADPR reaction and donor of ADP-ribose moiety in normal cellular metabolism, part of the radioactivity of ^{32}P-NAD$^+$ pool is transferred onto the newly synthesized poly-ADP-ribose polymer on a target protein (Saikia, 1997). This can be conveniently monitored by scintillation counting which indicates the level of cellular PADPR. There are, however, methodological problems with the assay (Schneeweiss *et al.*, 1995). In order to internalize ^{32}P-NAD$^+$ into the cell so that it mixes with the cellular pool of cold NAD$^+$, the cell membrane must be partially ruptured to create big enough holes for its passage. This necessitates several rather harsh treatments, a commonly used one being hypotonic shock. Therefore, single cell suspension also becomes an essential requirement for the assay. Therefore, application of this assay methodology on a tissue like liver becomes very difficult. Furthermore, any such treatments either to create single cell suspension from a tissue or hypotonic shock to create holes in the membrane for the internalization of ^{32}P-NAD$^+$ has been shown to very adversely affect the PADPR reaction which is likely to give misleading results (Schneeweiss *et al.*, 1995). As a result, the assay of PADPR has been limited to either in *in vitro* systems or in tissues which could be converted into single cell suspension (like bone marrow and spleen cells) without any chemical treatments. Since in the investigation embodied in this thesis tissue like liver was of interest, it was necessary to develop an assay system for PADPR which could be applied to liver and the results are reliable. Keeping this in mind, an immunoassay for PADPR has been developed.

5.1.3 Aims And Objectives

The process of carcinogenesis, DNA damage and its repair appear to be intricately related to PADPR (Boulikas, 1993). All these three processes are of prime concern to this investigation embodied in the thesis. Extending the information it seems logical to think that even regression of carcinogenesis, either under the influence of radiation (as in radiotherapy) or in chemo-radiotherapy (irradiation after MPG or LEM administration), may be mediated through the process of PADPR and the resulting effect on the chromatin superstructure. With this in mind, this piece of work envisaged to look into the following:

1. Level of liver PADPR in AEBN exposed mice, and that irradiated in the presence or absence of MPG either in its free form or as LEM.

2. Status of chromatin superstructure under those conditions.

5.2 METHODS AND MATERIALS

5.2.1 Chemicals And Materials

Raw betel nuts were bought locally and aqueous extract of betel nut (AEBN) was prepared from it as described in chapter 4 (section 4.2.4).

Nylon mesh, Triton-X-100, Dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), Glycerol, Bromophenol blue, Bovine serum albumin (BSA), Anti-IgG-ALP conjugate and Tris were procured from Sigma Chemicals Co., USA. Tween-20 and ALP colour developing kit were products of BioRad, USA. All other chemicals were of highest purity grade available from different indigenous sources. Glass double distilled was used for the preparation of solutions.

5.2.2 Animals And Carcinogen Treatment

Four weeks old female Swiss albino mice bred by a random breeding method from an inbred colony maintained in our animal house. A group of mice was maintained on AEBN in drinking water for 3 months as described in chapter 4 (section 4.2.3). These animals were used in this investigation.

5.2.2 Experimental Groups

The animals were divided into the following groups for this investigation:

(1) Control group - This group of mice were neither exposed to AEBN nor treated with MPG.

(2) AEBN control group - This group of mice were chronically exposed to AEBN for 3 months.

(3) Positive control group - This group of mice was chronically exposed to AEBN for 3 months and then irradiated to the dose of 8 Gy γ rays.

(4) MPG control group - This group of mice after being chronically exposed to AEBN for 3 months received intraperitoneal injection of MPG (20 mg kg^{-1} body weight).

(5) MPG group - The AEBN exposed mice was intraperitoneally injected with MPG (20 mg kg^{-1} body weight) for 30 min prior to 8 Gy γ ray irradiation.

(6) LEM control group - This group of mice after being chronically exposed to AEBN for 3 months received intraperitoneal injection of LEM (20 mg kg^{-1} body weight).

(7) LEM group - The AEBN exposed mice was intraperitoneally injected with LEM (20 mg kg^{-1} body weight) for 30 min prior to 8 Gy γ ray irradiation.

5.2.3 Dose And Mode Of Irradiation

To the mice of the experimental groups were whole body irradiated to 8 Gy of ^{60}Co gamma rays (dose of radiation = 19.37 Gy min^{-1}).

5.2.4 Isolation Of Total Chromatin From Liver

The procedure is essentially based on the method described by Marushige and Marushige (1978) with minor modifications.

5.2.4.1 **Preparation of required buffers/solutions:** The following reagents were prepared and stored refrigerated for use:

1. Homogenization buffer - It consisted of:

Tris-HCl buffer (pH 8.0)	10 mM
$MgCl_2$	5 mM
Sucrose	0.25 M

2. Detergent homogenization buffer - It contained 0.1 % Triton X-100 in homogenization buffer.

3. Overlaying buffer - The constituents of this buffer were:

Tris-HCl buffer (pH 8.0)	10 mM
$MgCl_2$	5 mM
Sucrose	2.2 M

4. Nuclei homogenization buffer - It contained the following:

Tris-HCl buffer (pH 8.0)	1 mM
DTT	0.5 mM
EDTA	0.1 mM
Glycerol	12.5 %

5.2.4.2 **Methodology**: Chromatin was isolated from the liver of the treated and the normal mice. The liver of the mouse killed by cervical dislocation was washed in tris-HCl buffer (pH 8.0) was homogenized in cold in 5 volumes of the homogenization buffer. The whole

homogenate was filtered through a nylon mesh and the filtrate centrifuged at 500 x *g* for 10 min at 4 °C in a Heraeus-20 RS centrifuge. The nuclei pellet was carefully recovered and purified. To do this the pellet was overlaid on a 4-volumes of the overlaying buffer in a centrifuge tube and was subjected to centrifugation at 61,000 x *g* for 60 min at 4 °C in a Hitachi RPS ultracentrifuge. The pure nuclei pellet was collected. The purity of nuclei was confirmed by microscopic observation in a Zena phase contrast microscope.

The nuclei were homogenized in 30 volumes of nuclei homogenization buffer. The homogenate was centrifuged at 30,000 x *g* for 10 minutes at 4 °C in the Heraeus centrifuge. The gelatinous portion was recovered as chromatin.

5.2.5 **Chromatin Activity Assay**

The general method described by Weisbrod and Weintraub (1979) was followed after suitably modifying it.

5.2.5.1 **Preparation of required buffers/solutions:** The following reagents were prepared and stored refrigerated for use:

1. 50 mM tris-HCl buffer (pH 7.5) containing 10 mM of $MgCl_2$.

2. 50 mg ml^{-1} aqueous solution of BSA.

3. 500 mM EDTA solution.

4. Loading buffer - It consisted of:

Sucrose	100.0 %
Bromophenol blue	0.4 %

5. Reaction buffer (R-Buffer): The composition of this buffer was:

Tris-HCl buffer (pH 7.5)	50 mM
$MgCl_2$	10 mM
BSA (50mg ml^{-1})	10 mM
DTT	0.1 M

5.2.5.2 **Methodology**: Chromatin preparation in a volume of 25 µl was mixed with 25 µl of the reaction buffer and 10 µl of 50 mg ml^{-1} BSA. The reaction mixture was incubated at 37 °C for 30 min after thorough mixing. One unit of DNase I was added to it and the mixtures were digested for 2 min, 5 min, 10 min or 20 min at 37 °C. The digestion was stopped by addition of 5 µl of 500 mM EDTA solution. The sample was then electrophoresed on an agarose gel.

5.2.6 Agarose Gel Eletrophoresis For DNA

The standard method described by Rickwood and Hames 91990) was used with some modification.

5.2.6.1 **Preparation of required buffer:** These buffers were prepared and stored refrigerated:

1. Electrophoresis buffer (TBE) - The buffer contained;

Tris-borate buffer (pH 8.3)	10 mM
EDTA	1 mM

Usually a 10 X concentrated stock was prepared which was diluted at the time of the gel run.

2. Loading buffer (6 X)

Sucrose	100.0 %
Bromophenol blue	0.4 %

5.2.6.2 **Methodology:**

(A) Casting of gel - Agarose at a concentration of 0.3 % was taken and dissolved in TBE by warming it in water bath at 60 °C. The agarose was then poured on the electrophoresis plate and the comb was put into place and allowed to cool down to room temperature.

(B) Electrophoresis run - To a sample of 60 µl. the loading buffer (10 µl) was added and mixed. From this 6 µl was loaded in a well of the 0.3 % agarose gel. The electrophoresis was carried out on a constant voltage of 20 V (10 mA) for 7 hours using TBE buffer.

The gel was then immersed for 15 min in TBE buffer containing 1µg ml^{-1} ethidium bromide. The gel was washed 4-5 times in distilled water. The digestion pattern of chromatin was then observed under UV illumination on a transilluminator.

5.2.7 Dot Blot Assay For Liver And Spleen Cell Poly-ADP-Ribosylation

5.2.7.1 **Preparation of the buffers/reagents**: The following buffers were prepared and stored refrigerated:

1. Antibody against poly-ADP-ribose - From mouse bone marrow cells, the ADP-ribose polymer was isolated and purified. The purity of the polymer was ascertained on HPLC. This polymer of ADP-ribose was used to raise antibody against it in a rabbit

by intramuscular immunization protocol. After 6-8 weeks, a booster dose was administered and on the third day, the rabbit was bled through its ear vein. The blood serum was collected which was used as the first antibody.

2. Tris buffered saline (TBS) - The composition of this buffer was:

Tris-HCl buffer (pH 7.5 at 4 °C)	20 mM
NaCl	500 mM

Usually a 10 X solution of TBS is made and diluted at the time of use.

3. Tween TBS (TTBS) buffer: To prepare this, 0.05 % Tween 20 was added to 1 X TBS buffer.

4. Blocking solution : A 5 % non-fat dry milk (NFDM) solution was made in TBS buffer with 0.2 % sodium azide in it.

5. First antibody solution : The serum (first antibody) was diluted 1:750 in TBS and was used for immunoprobing.

6. Anti-IgG-ALP conjugate solution: The conjugate (obtained from Sigma Chemical Co.) was diluted 1:5000 in TBS for use.

7. Colour development reagent: It was a commercial product and was used directly.

5.2.7.2 **Methodology**: The liver of the mice killed by cervical dislocation was washed in tris HCl buffer (pH 8.0) was homogenized in cold in PBS. Similarly, spleen cells were flushed out and homogenized in PBS. These two whole homogenates were used as source of poly-ADP-ribose or antigens.

On a nitrocellulose sheet, cut to size and appropriately marked, the liver or spleen cell whole homogenate was dotted under vacuum. After the antigen was applied, the membrane was immersed into the blocking solution for 45 min at 37 °C with occasional gentle shaking. The blocking solution was decanted and the membrane washed in TTBS for 10 min. After decanting TTBS, the first antibody solution was added and the membrane incubated for 45 min at 37 °C with gentle agitation. The first antibody was removed by washing TTBS for 10 min at 37 °C. This process was repeated twice. The anti-IgG-ALP conjugate solution was then added and the membrane was incubated for 45 min at 37 °C. The solution was removed and the membrane washed in TTBS for 10 min followed by a wash in TBS for 5 min with gentle agitation.

The membrane was immersed in colour development reagent for about 50 min with gentle agitation at 37 °C. The colour development was stopped by washing the membrane in distilled water for 10 min with gentle agitation.

