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BIRESH CHANDRA GUHA MEMORIAL LECTURE—1984

LIPOSOME TECHNOLOGY

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MR. President, Distinguished Fellows of the Academy, Ladies and Gentlemen !

It is indeed an honour and privilege for me to deliver the Professor Biresh Chandra Guha Memorial Lecture this year. I am grateful to the Indian National Science Academy for electing me for this Lecturership.

Professor Biresh Chandra Guha, the doyen of Indian biochemists, was my teacher and introduced me to the subject of biochemistry decades ago. Professor Guha was not only an ideal teacher and a renowned biochemist, he was also a futurist and a great seer. I remember as early as in the fifties he used to discuss with us about the potential use of enzymes in biological industries. At a time when the term biotechnology was unheard of and even modern biology was yet to penetrate into industrial sector, Professor Guha visualised the great potential of biotechnological revolution which is now knocking at our door these days. I imagine how happy he would have been to see his prophesy coming true had he been living today. I, therefore, embark upon the unique privilege of delivering this lecture with a deep sense of humility and gratitude.

Liposomes are used extensively in the studies on model membranes, as a drug and enzyme carrier and in immunology. In this lecture, I will discuss the work carried out in my laboratory in the following areas :—

(A) Liposome as a model membrane.

(B) Liposome as a drug and protein carrier.

(C) Liposome as an adjuvant in Immunology.

(D) Liposome as a haptenic carrier for small molecules.

A. LIPOSOME AS A MODEL MEMBRANE

Cell surface carbohydrates are implicated in a number of important cell surface properties such as cell-lectin, cell-toxin and cell-cell interaction. Since the intact cell is a very complicated system and as such the study on molecular level is very difficult, if not impossible. Accordingly, it is logical to study the individual component in a model membrane so that one can at least get an idea of a particular component in relation to the cell-ligand interaction under defined experimental condition.

The receptor properties of a number of glycoproteins have been studied. However, the study of the receptor property of glycolipids is difficult since they form micelles in aqueous solution. This difficulty was successfully resolved by incorporating the glycolipid in the lipsosome. We developed a very simple system to study. In collaboration with a biophysicist, Dr S K Poddar of Bangalore and one of my brilliant graduate students, Dr A Surolia, we developed a simple system to study the lectin-glycolipid interaction.[1–4] Ganglioside (GM_1) containing multilamellar and unilamellar liposomes were prepared and their interactions with galactose binding *Ricinus communis* lectin (RCA_1), was investigated. Measurement of various kinetic parameters e.g. association constant and rate of cluster formation led us to conclude that the system could be used as a simple model for the study of receptor-ligand interaction. A few interesting inferences were derived from this study; (1) About 60 per cent of the galactose residues of GM_1 is externally available on the surface of unilamellar liposomes; (2) in case of multilamellar liposomes only 25 per cent of the same is exposed on the surface; (3) the rate of lectin GM_1, liposome interaction is markedly affected by the surface density of the sugar residue (number receptor sites per um^2/liposomes) i.e. by GM_1 concentration in liposomes. Thus the rate of liposome aggregation increased 20-fold when the molar ratio of GM_1 to phospholipid was increased from 0.08 to 0.18. In another study, fatty acid.chain length as well as oligosaccharide chain length on the rate of interaction between RCA, and glycolipid liposomes were investigated. These studies suggest that the phase transition temperature of the phospholipid component of liposomes, length of the surface-bound oligosacharide chain and cholesterol concentration also affect the binding of the terminal sugar with the lectin.

These studies led us to conclude that (1) the terminal galactose residue is almost embedded into the lipid of liposomes containing Gal-Cer (2) lectin binding with Gal-cer and cytolipin H liposomes are strongly influenced by fatty acid chain length of the phospholipid compound as well as the cholesterol concentration of liposomes. The role of cholesterol was not clearly understood. It may be that cholesterol may affect the membrane fluidity. It was also noted that the increased receptor density can be predicted by the increased lectin cross binding. It may be of interest to mention here that although the lectin mediated agglutination of liposome was very marked with density of the receptor, the binding of the lectin linearly increased with the increased concentration of the receptor on the surface. The impact of our initial report on the glycolipid lectin interaction can be gauged from the following quotation from a recent review of the subject by Grant and Peters[5] : "*Surolia, Bachhawat and Poddar have been responsible for a series of very clever experiments which set the pace.*"

