CHAPTER - 3

KINETICS AND NATURE OF IMMUNE RESPONSE OF GALACTOSYLATED LIPOSOMES
3.1 Summary:

The kinetics of the immune responses to galactosylated liposomes in saline was studied in rabbits. The antibody level in the secondary immunization was found to be higher compared to that in the primary response. The antibodies were purified on an immunoadsorbent column of galactosylated bovine serum albumin. Antigalactosyl antibodies belonged to both IgM and IgG classes, the former being the major fraction. Both failed to agglutinate normal or neuraminidase treated human or rabbit erythrocytes but they agglutinated trypsinized cells.

3.2 Introduction:

Immune responses against liposome-associated dinitrophenyl hapten (206) and protein antigen (212,245) have been well characterized. Kinsky et al. have shown that dinitrophenyl (DNP) sensitized liposomes function as thymus independent immunogens in mice (206). Antibodies elicited were of the IgM class. Anti-DNP antibodies could be elicited through liposomes in T-cell depleted (thymectomized, irradiated, bone marrow reconstituted) and T-cell deficient (nude) mice. However, bovine serum albumin (BSA) entrapped in liposomes requires the participation of T lymphocytes in eliciting anti-BSA immune response (245). The role of liposomes in the antigenicity of the carbohydrate determinant was not known. Only recently liposomes have been used as carriers to produce antibodies in rabbits specific for isomaltosyl oligosaccharides (243) and for various monosaccharides e.g. D-galactose (Chapter 2), D-mannose (270) and N-acetyl-D-glucosamine (272). Wood and Kabat (243) elicited antibodies against isomaltosyl oligosaccharides by coupling the haptens to stearylamine and incorporating the stearyl oligosaccharides into sphingomyelin liposomes. We
have coupled the p-aminophenyl derivative of monosaccharides to phosphatidylethanolamine containing lecithin liposomes. Our studies have further demonstrated the adjuvant role of liposomes for saccharide determinants (Chapter 2). Galactosylated liposomes elicited a nearly equivalent immune response both in Freund's adjuvant and without it i.e. in saline. The aim of the present investigation is to study the immune response of galactosylated liposomes.

3.3 Materials and Methods:

3.3.1 Reagents:

The saccharide ligands, bovine sorum albumin (BSA) and dicetylphosphate were purchased from Sigma Chem. Co. USA. Egg lecithin (phosphatidylcholine), cholesterol and phosphatidylethanolamine were obtained from the CSIR Centre for Biochemicals Delhi, India. Other reagents used were of analytical grade. Coupling of p-aminophenyl-\(\beta\)-D-galactopyranoside to phosphatidylethanolamine incorporated liposomes (lecithin : Cholesterol : dicetylphosphate : phosphatidylethanolamine = 7:2:1:2, molar ratios) was performed as described in the Chapter-2 (materials and methods). p-aminophenyl-\(\beta\)-D-galactoside was conjugated to BSA by the method described in Chapter 2 and the conjugate was found to have 30 moles of sugar per mole of BSA (Gal\(_{30}\)-BSA). Sheep anti rabbit IgM and IgG antisera were obtained from Sigma Chem. Co. USA. Carrier free \(^{125}\)I was purchased from Bhaba Atomic Research Centre, India.

3.3.2 Immunization schedule:

Two groups of rabbits (New Zealand white strain) each containing five animals were taken. In both groups each animal was injected subcutaneously with 1 ml of galactosylated liposomes in saline (15 mg lipid and 3 mg sugar). In one group twentyone days after the first injection (Primary immunization), a second dose of liposomes containing the same amount of sugar was injected into
each animal. Blood samples for the determination of antibody titers were collected from the rabbits immediately before and at different time intervals after the first injection.

3.3.3 Quantitation of immune response:

The test antigen Gal$_{30}$-BSA was iodinated by the chloramine-T procedure as described in Chapter 2. Antigalactosyl immune response was measured by a modified radioactive antigen binding assay (344). Serially diluted samples (80 µl) were incubated with 25 µg Gal$_{30}$-$^{125}$I BSA (70 µl, 70,000 cpm) in 0.02 M sodium phosphate buffer containing 0.15 M NaCl (PBS) for 18 hrs at 4°C, then 100% saturated ammonium sulfate solution (150 µl) was added. After incubating further for 30 min, the tubes were centrifuged in the cold and the radioactivity was measured in the supernatants as described in Chapter 2. Calculations were made as 33% antigen binding capacity (ABC-33) and the values were expressed as the amount of Gal$_{30}$-$^{125}$I-BSA bound per one ml of undiluted serum. The values were corrected against appropriate blanks, without antiserum and with normal rabbit serum (NRS). The nature of specific antibodies was determined by gel filtration of the serum through a Sephadex G-200 column and by the ability to lyse haptenated liposomes as described in Chapter 2. The fractions were further checked with sheep antirabbit IgM and IgG antisera.