5.2.8 Protein Staining On Nitrocellulose Membrane

5.2.8.1 **Preparation of required buffers/solutions:** The following buffers were prepared and stored refrigerated:

1. PBS 0.1 M

2. Tween 20 solution (0.4 %) - This was prepared by adding 0.3 ml of tween 20 in 100 ml of PBS.

3. India ink - Commercially available India ink (0.1ml) was mixed with 100 ml of PBS. This was prepared immediately before use.

5.2.8.2 **Methodology** - After nitrocellulose membrane after blotting of the whole homogenates of the liver and of SC was air dried and washed in 0.4 % Tween 20 solution for 5 min. The membrane was then transferred to India ink solution and left at room temperature for 240 min with gentle agitation.

5.3 RESULTS

5.3.1. Organizational Status Of Chromatin

Fig. 5-1 depicts the results of chromatin activity assay performed on the chromatin of liver obtained from mice exposed to AEBN for 3 months (A) and that from AEBN exposed mice administered with 20 mg kg-1 body weight of MPG (B) or LEM (C) for 30 min.

It shows that the states of chromatin organization underwent changes by the mere presence of MPG and LEM (lanes 1 in panels A, B &C). The chromatin from AEBN exposed mice liver was progressively more fragmented by DNase I (lanes 2-5 in panel A). However, when MPG was present (panel B), the chromatin offered resistance to DNase I fragmentation up to 20 min of fragmentation time (lanes 2-5). The same was the situation in case of LEM (lanes 2-5 in panel C).

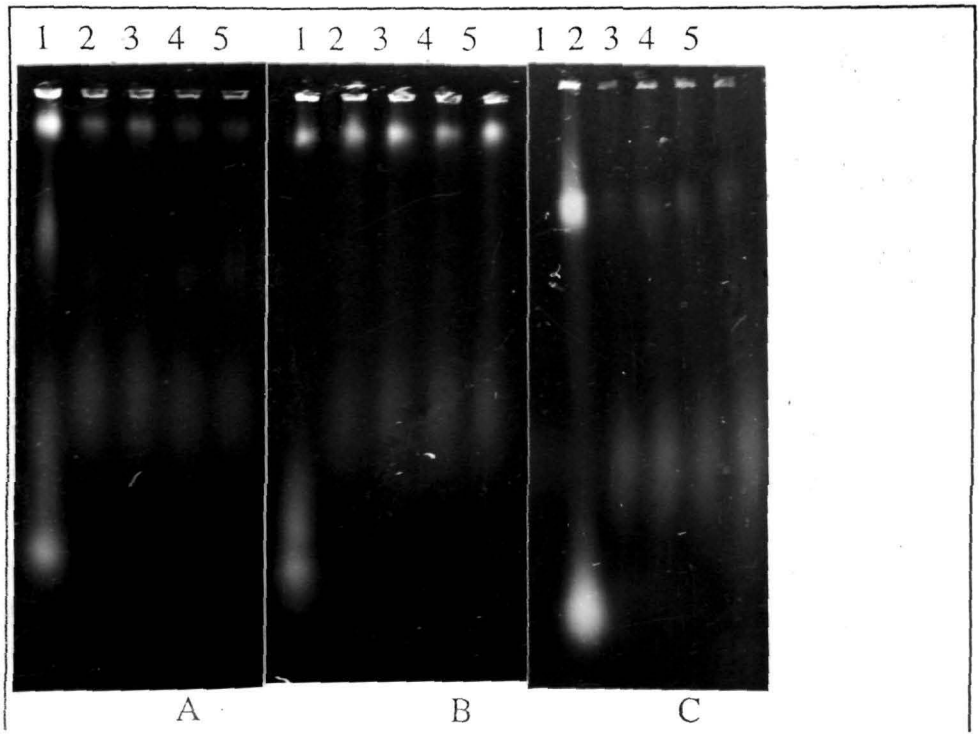

FIGURE 5-1: Agarose gel electropherogram of DNase I fragmented liver chromatin. The DNase I fragmentation patterns of chromatin of liver of the AEBN exposed mice, AEBN exposed mice administered with MPG and AEBN exposed mice administered with LEM are shown in panels A, B and C, respectively. Lane 1 = chromatin without DNase I, lanes 2 to 5 = chromatin with DNase I for 2, 5, 10 & 20 min, respectively.

Fig. 5-2 shows the results of chromatin activity assay performed on chromatin isolated from

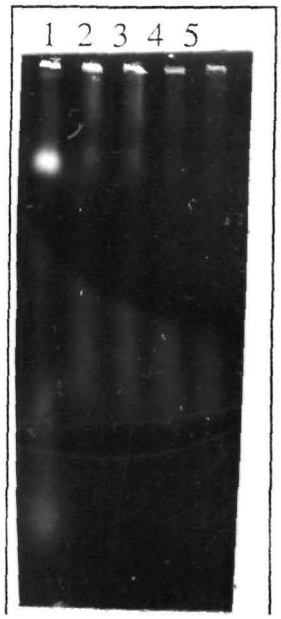

FIGURE 5-2: Agarose gel electropherogram of DNase I fragmented liver chromatin. The DNase I fragmentation patterns of chromatin of liver of the AEBN exposed mice irradiated to 8 Gy γ-rays. Lane 1 = chromatin without DNase I, lanes 2 to 5 = chromatin with DNase I for 2, 5, 10 & 20 min, respectively.

AEBN exposed mice which was irradiated to 8 Gy of γ-rays. Irradiation appears to have sensitized the chromatin to DNase I as even in 2 min (lane 2) the chromatin was extensively fragmented. Increasing time of DNase I action (lanes 3-5) did not make any difference.

Fig. 5-3 shows the results of DNase I fragmentation of chromatin isolated from AEBN exposed mice exposed to 8 Gy γ-rays 30 min after MPG administration. As expected, irradiation after the administration of MPG, did not cause extensive chromatin fragmentation by DNase I in 2 min (lane 2) as compared to that in Fig. 5-2. Nonetheless, with progressive

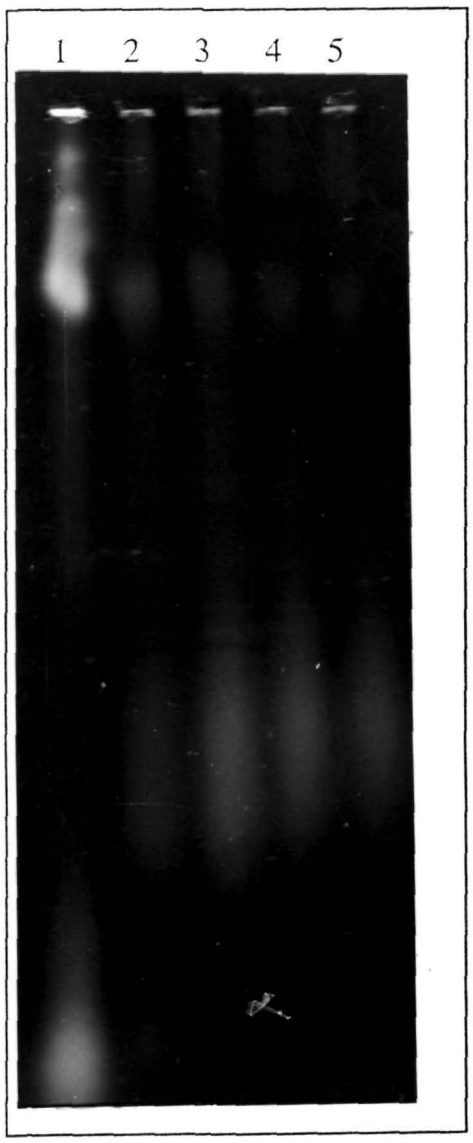

FIGURE 5-3: Agarose gel electropherogram of DNase I fragmented liver chromatin. The DNase I fragmentation patterns of chromatin of liver of the AEBN exposed mice irradiated to 8 Gy γ-rays 30 min after administration of MPG. Lane 1 = chromatin without DNase I, lanes 2 to 5 = chromatin with DNase I for 2, 5, 10 & 20 min, respectively.

increase in time of DNase I treatment (lanes 3-5) the fragmentation of chromatin was expectedly enhanced.

Fig. 5-4 shows the results of DNase I fragmentation of chromatin isolated from AEBN exposed mice exposed to 8 Gy γ-rays 30 min after LEM administration. Here also though DNase I was able to fragment chromatin, the extent of fragmentation was significantly low for all times of DNase I treatment (lanes 2-5).

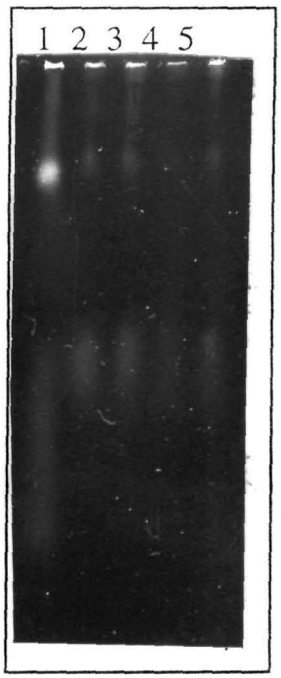

FIGURE 5-4: Agarose gel electropherogram of DNase I fragmented liver chromatin. The DNase I fragmentation patterns of chromatin of liver of the AEBN exposed mice irradiated to 8 Gy γ-rays 30 min following administration of LEM. Lane 1 = chromatin without DNase I, lanes 2 to 5 = chromatin with DNase I for 2, 5, 10 & 20 min, respectively.

5.3.2. **Level Of Total PADPR Of Spleen Cells (SC) and Liver**

Fig. 5-5 shows the results of dot blot assay of total cellular PADPR of SC of untreated mouse and that administered MPG and LEM. Almost identical amount of SC whole homogenates containing cellular proteins (panel B) showed very low level of PADPR in the untreated, normal mouse (lane 1) which increased in MPG treated mouse (lane 2) and reached even higher level in case of LEM administration (lane 3).

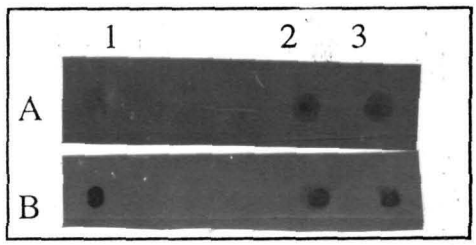

FIGURE 5-5: Results of dot-blot assay (A) and that of proteins in the dots (B) for spleen cells. Lane 1 = untreated, normal mouse, lane 2 = untreated mouse administered with MPG and lane 3 = untreated mouse administered with LEM.

Fig. 5-6 (panel A) shows the levels of PADPR of SC in mice transformed by a 3-month long AEBN treatment. The PADPR went down in the AEBN treated mouse SC (lane 2) as compared to the untreated normal mice (lane 1). It, however, went up a little when AEBN treated mice was irradiated with 8 Gy γ-rays (lane 3). The presence of MPG (lane 4) or LEM (lane 5) before irradiation again inhibited the PADPR of SC. That almost identical amounts of cellular proteins (whole homogenates) were dotted is apparent from panel B.

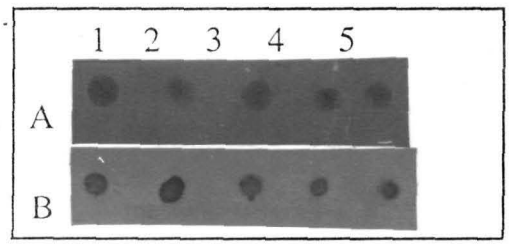

FIGURE 5-6: Results of dot-blot assay (A) and that of proteins in the dots (B) for spleen cells. Lane 1 = untreated, normal mouse, lane 2 = AEBN treated mouse, lane 3 = AEBN treated mice with 8 Gy irradiation, lane 4 = AEBN treated mice with 8 Gy irradiation following MPG administration and lane 5 = AEBN treated mice with 8 Gy irradiation following LEM administration.

