CHAPTER - 6

PRODUCTION OF SUGAR SPECIFIC ANTIBODIES (ANTIGALACTOSYL ANTIBODIES) USING HAPtenated LIPOSOMES WITHOUT THE INVOLVEMENT OF AN AROMATIC SPACER GROUP
6.1 Summary:
Nε-aminocaproyl-β-D-galactopyranosylamine was coupled to phosphatidylethanolamine containing liposomes by glutaraldehyde. The galactosylated liposomes when injected into rabbits in saline elicited an immune response specific for D-galactose. This method should prove useful in raising antisera specific for other saccharide haptens or polysaccharide antigens without employing Freund's adjuvant.

6.2 Introduction:
Immune response to haptenated liposomes has been studied extensively by the pioneering works of Kinsky et al. (216). Carrier and adjuvant role of liposomes in eliciting antibodies specific for carbohydrate determinants has been described in chapter 2. Aminophenyl derivatives of monosaccharides were conjugated to phosphatidylethanolamine containing phosphatidylcholine (lecithin) liposomes by glutaraldehyde. The glycosylated liposomes were found to generate a hapten specific humoral immune response in rabbits and the kinetics and nature of immune response have been studied as described in chapter 2 and 3. The noted adjuvanticity might be due to the presence of aromatic moiety of the glycosides since it is known that the aromatic amino acid residues contributes in the immunogenicity of synthetic polypeptides (384,385). In order to resolve this, non-phenylated glycosides were coupled to liposomes by glutaraldehyde under the similar conditions. Herein the immunogenicity of such liposomes is described.

6.3 Materials and Methods:
6.3.1 Reagents:
Nε-aminocaproyl-β-D-galactopyranosylamine and other saccharide ligands were obtained from Sigma Chemical Co., USA. All other reagents used
were of analytical reagent grade.

6.3.2 Coupling of N\textsubscript{6}-aminocaproyl-\(\beta\)-D-galactopyranosylamine to liposomes (galactosylated liposomes)

Liposomal suspension (Lecithin : Cholesterol : dicetylphosphate: phosphatidylethanolamine = 7:2:1:2, molar ratios) in 20 mM sodium phosphate buffer, containing 150 mM NaCl, pH 7.4 (PBS) was prepared as described in chapter 2. Aminocaproyl-\(\beta\)-D-galactopyranosylamine (15 mg) was added followed by slow addition of glutaraldehyde (15 mM final concentration). Uncoupled reagents were removed by dialysis against PBS. The extent of conjugation was calculated by titration of free amino groups of phosphatidylethanolamine with trinitrobenzene sulphonic acid, as described in chapter 2.

6.3.3 Preparation of antiserum:

A set of rabbits (New Zealand white strain; four numbers) were immunized subcutaneously with galactosylated liposomes (Each animal given 1.5 ml liposomal suspension containing 56 mg lipid and 3 mg sugar) in saline. Three injections were given 12 days intervals. The sera were collected by cardiac puncture seven days after the last injection and stored at -20°C after decomplementing at 56°C for half an hour.

6.3.4 Immunochemical techniques:

Preparation of Gal\textsubscript{30}-BSA: \(p\)-Aminophenyl-\(\beta\)-D-galactopyranoside coupled to bovine serum albumin (BSA) was used as test antigen (Gal\textsubscript{30}-BSA) as described in chapter 4.

Immunodiffusion: Double diffusion in 1% agarose gel was carried out as described by Ouchterlony (337).

Quantitative precipitation and hapten inhibition: These have been done as described in chapter 2. In brief antiserum (50 \(\mu\)l) was mixed with varied
Fig. 36  Immunodiffusion of antigalactosyl antiserum raised through galactosylated liposomes against the synthetic conjugate (Gal$_{30}$-BSA) (2 mg/ml in PBS). Antiserum was added in the wells 1 and 2; Well 3 contained normal rabbit serum. Central well (C) had the Gal$_{30}$-BSA.
TABLE - 13

PERCENT INHIBITION OF ANTIGALACTOSYL ANTISERUM (50 μl) PRECIPITATION WITH Gal30-BSA (300 μg)