B. Liposome as a Drug Delivery System

In a collaborative work with Dr James H Austin of Denver Colorado, USA and my group at that time at Vellore, we for the first time showed that the deficiency of arylsulphatase was the main factor for the accumulation of cerebroside-3 sulphate

in a sphingolipidosis, metachromatic leukodystrophy.[6,7] Arylsulphatase A, a lysosomal enzyme was found to be a glycoprotein.[8]

In 1971, Ryman and her coworkers initiated a number of studies where they used liposome encapsulated enzyme for the delivery into the cell.[9] During this period in a series of brilliant experiments, Ashwell as well as Morrell[10] and their groups in USA reported their work on the *in vivo* survival of glycoprotein and its removal through β-galactosyl moiety of the oligosaccharide chain of the glycoprotein by the liver. A specific receptor for β-galactoside moiety on the hepatocyte surface was recognised. A mannoside specific receptor was also recognised by Stahl *et al.* in 1978[11] on the cell surface of reticuloendothelial system of rats including the liver sinusoidal cells and macrophages.

These facts led me[12] to suggest that by modification of the liposomal surface by incorporating various glycosides it will be posssible to target different tissues *in vivo*. With this aim in view we were able to construct a liposome model from our work on the kinetics of lectin-glycolipid interaction. This model shows that a part of the oligosaccharide chain is on the surface of the liposome and is available for the binding with lectins (Fig. 1).

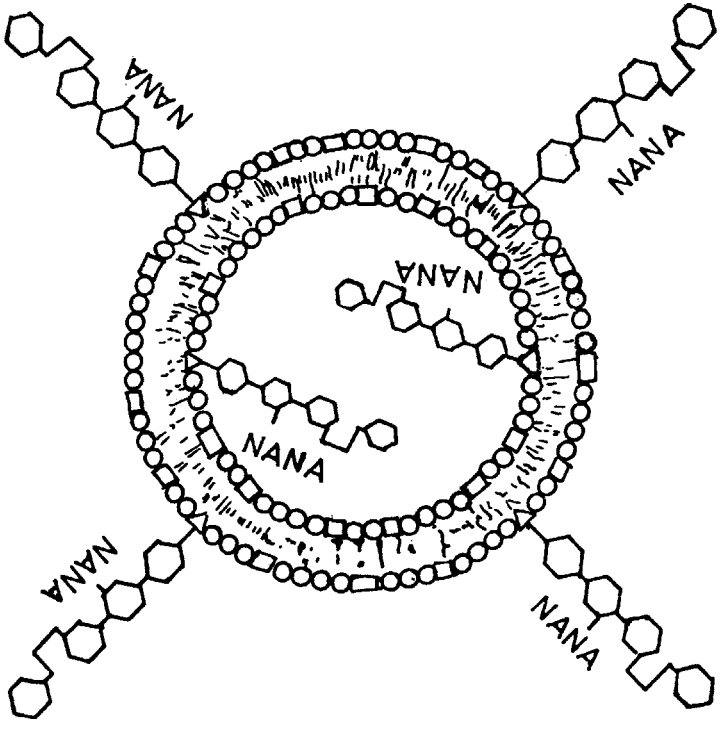

FIG 1 Schematic representation of GMI containing single compartment liposome

With this background information, we initiated our *in vivo* experiments with GM_1 ganglioside and asialo GM_1 ganglioside containing liposome with entrapped enzymes, proteins and drugs. Initially, we used a heterogeneous population of liposomes and we observed that the liposome-entrapped materials were very repidly taken up by the liver. Using appropriate oligosaccharide residues as *in vivo* inhibitors, we concluded that the uptake by the liver was through sugar specific receptors.[13,14] In an attempt to identify the types of the liver cells involved in this sugar-specific uptake, we administered *in vivo* asialo GM_1 liposomes and mannosylated liposome entrapped labelled γ-globulin. Livers were perfused and the different cells were isolated. It was observed that the galactosylated liposome entrapped material were enriched in the hepatocyte. This sugar specific enrichment was completely abolished[15] if asialofetuin was administered along with the asialo GM_1 liposomes. Similarly, mannosylated liposome entrapped materials were enriched in the Kupffer and endothelial cells and there was partial decrease in the uptake of the liposomes when mannan was a competitive inhibitor.