Fig. 5-7 (panel A) shows the levels of PADPR of liver in mice transformed by a 3-month long AEBN treatment. As compared to the untreated normal liver (lane 1), the AEBN treated mice exposed to 8 Gy γ-rays showed a lower level of PADPR. However, presence of MPG (lane 3) or LEM (lane 4) exhibited relatively higher level of PADPR; the former showing a significantly high value. A 100 X diluted liver homogenate from untreated normal mice showed significantly weaker signal (lane 5). The protein contents of these dots have been shown in panel B.

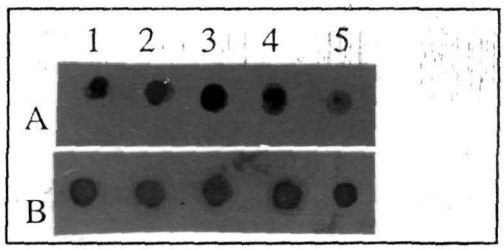

FIGURE 5-7: Results of dot-blot assay (A) and that of proteins in the dots (B) for liver. Lane 1 = untreated, normal mouse, lane 2 = AEBN treated mice with 8 Gy irradiation, lane 3 = AEBN treated mice with 8 Gy irradiation following MPG administration and lane 4 = AEBN treated mice with 8 Gy irradiation following LEM administration and lane 5 = 100 X diluted sample dotted in lane 1

5.4 DISCUSSIONS

Carcinogenesis process being a multistep process involving multiple alterations in the normal gene expression patterns as well as mutations is likely to involve alterations in the organizational state of chromatin in accordance with its requirements (Pitot, 1986; Boulikas, 1991; 1993). Consequently, any intervention to the process of carcinogenesis may also utilize the same molecular mechanism in opposite direction. Radiotherapy is one such intervention the aim of which is to reverse further development of cancer and, possibly, reverse the process. The molecular event involved in this reversal is only partially understood. The piece of work described in this chapter is an attempt to unveil some molecular events occurring after radiotherapy as well as when radioprotector like MPG is present during radiotherapy. As has been detailed in the introduction section, radiation induced damages to the genetic material, DNA, and its repair are under a great influence of the status of the organization of the chromatin superstructure (von Sonntag, 1981; Gillard *et al.*, 1986; de Murcia, 1991; Gillard *et al.*, 1996). A condensed chromatin structure prevents accessibility of DNA segment to radiation induced free radicals and other activated species. This also prevents DNA damaging effects of radiation. For repair systems to correct damaged DNA, likewise, proper accessibility of damaged sites of DNA to repair systems is essential. On this line, for effective radioprotection under the influence of MPG either in its free form or as LEM, necessitates that the chromatin is relaxed and, therefore, poised for efficient repair of the damages caused.

Figs. 5-1 to 5-5 depict the states of organization of chromatin under different situations. It is evident that mere exposure to free MPG (Fig. 5-1 B) or LEM (Fig. 5-1 C) affects the normal (Fig. 5-1 A) chromatin (compare lanes 1) wherein the chromatin becomes resistant to DNase I digestion. While with increasing time for DNase I action the AEBN treated mice chromatin is progressively more digested, the same was not true when MPG or LEM were administered for

30 min. The chromatin of AEBN transformed mouse after irradiation became hypersensitive to DNase I and the chromatin was significantly fragmented even after 2 min (Fig. 5-2). The sensitivity of chromatin towards DNase I was reduced by MPG (Fig. 5-3) as well as by LEM (Fig. 5-4). From the pattern of DNase I fragmentation, there seems to be no significant difference between MPG or LEM.

For radiotherapy, the basic operating principle is that radiation should cause enough damage to the genetic material and other components of a cell or tissue so that it does not survive. Among the two broad category of cellular targets, the genetic material and the other component, damage to DNA, the genetic material, is of utmost importance. It is obvious that the cell or the tissue envisaged to be killed by radiation is cancerous. From radioprotection view point, it is envisaged that the undesirable radiation induced damages to normal cell are kept to the minimum. It logical, therefore, to think that if the chromatin is in condensed state both radiation induced damage and the repair of damages will be slow. A relaxed status of chromatin will favour induction of damages to DNA. In this context, it may be worthwhile to look into the status of chromatin organization during radiotherapy and the influence of the presence of MPG, either in its free form or as LEM on it during radiotherapy.

The poly-ADP-ribosylation (PADPR) reaction is one of the most important post translational modification avenues for chromosomal proteins, that has been shown to influence the level of condensation/relaxation of chromatin (Althaus. 1987; Althaus, 1993; Sharan *et al.*, 1995; Schneeweiss et al., 1997). Figs. 5-5 to 5-7 show the dot-blot immunoassay results to represent the level of total cellular PADPR of SC and liver. As has been mentioned, the conventional assay of PADPR using radiolabelled NAD^+ had many limitations including it non-applicability to tissues like liver (Schneeweiss *et al.*, 1995; Saikia, 1997). The dot blot immuno assay developed in this work is not only very sensitive, it may be applied to tissues which can make single cell suspension easily but also to tissues like liver. The results show marked difference in the level of PADPR between the two tissues used in this investigation. While the SC showed a relatively low cellular level of PADPR (Figs. 5-5, 5-6), the liver showed a rather high value of it (Fig. 5-7). Of course, the amount of cellular proteins dotted were correspondingly different. Nonetheless, the levels of PADPR in SC and liver were different. It is in line with reports in the literature where differences of the level of PADPR are reported between different tissues (Althaus, 1987). The SC is a continuously dividing cell population in contrast to the liver which is pre-mitotically fixed tissue.

The presence of MPG or LEM before irradiation of the AEBN transformed mice significantly enhanced the level of PADPR of liver (lanes 3, 4 in Fig. 5-7) as compared to that in AEBN transformed and irradiated mice (lane 2 in Fig. 5-7) suggesting that the chromatin should be relatively more relaxed in its organization. This may be seen that the chromatin was poised for

better repair. If this situation is accepted, a molecular basis of MPG affordable radioprotection is evident. More work will be required to concretize this concept.

From this study, there seems to be only marginal difference between the radioprotective effects of MPG in its two forms - the free form of MPG and the liposome encapsulated MPG.

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The work embodied in this thesis opens up a possibility of use of liposome as a carrier for radio-modularoty drugs for use in chemo-radiotherapy. The results very emphatically establish that using liposome carrier may offer significant clinical gains in chemo-radiothrapy. The model drug used in this investigation was 2-mercaptopropionylglycine (MPG), a moderate radioprotective drug that has been shown to be a potential radioprotector both in experimental conditions and in clinical trials. The following are the salient findings emerging from this work:

1. The radioprotection affordable by MPG was enhanced when an equivalent amount of drug was administered through liposome vehicle (chapters 2-5).

2. Of several methods for preparation of liposome to encapsulate MPG, the reverse-phase evaporation method was found to be highly convenient, reproducible and effective (chapter 2). The preparation of liposome by this method involves common and simple laboratory equipments and may be performed at any place.

3. The method offers MPG encapsulation into liposome at a rate of over 50 % of the starting concentration of MPG, thus, qualifying to be an efficient method for MPG encapsulation. Five or 10 mM starting concentrations of MPG was found to be optimum for liposome encapsulation (chapter 2).

4. The liposome encapsulated MPG (LEM), as against its free form, afforded significantly higher protection to normal tissues on biological end-points such as, (a) viability of bone marrow and spleen cells and (b) on enzyme acetylcholine esterase (chapter 3).

5. The same was the case in cancer induced or transformed mice. The biological end-points tested for this investigation were cellular glutathione and enzyme g-glutamyltranspeptidase. On both these limits, the LEM afforded better radioprotection that MPG (chapter 4).

6. It has been shown that MPG was able to influence chromatin organization differently when it was administered in its free form or as LEM (chapter 5).

7. The presence of MPG or LEM was also reflected by the level of cellular poly-ADP-ribosylation, assayed by a new immuno-dot blot assay developed in this investigation (chapter 5).

8. The results point out to the fact that chromatin was poised for better repair in transformed mice when MPG or LEM were administered prior to irradiation (chapter

9. On the parameters of chromatin organization and poly-ADP-ribosylation, MPG and LEM affordable radioprotection was almost similar (chapter 5).

10. Overall, the radioprotection afforded by LEM was higher that free form of MPG (chapters 2-5).

The findings presented in this thesis may have significant impact on the clinical use of radiomodulatory drugs in chemo-radiotherapy as liposome encapsulation could enhance the effectiveness of MPG. A potential use of liposome carrier, however, will be with radiosensitizing drugs. It is known that most of radiosensitizing drugs are highly toxic to normal tissues. Notwithstanding the toxicity problem, use of radiosensitizing drug in chemo-radiotherapy will be more advantageous that use of radioprotective drugs. Liposome as a carrier may be an easy solution to this problem. By liposome encapsulation, the radiosensitizing drug will not be immediately available to other tissues, thus, reducing its toxicity. In addition, it could be visualized that a radiosensitizing drug after liposome encapsulation may be targeted to cancer tissue. Therefore, the cancerous tissue will be sensitized and even a lower dose of radiation may be able to produce enough damage to kill it. Some avenues of specific tissue targeting using immuno-liposome are being investigated.

Even though the work described in this thesis relates to MPG, a radioprotective drug, the findings are of significant clinical value and open up a new line of thinking for improvisation of chemo-radiotherapy.

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PUBLICATIONS .....

C-3

Liposome Mediated Delivery of 2-Mercaptopropionyl Glycine: Entrapment of MPG in Liposome.

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Introduction

2-mercaptopropionyl glycine (MPG), an amionothiol, has been widely used in experimental radioprotection in mice because the effective dose (20 mg/kg. body weight) is far below the toxic dose (2100 mg/kg. body weight) (1-2). The radio-protective effect of MPG was also reported *in vitro* for gamma induced radiolysis of catalase (3). However, gamma induced DNA strand breaks in human lymphocytes *in vitro* showed that depending on the dose of radiation, the MPG concentration must be adjusted for optimal radioprotection (4). Furthermore, DNA strand break analysis in human lymphocytes *in vitro* revealed that adjustment of optimal concentration of MPG in relation to the dose of radiation was a must otherwise MPG acted as a rediosensitizer instead of radioprotector (5). Therefore, in order to exploit the radioprotective potential of MPG in radiotherapy, it is highly desirable to deliver a calculated amount of MPG for the computed dose of radiation to the target tissue.

The widely used protocol of MPG administration for radiotherapy is intravenous injection of MPG 15 to 30 min prior to irradiation (6). Obviously, distribution of MPG in various tissues of the experimental animals or patients in directly controlled by the physiological conditions. In this situation, it is assumed that MPG concentration available in the tissue of interest is optimal for radioprotection. However, if this assumption is not correct, then, in light of our previous results (4-5), the desired radioprotective effect of MPG is undesirable and is likely to undermine the radioprotective potential of MPG.

In order to overcome this problem of delivery of calculated amount of MPG in specific target tissues, we have taken the approach of liposome-mediated delivery of MPG. In principle, liposomes can encapsulate drugs in its aqueous compartment. The liposomes then can be targeted to a particular tissue by linking specific antibodies on to the surface of the liposome. Upon administration, the modified liposomes specifically migrate to the desired tissue and deliver the drug (7).

Because MPG may act as radioprotector as well as sensitizer, liposome mediated MPG delivery system may offer several advantages over the conventional mode of administration. These may include (1) delivery of MPG to specific target tissues, (2) amount of MPG delivered in the tissue can be controlled, and (3) MPG delivered in the tissue can escape possible metabolism enroute the target tissue due to encapsulation.