3.3.4 Preparation of antigalactosyl antiserum:

This was performed according to the procedure described in Chapter 2. Briefly, four rabbits were taken for immunization. Each rabbit was immunized with 1 ml galactosylated liposomes (15 mg lipid and 3 mg sugar) in saline subcutaneously. Two more injections were given 10 days apart. Antisera were collected 7 days after the third injection by cardiac puncture and were decomplemented by incubating at 56°C for 30 min ; it was stored at -20°C.
3.3.5 Purification of antibodies

A gamma globulin rich fraction was isolated by precipitating the antiserum with 33% saturated ammonium sulfate. It was then loaded on an immunosorbent column prepared by conjugating Gal3g-BSA to CNBr-activated sepharose-4B essentially following the procedure of March et al. (345). The unbound fraction was removed by washing the column with PBS and the bound antibodies were eluted with 3 M NH4SCN, following the procedure of Hunter et al. (346). The pooled antibodies were dialyzed against PBS and concentrated by ultrafiltration. They were then fractionated into IgM and IgG classes through Sephadex G-200 chromatography and further characterized with sheep antirabbit IgM and IgG antisera by immunodiffusion in a 1% agarose gel as described in Chapter 2.

3.3.6 Neuraminidase and trypsin treatment of rabbit and human (A, B and O blood groups) erythrocytes:

These have been carried out following the procedure of Gahmberg et al. (347). The human A, B and O blood group erythrocytes were collected from human volunteers whose blood groups were pretested with specific blood group reagents.

(a) Treatment of cells with trypsin: Cell suspensions in PBS, pH 7.4 were mixed with twice the volume of 0.25% trypsin solution and incubated at 37°C for 30 min with continuous gentle shaking, transferred to an ice-water bath and washed by centrifugation with a large volume of ice-cold PBS, pH 7.0, three times before suspending finally in PBS, pH 7.4.

(b) Treatment with neuraminidase: The cells were suspended in
Each of the five rabbits was injected subcutaneously with 1 ml galactosylated liposomes (15 mg lipid and 3 mg sugar) in saline. Antisera were collected at regular intervals of time and assayed for antigalactosyl antibody level by radiolabelled antigen, Gal\(^{125}\)-labeled BSA, binding assay. From this, mean ABC-33 of the immunized sera was calculated and plotted as Log\(_{10}\) ABC-33 Vs. days after immunization.
Fig. 23 Each of the five rabbits was injected subcutaneously with 1 ml. galactosylated liposomes (15 mg lipid and 3 mg sugar) in saline. A second injection was given at 22nd day containing same amount of liposomes in saline. Antisera were collected and assayed as described in Fig. 22.
The magnitudes of the immune responses in the primary and secondary immunization with galactosylated liposomes were measured (Fig 22 and 23). Antibody titer could be easily detected from the 7th day and the highest titer was obtained at day 14 in the primary immune response (Fig 22). It was noticed that the antibody titer could be easily detected at an appreciable level up to about 35 days after the primary immunization. When a second dose of haptenated liposomes was injected on day 22, the antibody level increased further (Fig 23). The peak of the immune response (day 30) in the secondary response became three times higher than that of the primary immunization. The peak persisted at an appreciable level for a considerable time (day 40 from the secondary immunization). Gel filtration profile of the serum and further characterization with sheep antirabbit IgG and
AGGLUTINATION OF UNTREATED (U), NEURAMINIDASE TREATED (N) OR TRYPsin TREATED (T) RABBIT AND HUMAN (OF A, B, AND O BLOOD GROUP) ERYTHROCYTES BY ANTIGALACTOSYL IgM AND IgG ANTIBODIES. THE AGGLUTINATION EFFICIENCY WAS EXPRESSED AS THE MAXIMUM FOLD DILUTION OF STOCK IgM (1.2 mg/ml) AND IgG (1.2 mg/ml), WHICH EXHIBITED AGGLUTINATION. THE NUMBERS INDICATE THE FOLD DILUTION AND NO AGGLUTINATION IS INDICATED BY A DASH (-).