Using homogeneous small unilamellar liposomes (SUV), we carried out a series of experiments and developed a new method for the preparation of synthetic glycolipids.[16] In this method phosphatidyl ethanolamine was coupled with mellibiose or lactose through cyanoborohydride to form a synthetic glycolipid having terminal α-galactosyl and β-galactosyl residue. It was observed that β-galactosylated liposomes are perferentially taken up by the liver. During this period a number of workers, Gregoriadis,[17] Rahman and others[18] had attempted unsuccessfully to target the liposome having the glycoside residues. We suggested that their inability to target the liposome might have been due to the low level of glycolipids they had incorporated in their liposome as it was observed by us that the sugar specific uptake of the liposome was dependent on the density of the sugar residue on the surface of the liposome.[19]

However, without taking into account that we had reported the liposomal uptake using heterogeneous liposome preparation as well in other cases homogeneous small unilamellar liposomes, a number of reports[20,21] appeared in the literature which basically confirmed our observation that β-galactoside liposomes are specifically taken up by the hepatocytes. These investigators had used ceramide disaccharide having terminal β-galactoside. However, unfortunately since they have used different methods of preparation of liposomes and have not measured the available number of β-galactoside residues on the surface of these liposomes, the discrepancy regarding the rate of uptake of the liposomal entrapped material compared to our results would not be resolved.

A number of exciting probabilities of the use of β-galactosylated liposomes are now becoming apparent. We have been able to show that asialo GM_1 liposome entrapped material could be effectively used to prevent the hepatitis-like effect of D-galactosamine.[22] It is known that D-galactosamine can specifically destroy hepatocytes. This effect of D-galatosamine can be reduced or prevented by the administration of uridine. Since asialo GM_1 liposome is known to be enriched in the hepatocyte, asialo GM_1 liposome entrapped uridine administered just prior to

the administration of galactosamine could effectively prevent the galactosamine toxicity. Asialo GM_1 entrapped uridine was almost 10 times more effective compared to free uridine in combating the D-galactosamine toxicity.[23,24,25]

It may be mentioned here that asialo GM_1 liposome entrapped uridine was effective only when hepatocytes were intact i.e. prior to the administration of D-galactosamine and it was found to be comparatively ineffective when the hepatocytes were destroyed i.e. three hours after D-galactosamine administration. This may be due to the fact that D-galactosamine affects the hepatocyte leading to the probable loss of surface receptor for β-galactoside residue resulting in impaired receptor mediated uptake of the liposome.

Another interesting development of the application of β-galactosylated liposome had been in the direction of the introduction of genetic material into hepatocyte.[26] Scherphof and Nicolou jointly using ceramide lactoside were able to introduce pre-proinsulin gene into hepatocyte. They have further shown that if the non-glycosylated liposome entrapped pre-proinsulin gene was administered *in vivo* it generally accumulated into Kupffer cells in a degraded form. As has been pointed out by these investigators this is an interesting possibility as the hepatocytes are secretory cells.

In view of the above exciting possibilities of β-galactosylated liposomes, it was felt that further investigation of the nature of uptake of the liposome was important. Accordingly, the intracellular localization of liposome entrapped material both in the parenchymal and non-parenchymal cells of the liver was examined.[27] After taking proper precaution during the isolation of the liver cells to minimize the receptor mediated uptake and lysosomal degradation of the liposomes during the liver perfusion it was observed that the enhanced uptake of asialo GM_1 liposome by parenchymal cells was due to the β-galactosylated liposomal cells. Sub-cellular studies indicated substantial lysosomal localization of the liposome entrapped material both in parenchymal and non-parenchymal cells. Asialofetuin inhibited specifically the uptake of asialo GM_1 liposome. Negatively charged liposome had also the enhanced uptake by the liver cells compared to neutral liposome but lower than galactosylated liposome. In the case of negatively charged liposome both parenchymal and non-parenchymal cells showed increased uptake. It was of interest that although there was inhibition of uptake of asialo GM_1 liposome by asialofetuin there was increased amount of the entrapped material in the lysosomal fraction. The asialofetuin effect may be due to either that asialo GM_1 liposomes are cointernalized with asialofetuin through the common lysosomal route of ligand internalization or the desialated glycoprotein in the lysosome may somehow protect the liposome entrapped material from the highly metabolic mileu of lysosome. The exact mechanism of this phenomenon is yet to be known.