The present investigation was aimed to enhance the radiomodulatory potential of MPG. This report brings out the preliminary results of attempts to encapsulate MPG in liposomes.

Methods and Materials

Chemical: MPG (tiopronine) was a gift from Prof. T. Sugahara and Santen Pharmaceutical Co., Japan. Dipalmitoyl phosphatidyl choline (DPPC), dicetylphosphate (DCP), cholesterol and dithiobis 2-nitro benzoic acid (DTNB) were obtained from Sigma Chemical Co., USA, Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden and triton X-100 from Glaxo Labs., India. All other reagents used were of anlytical grade.

Preparation of liposomes:

A. Wet method: Liposomes were prepared as described earlier (8). In short, DPPC: Cholesterol:DCP were taken in test tube in molar ratio of 1.0:0.9:0.25 and dissolved in 0.2 ml chloroform by vortexing. 1.0 ml aqueous solution, phosphate buffered saline (PBS), pH 7.4 or 10 mM MPG in 10 mM PBS, pH 7.4 was added in 0.2 ml aliqotes followed by vortexing. The mixture was transferred in a round bottom flask and chloroform was removed in a rotary evaporator at 30-35°C. The remaining trace of chloroform was removed during gel filtrating chromatography.

B. Dry film method: Liposomes were made as described by Bangham et al (9). In short, DPPC: Cholesterol: DCP mix in the same molar ratio, as above, were dissolved in 0.2 ml chloroform by vortexing. The chloroform was removed by nitrogen flushing at 40°C while continuously rotating the tube. This resulted in a thin and uniform film on the walls of the tube. The dried film was dispersed in 1 ml of PBS or 10 mM MPG solution by vortexing.

Separation of liposomes from the free MPG: The liposomal suspensions were passed through a Sepharose CL-4B column (30x15 cm) equilibrated with 10 mM PBS, pH 7.4 containing 0.02 % sodium azide. The column was developed with the same buffer. Fractions were collected and read at 280 nm.

Determination of MPG concentration: The MPG was quantified by the assay of -SH group using the method of Ellman (10) with minor modifications. In short, 2.9 ml of reagent (flushed with nitrogen) consisting of 10 mM DTNB in 100 mM phosphate buffered saline (pH 7.9) containing 0.1 mM EDTA was added to 0.1 ml of sample and absorption monitored at 412 nm immediately after mixing. Cystein was used as a standard.

Results and Discussion

Lipid composition and lipid/MPG ratio used in liposome preparation is shown in Table 1. 10 mM MPG solution in PBS (10 mM, pH 7.4)was employed for encapsulation. Liposomes containing 10 mM MPG or PBS (blank liposomes), prepared as discussed in Methods and Materials section, were separated from the unentrapped MPG by gel filteration chromatography on Sepharose CL-4B column. Liposomes eluted in the void volume were assayed for the content of -SH group to quantitate encapsulated MPG into the liposomes. The percent entrapment of MPG in liposomes prepared by both the method are shown in Fig. 1. 56 % entrapment

was obtained by the wet method, whereas 30 % by dry film method. Poor entrapment efficiency has been reported by dry film method earlier (9). We have reported 75% entrapment efficiency by the above wet method for a plant protein, gelonin, of molecular weight 30 kd (8). The decrease in entrapment efficiency of MPG, as reported here, may be because of its smaller molecular weight. However, the entrapment value of MPG reported by wet method is very close to the high entrapment efficiency methods for proteins and drugs described in literature (11-14). Our procedure is not only easy but also reproducible compared to any other high entrapment techniques described so far (11). Each experiment was repeated 3 to 4 times and the value of liposome encapsulation of MPG are meant.

The results of our studies open the possibility to develop a more effective MPG delivery system. It must be emphasized here, however, that these findings are still of a preliminary nature and further investigations are continuing.

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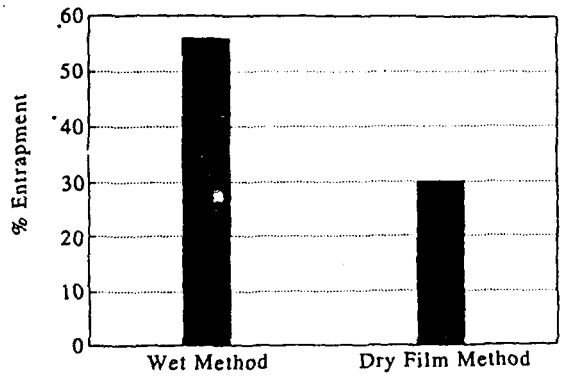
Table 1 Lipid Composition and Lipid/MPG Ratio of Liposomes.

Composition	Amount of lipid(mg)	Molar ratio	Lipid/MPG ratio
DPPC:Cholesterol: DCP	5:2.5:1	1:0.9:0.25	5.21

Fig. 1 MPG Entrapment Efficiency in Liposomes



I-12

LIPOSOME ENCAPSULATED MPG IN RADIOTHERAPY: A NEW APPROACH TO USE OF DRUGS.

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2-mercaptopropionyl glycine (MPG), an aminothiol, has been widely used in experimental radioprotection in mice because the effective dose (20 mg/kg body weight) is far below the toxic dose (2100 mg/kg body weight) [1,2]. The radioprotective effect of MPG was reported in vitro for (a) gamma induced radiolysis of catalase [3] and (b) DNA strand breaks in human lymphocytes [4]. The DNA strand break analysis in human lymphocytes revealed that adjustment of optimal concentration of MPG in relation to the dose of radiation was a must otherwise MPG acted as a radiosensitizer instead of radioprotector [5]. Therefore, in order to exploit the radioprotective potential of MPG in radiotherapy, it is highly desirable to deliver a calculated amount of MPG for the computed dose of radiation to the target tissue.

The standard protocol of MPG administration for radiotherapy is intravenous injection of MPG 15 to 30 min prior to irradiation [6]. Obviously, the pharmacokinetic distribution and metabolism of MPG in the experimental animals [7] or patients are directly controlled by the physiological conditions which may result in reversal of the radioprotective effect [4,5]. This will be highly undesirable and is likely to undermine the radioprotective potential of MPG. To overcome this problem of (a) delivery of calculated amounts of MPG and (b) physiology dependent possible metabolization of MPG, we have taken the approach of liposome delivery system for MPG. In principle, liposomes can encapsulate MPG or other drugs in its aqueous compartment. The liposomes then can be directly administered, as in this report, or targeted to a particular tissue by linking specific antibodies on to the surface of the liposome [8]. The first step in this direction was to encapsulate MPG in liposome. It has been achieved showing a high entrapment efficiency [9]. Using this liposome encapsulated MPG, we report an enhanced radioprotective effect of MPG.

Different methods of encapsulation of MPG (tiopronine) in liposome, separation of encapsulated MPG from free MPG and quantitation have been discussed earlier [9]. The entrapment method was simple [9,10] and entrapment efficiencies were over 70%, significantly higher than that by other conventional methods.

The biological radioprotective effect of liposome encapsulated MPG was tested in 6-8 week-old Swiss albino mice. 1.0 mL of 2.0 mM MPG encapsulated in liposome (equivalent to effective dose) was intraperitoneally injected to mice and 30 min later the mice were irradiated to 4 or 8 Gy in a gamma source (dose rate: 26 Gy/min). The positive control groups received free MPG on the same protocol. After irradiation, mice were killed and viability of spleen and bone marrow cells (BMC) were tested by dye exclusion technique [11]. Each point had 9 to 21 replicates.

A gamma-dose-dependent radioprotection in MPG as well as Lip-MPG group is evident from the figure. The most noticeable result is significantly higher radioprotection (4-5 folds) by liposome encapsulated MPG as compared to that by free MPG at the same dose level. One reason for this higher radioprotection could be that liposome encapsulated MPG escaped metabolization and delivered active form of MPG to tissues. The free MPG may have lost some of its radioprotective potential due to metabolization enroute the tissues. Higher radioprotection in spleen cells as compared to that in BMC could be due to preferential uptake by spleen of

drugs in general and liposomes in particular.

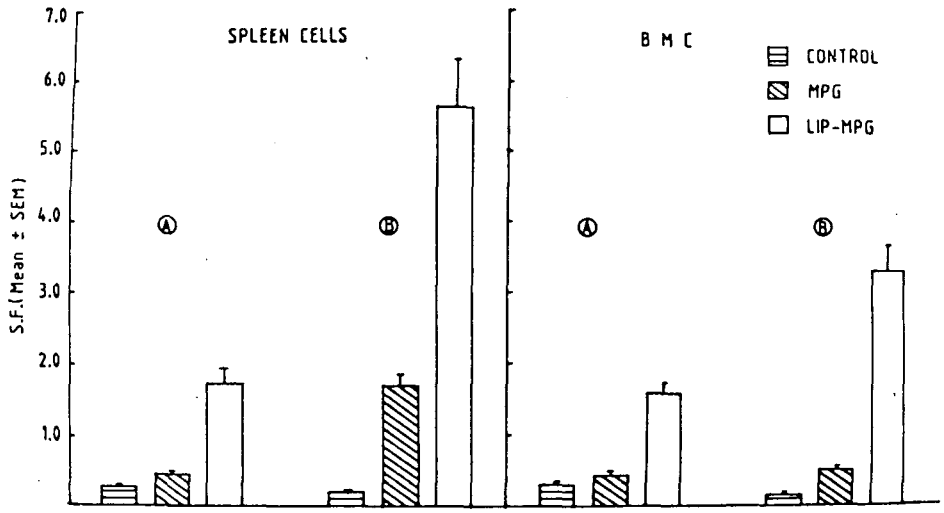

Fig. Survival fraction (SF) of spleen cells and bone marrow cells (BMC) after 4 Gy (A) and 8 Gy (B) gamma-irradiation. Control group received no drugs while free MPG and liposome-encapsulated-MPG were injected at same dose level 30 min prior to irradiation to MPG and Lip-MPG groups respectively.

Therefore, MPG could be highly effective radioprotective drug when delivered through liposome. In addition, the dose of MPG can further be reduced by liposome mediated targeting of MPG to specific tissues. More work is under way. This opens up new possibilities of use of drugs for radiotherapy. Loss of effectivity of drugs due to metabolization can be avoided as well as drugs can be targeted to specific tissues and toxicity of drugs to other tissues could be reduced significantly.

Acknowledgements: MPG was a gift from Prof. T. Sugahara and Santen Pharmaceutical Co., Japan.

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Liposome as a Carrier for Delivery of Radiomodulatory Drugs and Its Advantages in Chemo-Radiotherapy

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Asbtract

Radiomodulatory effects of free and liposome encapsulated radioprotector, MPG, and radiosensitizer, AK-2123, have been studied in mice. The drugs were intraperitoneally administered in mice 30 min prior to whole body irradiation by ^{60}Co γ-rays. Liposome encapsulated drugs, in comparison to their free forms, significantly enhanced the radiomodulatory effects in spleen and bone marrow cells. The enhancement of radiomodulation due to administration of liposomal drugs was more pronounced in bone marrow cells than in spleen cells. Liposomal MPG showed higher degree of radioprotection, whereas liposomal AK-2123 offered relatively less effect. Nonetheless, results indicate that liposomal drugs afforded enhanced radiomodulation. This may be because of delayed metabolic alteration of the encapsulated drug and enhanced concentration of drugs in bone marrow and spleen due to preferential accumulation of liposomes. As liposomes can be potentially targeted to specific tissue, this report discusses possibility of use of liposome carriers for radiosensitizing drugs for better therapeutic yields in chemo- radiotherapy of cancer.