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>AGGLUTINATION</th>
<th>EFFICIENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RABBIT</td>
<td>HUMAN</td>
</tr>
<tr>
<td></td>
<td>U  T  N</td>
<td>A  B  O</td>
</tr>
<tr>
<td>IgM</td>
<td>-  540</td>
<td>-  540 -  1215 -  180</td>
</tr>
<tr>
<td>IgG</td>
<td>-  162</td>
<td>-  150 -  81 -  54</td>
</tr>
</tbody>
</table>
Fig. 24 Elution profile of antigalactosyl antibody from immunoadsorbent column (0.1 x 15 cm) prepared with Gal3-BSA. After eluting the unbound fraction with PBS bound antibodies were eluted with 3M NH4SCN solution.
Fig. 25 Immunodiffusion of purified antigalactosyl IgM (a) and IgG (b) against sheep antirabbit IgM and IgG respectively. The central wells in (a) and (b) received the sheep antirabbit IgM and IgG respectively and the purified IgM and IgG were added in the peripheral wells.
IgG antibodies showed the formation of mostly antigalactosyl IgM antibodies in the primary immunization whereas a minor IgG fraction in addition to a major IgM fraction were obtained in the secondary response.

3.4.2 **Fractionation of antigalactosyl antibodies**

Antibodies were purified on a Gal$_{30}$-BSA immunoabsorbent column (Fig 24). The specific antibodies were then passed on a sephadex G-200 column for fractionation into IgM and IgG classes. The IgM and IgG fractions were further characterized with sheep antirabbit IgM and IgG (Fig 25) antibodies. The amount of IgM (70%) predominated over IgG (30%). On an average 1.0 ml of antiserum had 3.5 - 5.0 mg specific antibodies, including both IgM and IgG fractions.

3.4.3 **Agglutination of erythrocytes by purified IgM and IgG antibodies**

To study the interaction of IgM and IgG with cells, their agglutination ability with human and rabbit erythrocytes was examined. Trypsinized or neuraminidase treated erythrocytes were also included. Both types of antibodies did not agglutinate normal or neuraminidase treated erythrocytes (Table 9). The cells became agglutinable only after the action of trypsin. The agglutination titer of IgM was found to be higher than that of IgG, with trypsinized rabbit cells. Among human erythrocytes IgM has the highest specificity for blood group B cells whereas IgG best agglutinates blood group A cells. Mercaptoethanol treated IgM was incapable of causing agglutination, in contrast to IgG.

3.5 **Discussion**

In chapter 2 we have shown that galactosylated liposomes in saline are capable of generating an antigalactosyl immune response whose magnitudes is comparable to that obtained with Freund's adjuvant. For further
characterization of the immunological properties of galactosylated liposomes, the kinetics of the immune response and the properties of the antibodies were studied. It was noted that the antibody level in the secondary immunization was considerably higher than that of the primary immunization. The antibodies were mostly IgM in the primary response whereas both IgM and IgG could easily be detected after the secondary immunization. The majority of the antibody population was of the IgM class. It is known that the hapten coupled liposomes can act as T-independent immunogens in mice, eliciting only IgM antibodies (348,232). Haptenic molecules on liposomal surfaces can trigger B-cells directly without requiring T-cells. Production of IgM response is widely held to be a criterion of T-independent immunogens, although there are exceptions. For example, DNP-Lys-Ficoll, a T-independent antigen, can produce antibodies of other classes in addition to IgM (349, 350). DNA-liposomes also give rise to antiDNP IgM and IgG antibodies in guineapigs (221). It may be added that rabbit antibodies against isomaltosyl oligosaccharides obtained through liposomes are both of IgM and IgG types (243). Our observation of enhanced antibody levels in the secondary immunization, compared to the primary response, suggests the existence of immunological memory for galactosylated liposomes. Induction of IgM memory to tripeptide enlarged haptens, 3-(p-azobenzenearsenonate)-N-acetyl-L-tyrosylglycylglycine and N-(2,4-dinitrophenyl)-β-alanylglucylglycine, coupled to liposomes was described in mice (351). However, Yasuda et al. (348) did not observe such an effect in DNP-Coupled liposomes, where the antibody levels in primary and secondary immunization remained similar. An augmented secondary immune response against proteins entrapped in liposomes has been described (245,297). These antigens however act as the T-dependent type (245). The ability of liposomes to generate immunological memory for saccharide determinants may be
useful in the production of bacterial vaccines. The property of antigalactosyl antibodies to interact with intact cells was studied. IgM and IgG fractions do not agglutinate normal rabbit and human erythrocytes. The inability of the antibodies to agglutinate A and B blood groups comply with the fact that the antibodies did not recognize the α-galactosyl moiety. The action of neuraminidase which is assumed to expose the penultimate galactosyl moiety by removing sialic acid (352) on the erythrocytes did not make them agglutinable. On the other hand, the antibodies could easily agglutinate trypsinized cells. The interaction of the antibodies with the trypsinized erythrocytes becomes higher presumably because the trypsinization enhances the accessibility of the cell surface ligands for antibody binding. It is relevant to note that a β-D-galactoside specific lectin from the electric organ tissue of Electrophorus electricus (353) or antigloboside antisera/ could agglutinate only the trypsinized erythrocytes.