As mentioned earlier, although the pattern of uptake β-galactoside liposome is in essence similar in all the reported work by various investigators in relation to its preferential uptake by the parenchymal cells, there exists some difference as to the rate of uptake between our observations and that of Scherphof and Nicolou.

In this context, it will be of interest to mention that these investigators employed phosphatidyl serine containing lactosylceramide liposomes. It was reported by Hampton *et al.*[30] in their *in vitro* experiment that phosphatidyl serine containing liposomes does not bind with the galactose binging lectin from castor been unless Ca^{++} is present. In view of this *in vitro* effect, it may be suggested that the phosphatidyl serine may have some effect on the receptor mediated uptake. This will explain the slow rate of uptake observed by these investigators. This point needs to be further investigated.

It is of interest to mention here that about 10 years ago we started with the concept that it should be possible to the deliver drugs or enzymes intracellularly into lysosome in the case of diseases associated with lysosomal enzymes e.g. sphingolipidosis. It is apparent that we have been able to develop a very interesting model system using β-galactosylated liposomes to do this job (Fig. 2).

It may, however, be noted that it is yet to be ascertained about the stability of the external enzyme and expression of its activity after the delivery into the lysosomes. It is gratifying for me that it has been possible to extend our *in vitro* kinetic studies on lectien-liposome interaction to the use of glycolipid liposome or the targeting of specific liver cell types *in vivo* simply by varying the sugar residue on the surface of liposome.

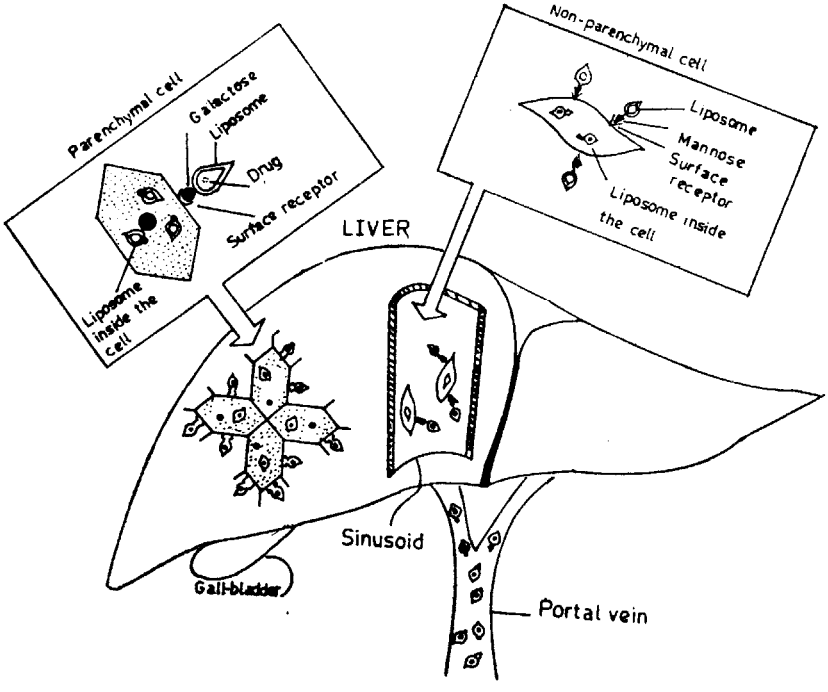

FIG 2 Targeting of drug to specific liver-cell types

Oral Administration of Liposome

In an earlier section, I have discussed *in vivo* administration of liposomes through intravenous route. In recent years attempts have been made to administer liposome entrapped material orally.[29,30] This route of administration has obvious advantages.

In a number of experiments, it was observed that orally administered liposome entrapped protein such as labelled gamma globulin was completely degraded when the radio activity was detected in cardiac blood.

Thus, these experiments were of inconclusive nature as it was not definite whether this degradation was in the intestine along with the disruption of liposome or in the organs. In later experiments, it was observed that a significant amount of liposome entrapped material could be detected in the portal blood. It was further observed that in the portal blood 75 per cent of the entrapped protein was intact and 50 per cent of the total gamma globulin in the portal blood was still intact as the liposome entrapped material. At the same time all the gamma globulin in the cardiac blood was found to be in the degraded form. This observation may be explained by assuming that liposome present in portal blood was taken up by the liver, processed there, and then circulated.