Key words : Liposome, Liposomal Drug, MPG, AK-2123, Radiomodulation

Introduction

Radiotherapy of cancer has reached its zenith and it is obvious that radiations of different qualities and quantities as well as dose fractionation protocols can only marginally improve their present clinical efficacies. It is because radiation interaction with matter is random. Consequently, radiations cause damage to cancerous and healthy cells alike. Thus, radiations, while damaging and killing cancerous cells, also inflict damage of various kinds to normal cells which, in

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many cases, may transform these cells. This poses serious constraints on the use of radiations in therapy of cancer. In order to overcome these limitations and to improve upon clinical gains of radiotherapy of cancer, use of radiomodulatory drugs along with radiations (chemo-radiotherapy) have yielded positive therapeutic advantages. Two classes of such drugs, namely, radioprotective and radiosensitizing drugs, are relevant to chemo-radiotherapy protocols. In principle, a radioprotector shall protect normal tissues from radiation induced damages paving way for application of increased doses of radiation to kill cancerous cells more efficiently. On the other hand, a radiosensitizer shall sensitize cancerous cells so that even a lower dose of radiation could effectively kill them; thus, normal cells sustain relatively less damage. Chemo-radiotherapy, indeed, improved rate of cancer cure by radiations. However, toxicity of drugs to other tissues, lack of control on the quantity of drug in cancerous tissues after administration, non-specific sensitization of normal tissues and chemical alterations of drug in metabolism have been serious impedance to the efforts of betterment of therapeutic index of cancer chemo-radiotherapy. During the last few decades further improvements of drug efficacy in chemo-radiotherapy have relied on hybridomas and recombinant DNA technology on one hand and, on the other, on the discovery and understanding of a number of cell membrane vesicles/receptors and their interactions with ligands.

The concept of drug delivery system is based on the fact that certain carriers inherently possess or can be made to acquire selective migration to biological tissues or targets. Therefore, a drug attached onto or within this specific carrier will also be delivered to or around a specific target tissue. As expected, this approach has been shown to enhance the efficacy of drugs in a number of experimental, sub-clinical as well as in limited clinical trials[1,2].

Of the several carriers or drug delivery systems that have been studied, colloidal microspheres and liposomes have come out to be the systems with potentials to be used on humans; the latter offering several advantages over the former. We have attempted to study liposome drug delivery system as a carrier of two radiomodulatory drugs with the aim that such studies will lead to better use of these drugs in cancer chemo-radiotherapy. This report, therefore, deals only with liposome as a drug delivery system; readers are referred to a review[3] for colloidal microspheres and other drug delivery systems.

Liposomes are biomembranous sacks of 0.5 to 5 μm diameter. These are made up of lipids, phospholipids and other polar amphiphiles forming a closed concentric single (unilamellar) or multiple (multilamellar) bilayer vesicles. The vesicle can entrap with in it water and water soluble solutes as well as insoluble materials. In addition, lipid soluble or lipid bound materials can also be accommodated on the vesicle membrane. Therefore, this uniquely versatile

carrier system is very flexible vehicle for delivery of drugs of practically any chemical nature, composition, or type. The preparation can be lyophilized and stored conveniently and may be reconstituted with water. Furthermore, by using appropriately charged amphiphiles, liposomes may be tailor-made to display charge groups in its surface. This can help direct liposomes to a reciprocally charged tissue achieving limited tissue targeting. Another approach for specific tissue targeting may be tagging of antibodies on liposome surface (immuno-liposome) directing the liposomes to seek tissues with complementary antigen. Biodegradable and innocuous nature of liposomes adds to the list of advantages of liposomes as drug delivery system. The circulation time of liposomes may be varied depending on the lipid composition of the vesicle. Thus, liposomes are imminently suitable drug delivery system for use in cancer chemo-radio-therapy protocols.

Several methods of preparation of liposome vesicles are now available producing liposomes with specific characteristics such as size, surface charge, drug entrapment efficiency, fluidity, stability, drug retention time, drug release kinetics, physiological half life and pharmacokinetic behavior in different tissue sytems[2,4-6]. Despite these advantages, liposome has a serious drawback in its selective and preferential migration to tissues rich in reticuloendothelial cells like liver, spleen and bone marrow[2]. This leads to selective accumulation of normal liposomes along with its contents into these tissues. We have exploited this characteristic of liposome for simulating tissue targeting by selecting spleen and bone marrow for investigation in the present study.

In order to test suitability of liposome drug delivery system in chemo-radiotherapy, we report here an optimized and convenient method of preparation of liposomes, the entrapment efficiencies of two model radiomodulatory drugs in the liposome and the resulting radiomodulatory effects of these drugs. 2-mercaptopropionylglycine (MPG), a representative radioprotector, and N-(2'-methoxyethyl)-2-(3"-nitro-1"-triazolyl) acetamide (AK- 2123), a hypoxic cell sensitizer, have been used in this study on mice. ^{60}Co γ-radiation has been used as a source of radiation.

Materials and Methods

Chemicals

Dipalmitoyl phosphatidyl choline (DPPC), diacetylphosphate (DCP), cholesterol and trypan blue were obtained from Sigma Chemical Co., USA; dithionitrobenzoic acid from SRL, India; and Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden. MPG (Tiopronine) was supplied by Santen

Pharmaceuticals Co., Japan and AK-2123 by Dr. V.T. Kagiya, Japan. All other chemicals were of highest purity grade available from Indian sources. Glass double distilled water has been used in all preparations.

Animals

Inbred, yound adult (6-8 weeks old) Swiss albino mice, colony maintained in our animal house, were used in all experiments. They were housed in polycarbonate cages with husk bedding and maintained on standard mouse pellet and drinking water *ad libitum*.

Preparation of Liposomes

Liposomes were prepared by a modified reverse-phase evaporation method reported earlier[7,8]. Briefly, 5, 2.5 and 1 mg of DPPC, cholesterol and DCP, respectively, were dissolved in 0.25 ml of chloroform. Aliquots (0.2 ml) of 1 ml of the aqueous solutions of radiomodulatory drugs to be encapsulated were added to this lipid solution while vortexing. Separation of free drug from the liposome encapsulated equal amount of drug was achieved either by centrifugation or by Sepharose CL-4B column chromatography[7]. The liposome preparations were stored refrigerated until use.

Drug Administration

Equal amount of free and liposome encapsulated drugs were administered by intraperitoneal route into mice 30 min before irradiation. The administered doses of MPG and AK-2123 were 20 mg kg^{-1} body weight and 200 mg kg^{-1} body weight, respectively.

Irradiation

Animals were acutely whole body irradiated at doses of 1, 2, 4, 6, 8, or 18 Gy in Gamma Chamber 900 (BARC, Bombay), delivering ^{60}Co γ-rays at a dose rate of less than 23 Gy min^{-1}. Animals were sacrificed for cell viability test within 60 min after irradiation.

Quantification of MPG

Assay of -SH group of MPG (Fig.1) was made to quantify the free and liposome encapsulated MPG. The method of Ellen was used with minor modification as described previously[8]. In short, 0.1 ml of test sample was added to 2.9 ml of N_2 flushed assay mixture containing 10 mM DTNB in 100 mM PBS (*p*H 7.9) and 0.1 mM EDTA. After thorough mixing, absorption was read immediately at 412 nm in a Shimadzu spectrophotometer.

Quantification of AK-2123

In the absence of a suitable chemical group on AK-2123 (Fig. 1) for spectrophotometric quantification, LASER Raman spectroscopy was employed for quantification of AK-2123. The principle of this assay lies in the presence of symmetric stretch of bonds on -NO_2 group of AK-2123 (Fig. 1). At a known wave number of 1313 cm^{-1} for these bonds in Raman spectroscopy, the intensity (I band) of -NO_2 groups of AK-2123 was measured. To avoid any errors due to sample to sample variations or instrumental errors, an internal standard of $(NH_4)_2SO_4$ was used because it also exhibits symmetric mode on -SO_4 with 986 cm^{-1} wave number. The relative I band of -NO_2 in relation to -SO_4 vs. concentration of AK-2123 shows a linear relationship. Using this, it has been possible to quantify AK-2123 in aqueous solution as well as in a liposome preparation.

Cell Viability Assay

The viabilities of spleen cells (SC) and bone marrow cells (BMC) of mice from different treatment groups were calculated by dye exclusion technique as

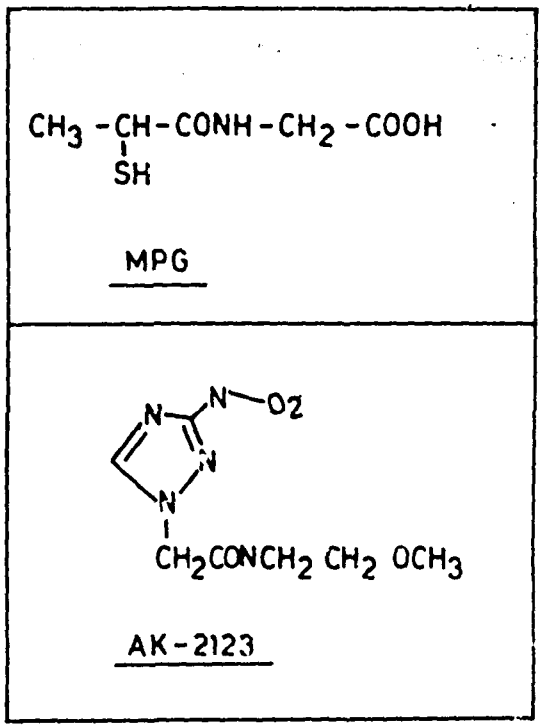

Fig.1 : Chemical structures of 2-mercaptopropionylglycine (MPG) and N-(2'-methoxyethyl)-2-(3''-nitro-1''-triazolyl) acetamide (AK- 2123).

described earlier[8]. Briefly, animals were sacrificed by cervical dislocation after irradiation and suspensions of SC and BMC in minimal essential medium were prepared. The cells were counted on a Burker chamber in phase contrast using Zena light microscope after 5 min incubation at 37 °C of cells with 1% trypan blue. The percentage of surviving cells was calculated.

Statistical Analysis

Each data point represents a minimum of 4 independent experiments each with 4 replicates. Data falling within Poissions distribution were used to calculate the mean with standard deviation (SD). Student's t-test was applied to calculate the significance of differences between different experimental groups.

Results

Preparation of Liposomes

The microphotograph of liposome preparation is shown in Fig.2. A majority of the vesicles appeared to be large-sized and the preparation was homogenous.

Entrapment Efficiency of Drugs in Liposomes

The modified reverse-phase evaporation method employed for preparation of liposome[7,9] offered high entrapment efficiencies for MPG and AK-2123. As compared with other methods available in the literature[10], this is a simple and

Table 1: Molar Ratios and lipid-drug Ratios of the two Preparations

Material	Molar ratio of DPPC: cholesterol: DCP	Ratio of lipid/drug	% entrapment
MPG	1:0.9:0.25	5.21	52
AK-2123	1:0.9:0.25	5.21	58

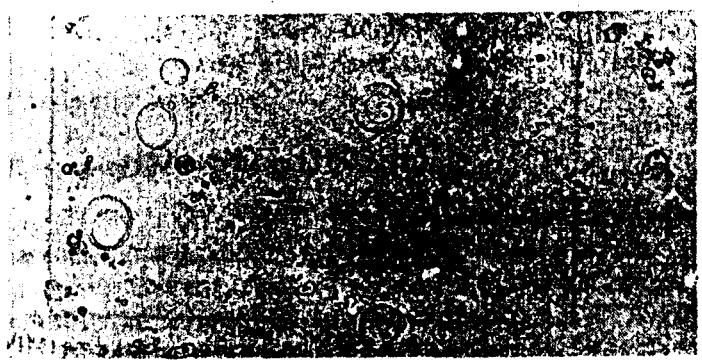

Fig.2 : Microphotograph of liposomes (Magnification X 150).

convenient method for entrapment. It shows that entrapment for MPG and AK-2123 in liposome was between 50% and 60%. The molar ratios and lipid-drug ratios for the two preparations are shown in Table 1.

Quantification of MPG

The quantification of MPG entrapped in liposomes was based on assay of -SH group of MPG (Fig. 1). The assay of -SH group was appropriate for assessment of MPG and its quantification to calculate the entrapment efficiency in liposome. The method worked for liposome entrapped MPG after lysing liposome by mild detergent treatment (2% of Triton X 100). Accordingly, it was estimated that 0.408 mg of MPG was administered to each mouse in the present investigation. Since the entrapment efficiency of MPG in liposome was about 50%, the dose of liposome encapsulated MPG was accordingly adjusted to get the same dose as free MPG.

Quantification of AK-2123

The relationship between relative I band and quantity of AK-2123 in aqueous solution was linear (Fig.3). This has been used for quantification of AK-2123 in liposomes without disintegrating liposomes. Hence, the amount of free and encapsulated AK-2123 for administration was appropriately calculated

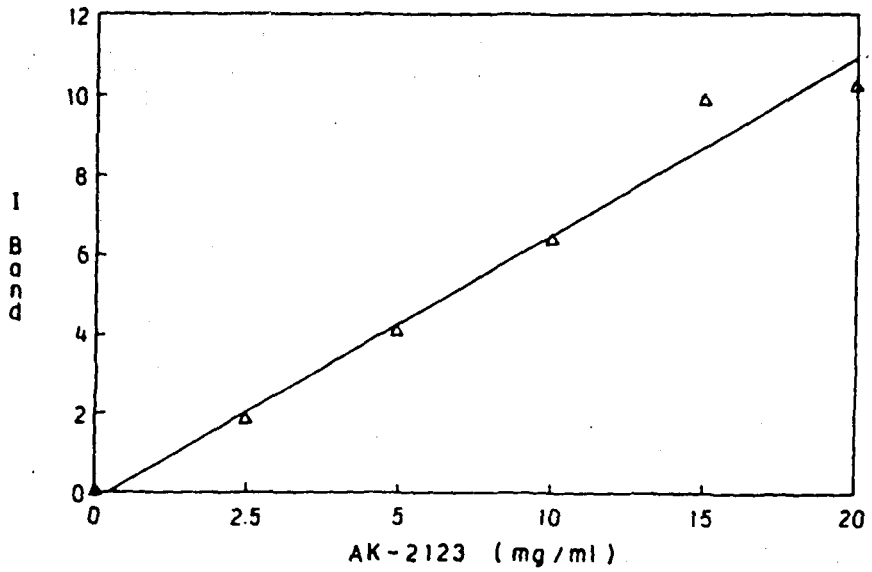

Fig.3 : The plot between quantity of AK-2123 and its relative I band showing a linear relationship (see text for details).

to deliver the equivalent effective dose of AK-2123 for radiosensitization. The method offers a very convenient way for precise quantification of AK-2123.

Effect of Radiation on MPG Treated Animals

The viabilities of SC (Fig.4) and BMC (Fig.5) decreased in a radiation dose-dependent manner. This trend was rescued by free MPG administration 30 min prior to irradiation. The presence of liposome encapsulated MPG, however, abolished the radiation dose-dependent decrease in survival of both the cell types. The enhancement of viabilities of SC and BMC was significantly higher ($p \geq 0.01$ - 0.0001) when same dose of MPG was administered as

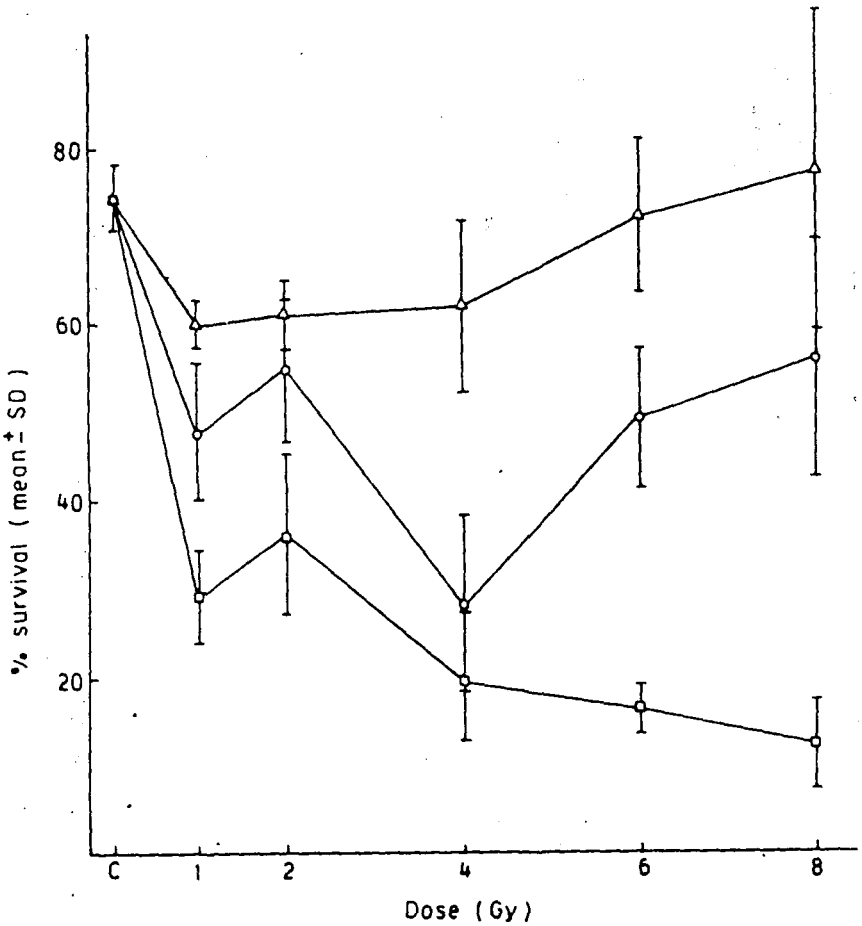

Fig.4 : Radiomodulatory effects of MPG on the survival of mouse spleen cells (SC) as revealed by dye exclusion assay (◊——◊ : radiation alone; 0——0: MPG + radiation; Δ——Δ : (liposome encapsulated MPG + radiation).

liposome encapsulated MPG prior to irradiation (Figs 4 & 5). It is to be noted that the relative protection afforded by liposome encapsulated MPG was higher at 8 Gy as compared with that at 1 Gy of gamma radiation, indicating radiation dose-dependent increase in MPG affordable radioprotection after liposome encapsulation.

Effect of Radiation on AK-2123 Treated Animals

Free AK-2123 afforded significant sensitization of cells to radiation as revealed by the radiation dose-dependent reduction in viability of SC (Fig. 6) and BMC (Fig.7). Administration of liposome encapsulated AK-2123 marginally but significantly ($p \geq 0.1$-0.01) increased sensitivity of cells to radiation. The sensitization was higher in case of BMC than SC (Figs 6 & 7).

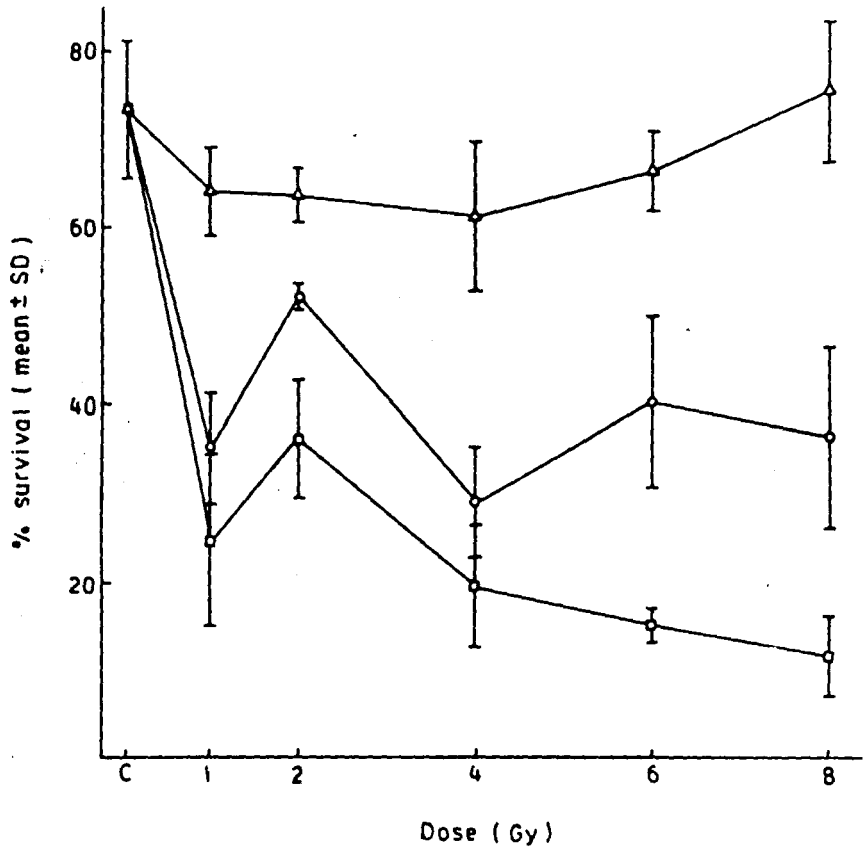

Fig.5 : Radiomodulatory effects of MPG on the survival of mouse bone marrow cells (BMC) as revealed by dye exclusion assay (δ——δ : radiation alone; 0——0: MPG + radiation ; ——— : liposome encapsulated MPG + radiation).

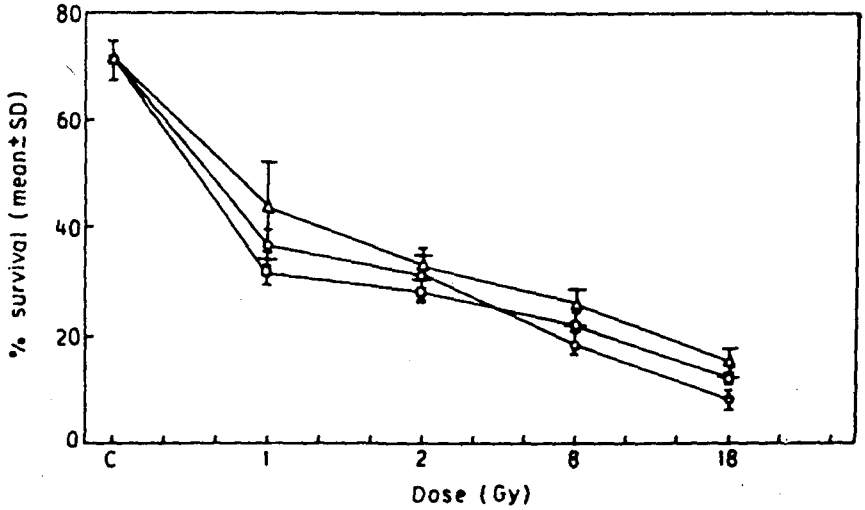

Fig.6 : Radiomodulatory effects of AK-2123 on the survival of mouse spleen cells (SC) as revealed by dye exclusion assay (Δ——Δ : radiation alone; 0——0: AK-2123 + radiation; ◊——◊ : liposome encapsulated AK-2123 + radiation).