When asialo GM_1 liposomes were employed there was a very rapid uptake by the liver. This uptake was actually inhibited by asialo fetuin administration at a suitable time interval. The presence of asialofetuin also increase the concentration of undegraded material in the portal blood. This was the first observation to show that orally administered liposomes enter into circulation through portal route. This study also indicates that although total amount of intact liposome is only 2–3 per cent of the administered dose, this mode of administration has a considerable potential as a drug delivery system. The surface modification of liposomes will also help in directing these liposomes even through oral route.[33]

It is hoped that in future it will be possible to increase the stability of liposome in the intestine. This will have an influence on the orally administered liposome entrapped drug.

Oral administration of liposome and its uptake by the liver suggests a rather exciting possibility with respect to indigenous Ayurvedic drug. It is well known that Ayurvedic drugs are effective when administered orally and quite a few of these drugs are in the form of glycosides. The terminal sugar of the drug and the way it is prepared and administered may have a profound effect on the passage of this drug through intestine and uptake of the drug by various organs.

C. Liposome in Immunology

The adjuvant effect of liposome was first reported by Allison and Gregoriaidis.[34] Since then the immunopotentiating effects of liposome have been the subject of intensive study. Liposome has an advantage over adjuvants currently used for human and animal immunization since they are prepared from biodegradable phos-

pholipids which does not produce any granuloma. Further, liposomes protect the antigens from the hypersensitivity reaction.

In the laboratory of the Indian Institute of Chemical Biology, we have initiated a series of research work on the immunopotentiating effect of liposomes. Using protein as an antigen we have confirmed that liposome entrapped antigen also can be used when liposomes are employed as an adjuvant. We made the important observation that the nautre of the surface of the liposome has a significant effect on the level of antibody titre. It was observed that a lysozyme (a protein antigen) entrapped in neutral and negatively charged liposome induced significant levels of antibodies. However, the antibody titre obtained with lysozyme entrapped in positively charged liposomes is higher than that with negatively charged or neutral liposomes or even with complete adjuvant. The strong immune response was found to be accompanied by mild granuloma[37] formation at the site of the injection. The exact mechanism of adjuvant action of the liposome is not known. There is a possibility that positively charged liposomes interact differently with cells *in vivo* in comparison to that of neutral and negatively charged liposomes. Similar immunopotentiating effect were also observed when synthetic phospholipids such as dipalmitoyl phosphatidyl choline and distearyl phosphatidyl-choline were used during the preparation of liposomes instead of egg lecithin. The best route of administration of liposome entrapped antigen to produce maximum antibody titre was found to be subcutaneous.

In order to understand the mechanism of action of liposome as an adjuvant, the vesicles were designed to specifically interact with macrophages, the phagocytic cell responsible for the clearance of liposomes. When antigen entrapped liposomes with mannose and galactose exposed on the surface were injected, liposome with terminal galactose residues induced an immune response comparable to adjuvant effect of sugar free neutral liposomes,[38] whereas the immune response of mannosylated liposomes was equal to that of the free antigen. It is tempting to postulate that mannose-liposomes are taken by the macrophage *via* a receptor mediated process recognising the mannose residue resulting in the rapid degradation of the entrapped antigen and accordingly not available for eliciting antibody response.

D. Liposome as a Haptenic Carrier for Small Molecules

In addition to their adjuvant effect, liposomes have been recognised as an efficient haptenic career of antigens in recent years. Haptenic groups are attached to liposomes by coupling them to phosphatidyl ethanolamine. With such liposomes dual properties of liposomes as carrier and adjuvant in eliciting antisaccharide (antigalactosyl and antimannosyl) immune response[40,41,42] were apparent.

Liposome mediated antigaladtosyl antibody response when compared was found to be as good as either in the presence of complete Freund's adjuvant or through the conventional method of protein carrier.

This use of liposome as an haptenic carrier may be extended to various peptide fragments or small peptides. This will minimize the cross reactivity of the carrier protein.

The work presented in this review highlights the potential applications of liposomes in clinical science. This potential can be fully realized once we can develop stable unilamellar liposomes, stable *in vitro* and *in vivo*.

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