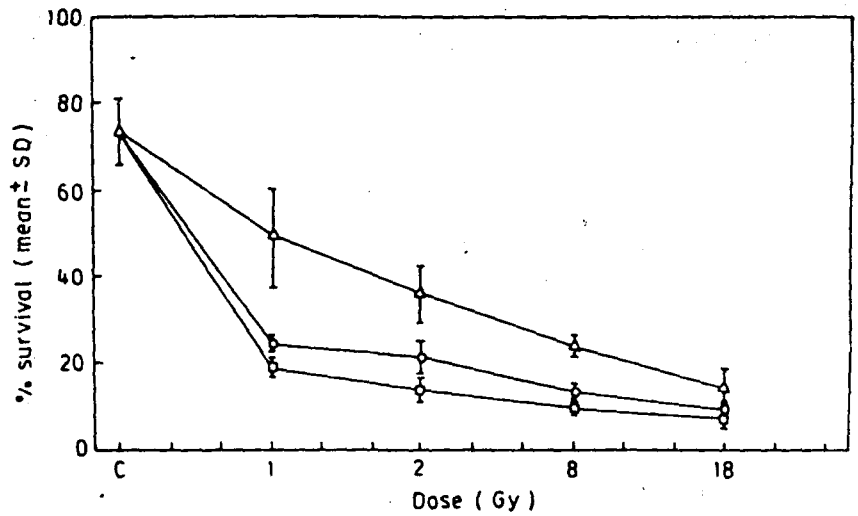

Fig.7 : Radiomodulatory effects of AK-2123 on the survival of mouse bone marrow cells (BMC) as revealed by dye exclusion assay (Δ——Δ : radiation alone; 0——0: AK- 2123 + radiation; ◊——◊ : lipsome encapsulated AK-2123 + radiation).

Discussion

The choice of two representative drugs, MPG and AK-2123, was made to test suitability of liosome encapsulation of radiomodulatory drugs and advantages it offers in chemo-radiotherapy. MPG is a known radioprotector with well

defined characteristics[11]. Similarly, AK-2123 has been shown to be of advantage over other such radiosensitizing drugs in experimental and limited clinical studies involving human patients[12,13]. These drugs were chosen to see whether liposome encapsulation, which involves contact of drug with organic solvent, alters the chemical nature of drugs. The results shown in this report (Figs 6 & 7) clearly indicate that the modified reverse-phase evaporation method of liposome preparation did not alter chemical nature of both the drugs as they remained more effective radioprotector and radiosensitizer, respectively, after liposome encapsulation. It is to be emphasized that in the method used in this report, the contact of drugs with organic solvent, chloroform, in the preparation of liposome has been small, offering this advantage; in other methods of preparation of liposome longer time of contact of organic solvents with drugs is required[10]. Furthermore, this method of liposome preparation was highly reproducible and involved mild conditions for large scale production.

The entrapment efficiency of drugs into liposome prepared by modified reverse-phase evaporation method was over 50% for the model drugs used in this investigation (Table 1), making this method highly efficient and suitable for drug encapsulation as compared with other available methods[14]. The liposome had an appropriate molar ratio of its constituents and a convenient lipid to drug ratio (Table 1).

The encapsulated radiomodifiers used in this investigation, as compared to their free forms, afforded greater radiomodulation in two tissue systems examined (Figs 4-7). The degrees of radiomodulation, however, were different for the two model drugs and for the two tissues examined. For both drugs BMC exhibited better radiomodulation (Figs 5 & 7) than SC (Figs 4 & 6). This could be due to physiological, metabolic and liposome uptake differences between BMC and SC.

The possible reasons for enhancement of radiomodulatory effects by liposomal MPG and AK-2123 could be: (1) delayed metabolic alteration of encapsulated drug, thus maintaining the active chemical forms of the drugs for a longer time, (2) reduced non- specific interaction of drugs with other metabolites which has been shown to influence radiomodulatory effects of MPG[11,15], and (3) increased concentration of drugs in these tissues due to preferential accumulation of liposomes in SC and BMC[2]. The present investigation does not shed light on the relative contributions of these three possibilities for enhancement of radiomodulation by liposome encapsulation. Nonetheless, the results show that liposome encapsulation enhanced radiomodulatory potentials of MPG and AK-2123. Gabizon[16] has suggested for DOX, an antitumor drug, that qualitative rather than quantitative differences were the cause of enhancement of effectiveness of DOX after liposome encap-

sulation. Papahadjopoulos *et al.*[17] have also reported increased efficacy of antitumor drugs after liposome encapsulation. To the best of author's knowledge, no such investigation has been carried out for radiomodulatory drugs. Therefore, This report brings out a novel finding to increase clinical efficacy of drugs for radiotherapy.

These findings may have significant impact on the clinical use of radiomodulators in cancer chemo-radiotherapy, since after liposome encapsulation the effectiveness of radiomodulatory drugs could be enhanced. This is particularly relevant for radiosensitizers which can increase clinical gains if it selectively sensitizes tumors (targets). This can be potentially achieved by using liposome drug delivery system for radiosensitizers. As emphasized earlier, liposomes could be targeted to specific cancer tissues through immunoliposomes having specific antibody against tumor antigens. Thus, radiosensitizer may be targeted to cancer tissues. This will reduce the toxicity of drugs to normal tissues, a serious problem associated with most radiosensitizers. Since cancer tissues will be sensitized, lower doses of radiation will be required to damage and kill cancer tissues. This will also reduce non- specific damages to healthy tissues. In conclusion, this approach of liposome encapsulated radiosensitizers may revolutionize the clinical practice of use of drugs in radiotherapy by enhancing efficacy of drug.

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LIPOSOME ENCAPSULATED MPG HAS VERY HIGH RADIOPROTECTION EFFECT

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2-mercaptopropionyl Glycine (MPG), an aminothiol is widely used as a radioprotector in radiotherapy. The effective dose of MPG (20mg/kg body weight) being significantly less than the toxic dose of MPG (2100mg/Kg body weight), offers an added advantage to the use of MPG as a radioprotector. However in our experience the amount of MPG must be optimal for the dose of radiation in radiotherapy for effective radio protective effect. When it is not so, we have found radiosensitization effect of MPG instead of expected radioprotective effect. Therefore in order to use MPG as radioprotective drug it becomes necessary to deliver a calculated amount of MPG to the target tissues.

To achieve this we have used Liposome drug delivery system for specific delivery of MPG to target tissues. This presentation shall discuss the results of encapsulation of MPG in Liposomes and radioprotective effect of Liposomes encapsulated MPG. This MPG is used in Swiss Albino mice.

P31 17 IN VIVO RADIOPROTECTION BY LIPOSOME ENCAPSULATED 2-MERCAPTOPROPIONYLGLYCINE.

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2-mercaptopropionylglycine (MPG), an aminothiol, has been widely used in experimental radioprotection because its effective dose (20 mg/kg b.w.) is significantly below its toxic dose. In certain cases, however, MPG afforded radiosensitization. This reversal could be due to non-optimal dose of MPG in the target tissue and/or due to metabolic alterations of the drug. In order to overcome these limitations of conventional mode of administration of MPG, a liposome drug delivery system for MPG has been developed. The liposome entrapment efficiency for MPG was over 50%. Swiss albino mice were exposed to different concentrations of liposome encapsulated MPG by ip injection prior to various doses of whole body gamma exposure. In general, the MPG affordable radioprotection was significantly elevated for different doses of radiation. Use of liposome as a drug delivery system offers a significant clinical advantage in radiotherapy.

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2-Mercaptopropionylglycine Affords Enhanced Radioprotection After a Liposome Encapsulation

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2-mercaptopropionylglycine (MPG)/Liposome/Drug delivery system/Radioprotection/Cell viability/ Acetylcholine esterase

Use of radioprotective drugs in radiotherapy is desirable to protect normal tissues. 2-mercaptopropionylglycine (MPG) has shown promising results in experimental radioprotection. In this report, a liposome drug delivery system for MPG has been used in Swiss albino mice exposed to 1 to 8 Gy whole body Gamma-irradiation to test whether or not this modality enhances the MPG afforded radioprotection. A statistically significant, dose dependent enhancement of protection by liposome encapsulated MPG (LEM) was observed. LEM, as compared to free MPG, improved the viabilities of spleen and bone marrow cells by factors between 1.11 and 2.23 for different doses of radiation.

INTRODUCTION

2-mercaptopropionylglycine (MPG), an aminothiol, has been extensively used in experimental radioprotection *in vivo* and *in vitro*[1-3] because it affords moderate radioprotection at an effective dose, 20 mg kg b.w.$^{-1}$, far below its toxic dose, 2100 mg kg b.w.$^{-1}$. However, MPG showed radiosensitization effect in cases of Gamma-induced microsomal lipid peroxidation[4], catalase radiolysis[5], and *in vitro* human-lymphocyte-DNA damage[6,7]. Two main reasons could explain this undersirable reversal of effect of MPG. At first, free MPG is distributed to various tissues depending on the physiological conditions[8]. Thus, the quantity of MPG available in a particular tissue may be other than optimum for protection. Secondly, MPG metabolism to its oxide, disulfide and other derivatives after its administration[8-10] may render MPG less effective radioprotector. It has been demonstrated that MPG caused radiosensitization

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of microsomal lipid peroxidation[4], and of catalase radiolysis[5] due to MPG/Fe complex formation. Therefore, it is desirable to design modalities wherein MPG, or other similar drugs, may continue to be a radioprotector, possibly with an enhanced efficiency.

In this report, a liposome drug delivery system has been tested for MPG to see whether or not this modality increase radioprotection afforded by MPG to spleen, bone marrow and liver of Swiss albino mice at its non-toxic effective dose.

MATERIALS AND METHODS

Chemicals: MPG (Tiopronine) was obtained from Santen Pharmaceuticals Co., Japan.; dipalmitoyl phosphatidyl choline (DPPC), dicetylphosphate (DCP), cholesterol, trypan blue and acetylcholine from Sigma Chemical Co., USA; dithionitrobenzoic acid (DTNB) from SRL, India and Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden. All other reagents and chemicals were of analytical grade.

Animals and Gamma-irradiation: Female Swiss albino mice (6–8 weeks old), randomly inbred colony housed at $21 \pm 2^{\circ}C$ with water and pellet dry feed *ad libitum*, were acutely irradiated at doses of 1, 2, 4, 6, and 8 Gy of Gamma-rays (^{60}Co Gamma source; 23.65 Gy min^{-1}) using Gamma Chamber 900 (BARC, Bombay, India).

Preparation of liposomes: Liposomes were prepared by a reverse phase evaporation method reported earlier[11,12]. Briefly, 5, 2.5 and 1 mg of DPPC, Cholesterol and DCP, respectively, were dissolved in 0.25 ml of chloroform by vortexing. To this lipid solution, 1 ml of 5 mM aqueous solution of MPG was added in alipuots of 0.2 ml while vortexing. Separation of liposome encapsulated MPG (LEM) from free MPG was done by Sepharose CL-4B column chromatography or by centrifugation[12].

Determination of MPG concentration: The concentration of free MPG as well as LEM was calculated by the assay of -SH group and has been described elsewhere[11]. Briefly, 0.1 ml of test sample was added to 2.9 ml of N_2 flushed assay mixture containing 10 mM DTNB in 100 mM PBS (pH 7.9) and 0.1 mM EDTA, mixed and absorption was immediately read at 412 nm. Cystein was used as a standard.

Administration of free MPG or liposome encapsulated MPG (LEM): Mice were intraperitoneally injected with 0.408 mg equivalent of MPG either as 0.5 ml of 5 mM aqueous solution of MPG or 1 ml of LEM 30 min prior to Gamma-irradiation. The control animals were irradiated without MPG.

Cell viability test: The viabilities of spleen cells (SC) and bone marrow cells (BMC) were calculated by dye exclusion technique as described earlier[13]. Immediately after irradiation, animals were killed by cervical dislocation and spleen and bone marrow cell suspensions were prepared in minumum essential medium. Viable and dead cells were counted on a Burker chamber under a Zena light microscope after 5 min incubation of cells with 1% trypan blue at 37°C. Each data point represents a minimum of 3 independent experiments, each with 3–5 replicates.

Calculation of viability modification factor (VMF): Dividing % viabilities of cells after irradia-

tion in the presence of either free MPG or LEM by that of radiation alone gave this factor:

$$\frac{\%\text{ viability in the presence of either MPG or LEM after} \times \text{Gy Gamma-irradiation}}{\%\text{ viability after} \times \text{Gy Gamma-irradiation}}$$

Student's t-test was applied to calculate the significance of differences.

Assay of liver acetylcholine esterase (AchE): Immediately after irradiation, animals were killed, livers removed and homogenized in 0.2 M sucrose solution. The whole homogenate was centrifuged at 2,000 *Xg* for 30 min at 4°C. Resulting supernatant was used for the assay of AchE following the method of Ott *et al.*[14] with minor modifications. The assay mixture in 3 ml contained 1 mM acetylcholine, 0.125 mM DTNB, 0.05% Triton X100 and 0.5 ml enzyme preparation. The reaction was followed on a spectrophotometer at 412 nm and increase in absorption min^{-1} was calculated. The activity of enzyme has been expressed as a specific unit which consumes 1 μmole of substrate at room temperature min^{-1} mg $protein^{-1}$. For controls, 6 mice were used in six independent experiments while the number of mice in MPG and LEM groups were 3 and 4 respectively.

RESULTS AND DISCUSSION

The biodegradable nature of liposome and its high potential of encapsulating target molecules[15,16], were main reasons for the selection of this drug delivery system. Reverse phase evaporation method of encapsulation of MPG into liposomes, used in this report, is simple and has been shown to be reproducible[11,12]. The molar ratio of DPPC:Cholesterol:DCP in the liposomes was 1:0.9:0.25 and the lipid/MPG ratio was 5.21. The percent entrapment of MPG

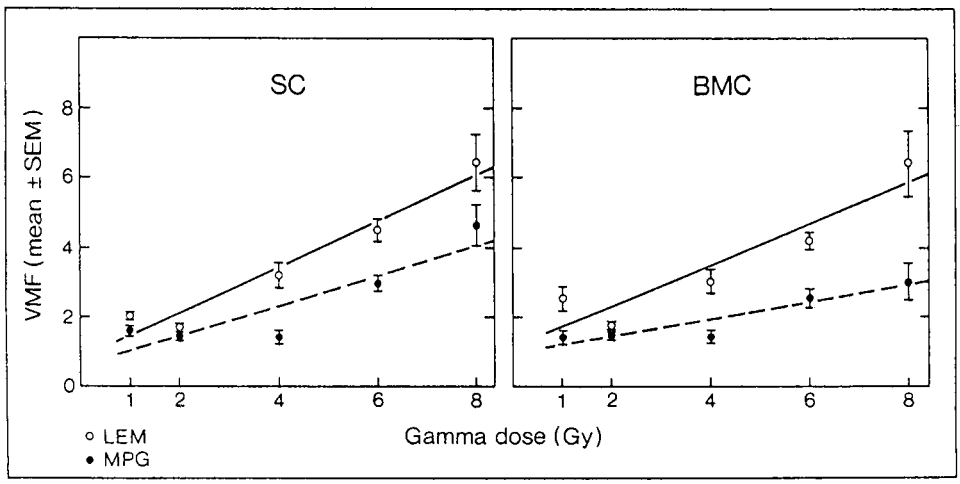

Fig. 1. Viability modification factor (VMF) for spleen cells (SC) and bone marrow cells (BMC) at different doses of whole body Gamma-irradiation. Free MPG and LEM were intraperitoneally injected to Swiss albino mice at the same dose level 30 min prior to irradiation.

was found to be 50±2.9. Administration of 0.5 ml of 5 mM MPG aqueous solution or 1 ml of LEM (0.408 mg MPG equivalent) delivered 20 mg MPG kg b.w.$^{-1}$ in each mouse (average weight=20 g) which is the effective radioprotective dose of MPG[2-7].

The viability modification factor (VMF) for spleen and bone marrow cells after various doses of whole body Gamma-irradiation in the absence (control) or the presence of equal amount of MPG (0.408 mg) administered as such (free MPG), or as LEM 30 min prior to irradiation showed a radiation dose dependent increase (Fig. 1). The numerical data on viability, with statistical evaluations, are given in Table 1. The LEM group displayed higher viability over the respective free MPG group by factors between 1.11 and 2.23 (for SC) and between 1.18 and 2.07 (for BMC) for different doses of radiation (Table 1, Fig. 1). For 4 & 8 Gy Gamma doses, the release of liver enzyme AchE into supernatant, signifying radiation induced membrane damage, was significantly reduced by MPG in both free MPG and LEM groups; the latter showing a higher tendency of protection (Fig. 2). The dose of MPG used in these experiments was far below its toxic dose, therefore, it is assumed that the drug did not influence the metabolism or the survival of the treated animals. Because the assays were performed for all groups, including the controls, identically, the immediate postirradiation protection of MPG, reported here, is likely to represent the normal situation of MPG affordable radioprotection.

Upon administration, free MPG (a) migrates to different tissues, consequently limiting the quantities of MPG available to SC, BMC and liver, and (b) is subjected to normal metabolic alterations[8-10]. The behaviour of LEM is likely to differ from the MPG on both these criteria because liposomes preferentially migrate to tissues rich in reticuloendothelial cells and fenstrated capillaries, viz. liver, spleen, bone marrow etc.[15] and the encapsulation delays metabolic

Table 1. Spleen cell (SC) and bone marrow cell (BMC) viabilities as a function of Gamma dose in the absence (control) and presence of equal quantity of free MPG (MPG), or liposome encapsulated MPG (LEM)

Tissue	Treatment groups	% viable cells (mean±SD)/dose of radiation				
		1 Gy	2 Gy	4 Gy	6 Gy	8 Gy
SC	Control	29.12 ±5.3(9)*	35.80 ±9.1(9)	19.31 ±6.7(11)	16.05 ±2.8(9)	11.98 ±5.3(16)
	MPG	47.35 ±8.1(9)	54.59 ±8.2(9)	27.70 ±9.5(11)	48.02 ±8.0(9)	55.73 ±13.8(17)
	LEM	59.73♣ ±2.8(9)	60.69◆ ±4.0(9)	61.86♥ ±9.8(11)	72.04♥ ±8.7(9)	77.14♠ ±18.1(19)
BMC	Control	24.73 ±9.8(9)	35.09 ±6.6(9)	19.54 ±6.8(11)	15.00 ±1.9(9)	11.45 ±4.7(19)
	MPG	35.25 ±6.2(9)	54.02 ±1.5(9)	29.30 ±6.2(11)	40.02 ±9.7(9)	36.20 ±10.6(9)
	LEM	63.74♥ ±4.9(9)	63.57♥ ±3.1(9)	60.78♥ ±8.4(11)	65.65♥ ±4.5(9)	74.63♥ ±7.9(11)

Significantly higher viability as compared to respective MPG groups: ♥($p \leq 0.0001$), ♣($p \leq 0.002$), ◆($p \leq 0.1$) and ♠($p \leq 0.001$)

* Numbers in paranthese indicates number of mice used.

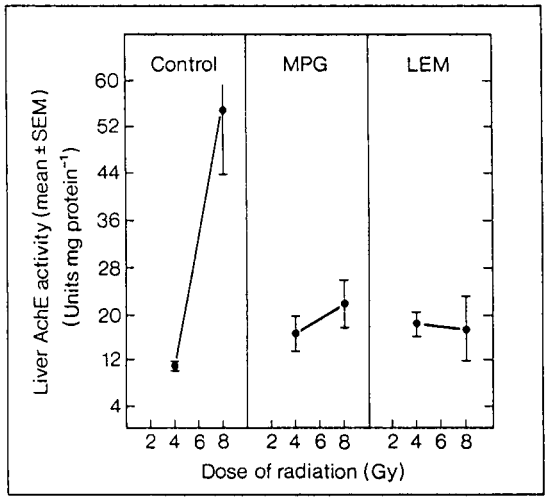

Fig. 2. Liver Acetylcholine esterase (AchE) released in the supernatant fraction after 4 and 8 Gy whole body Gamma-irradiation. Control group did not receive MPG while free MPG and LEM were intraperitoneally injected to Swiss albino mice at the same dose level 30 min prior to irradiation.

alterations of the entrapped drug[15,17]. Therefore, LEM afforded significantly higher radioprotection than free MPG, to SC and BMC (Fig. 1, Table 1) with liver AchE showing supporting trend (Fig. 2). The relative contribution of the two factors in LEM radioprotection has not been ascertained and is subject of further investigations.

LEM, as compared to MPG, afforded greater protection for all doses of radiation-statistically highly significant ($p \leq 0.001$) in case of BMC and at lower levels of statistical probability (p between 0.1 and 0.0001) in case of SC (see Table 1). BMC and SC exhibited higher viabilities with increasing doses of radiation in LEM treated groups but not in case of free MPG. Since MPG and LEM were administered to mice 30 min prior to irradiation, a part of the free MPG could be metabolically altered[8–10] making them less effective radioprotector. Thus, free MPG afforbable viability was nearly the same for different doses of radiation. In case of LEM, on the other hand, the metabolic alterations of MPG was delayed[15,17] and MPG remained longer in its protective form. Furthermoer, the encapsulation of MPG also prevents circumstantial interaction of MPG with Fe which has been shown to be the cause of its radiosensitizing effect[4,5]. To explain LEM induced increasing viability with higher doses of radiation, it may be reasonable to assume that release of MPG from liposomes into the cells is enhanced by radiation as liposomes are essentially like biomembranes. Thus, increasingly more MPG leaked out from liposomes into the cells at higher doses of radiation and could offer increased protection (higher viability). A relatively poor viability in 1 Gy controls is attributed to differences in batch of randomly inbred mice.

In conclusion, we have found that liposome encapsulation of MPG not only prevented undesirable radiosensitization effects of MPG reported earlier[4–7] but noticeably enhanced protective effects of MPG in our experimental conditions. These results agree with the earlier

observations of Papahadjopoulos *et al.*[17] that liposome encapsulation increased efficacy of antitumour drugs in mice. In an attempt to explain the reasons for it, Gabizon[18] has reported that metabolic alterations rather than quantitative differences caused liposome encapsulated DOX, an antitumour drug, to be more effective as compared to its free form. Whatever may be the reason, the efficiency of radioprotection afforded by MPG was significantly enhanced by liposome encapsulation. Thus, our results open up a new possibility of use of liposome drug delivery system for enhanced radiomodulation. Use of liposome drug delivery system offers an additional advantage of tissue targeting[15]. Liposomes may be directed to selective tissues with the help of specific antibody (against an antigen of the target tissues) tagged onto the liposome. Thus, the liposome vehicle can deliver calculated amounts of MPG to specific tissues in relation to the dose and protocol of irradiation and avoid a possible radiosensitization reported earlier[4–7]. Furthermore, liposome vehicle can be successfully used for other drugs in radiotherapy, whose use is presently limited due to high toxicity or metabolism problems. More work is underway in this laboratory on these lines.

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