<table>
<thead>
<tr>
<th>Ligands (50 mM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE-aminocaproyl-β-D-galactopyranosylamine</td>
<td>33</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>30</td>
</tr>
<tr>
<td>Methyl-β-D-galactopyranoside</td>
<td>41</td>
</tr>
<tr>
<td>p-Aminophenyl-β-D-galactopyranoside</td>
<td>26</td>
</tr>
<tr>
<td>p-Aminophenyl-α-D-mannopyranoside</td>
<td>5</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>38</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine</td>
<td>26</td>
</tr>
<tr>
<td>Lactose</td>
<td>37</td>
</tr>
<tr>
<td>Melibiose</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 37. Quantitative precipitation curve of the antagalactosyl antiserum (50 μl) raised through galactosylated liposomes with the Gal$_{30}$-BSA conjugate.
amounts of Gal₃₀-BSA in a final volume of 250 µl. After preliminary incubation at 37°C for 1 h, the tubes were incubated at 4°C for 4 days with gentle agitation daily. The precipitates were analyzed for total protein according to Lowry et al. (336). Fifty mM concentrations of various ligands were used to inhibit the interaction of antiserum (50 µl) and Gal₃₀-BSA (300 µg) in a final volume of 250 µl.

Spectrophotometric agglutination of galactosylated liposomes: This is carried out according to Hamers et al. (341). In brief, antiserum (100 µl) was added to galactosylated liposomes (10 µl) in 1.5 ml PBS in a cuvette and the increase in absorbance with time was determined at 600 nm in a Beckman model 24 spectrophotometer. Normal rabbit serum was used as a control.

6.4 Results:

Antiserum raised against galactosylated liposomes was checked in immunodiffusion with Gal₃₀-BSA (Fig 36). It is seen that the antiserum gives precipitin line whereas the normal rabbit serum does not. Fig 37 depicts the quantitative precipitin reaction of the antiserum with Gal₃₀-BSA. The specificity of the antiserum was determined by the ability of various ligands to inhibit the precipitation of Gal₃₀-BSA (Table 13). D-Galactose and its derivatives were found to be active as inhibitor. The highest inhibition was noted with methyl-β-D-galactopyranoside followed by D-Galactose and lactose. N-α-aminocaproyl-β-D-galactopyranosylamine and D-lactose were able to inhibit almost equally. D-mannose did not inhibit the precipitation of Gal₃₀-BSA, although a feeble inhibition was noted with p-aminophenyl-α-D-mannopyranoside. The antiserum was able to agglutinate the galactose coupled liposomes as studied spectrophotometrically (Fig 38). Normal rabbit serum failed to cause any agglutination. Similarly control liposomes without galactose ligand could not be agglutinated by the antiserum.

6.5 Discussion:

Antibodies against saccharide determinants were raised generally
Fig. 38 Agglutination of galactosylated liposomes (10 μl) with the antigalactosyl antiserum (100 μl) at 600 nm.
by conjugating aromatic derivatives of glycosides to protein carriers (48,386,44) although coupling by other methods are also available (29). In these works antibodies generated recognize the aromatic moiety of the aglycone significantly (48). In other words, aromatic residue constitute as a major determinant in the coupled saccharide hapten. The antibodies raised thus cross react with other heterologous haptens having aromatic residues. In chapter 2 a method to raise carbohydrate specific antibodies by coupling phenylated glycosides to liposomes has been described. Adjuvant activity of liposomes was noticed in such cases. The aim of this work is to couple saccharide haptens without having an intervening phenyl moiety to liposomal surface and to check for the adjuvanticity of liposomes. Results presented here show that galactose coupled liposomes as prepared could induce hapten specific immune response. Appreciable response was observed in the absence of any added adjuvants. Liposomal membrane is evidently acting as the adjuvant. The adjuvanticity of liposomes as noted could not be thus ascribed fully to aromatic phenyl residues of saccharide haptens as described in chapter 2. A lesser inhibition was noticed with aminophenyl-\(\beta\)-D-galactoside compared to methyl-\(\beta\)-D-galactoside or D-galactose while elucidating the specificity of antigalactosyl antiserum. Thus the antiserum did not cross react significantly with aminophenyl-\(\alpha\)-D-mannoside. This is in contrast to our previous observations (chapter 2) with an antiserum raised using aminophenyl-\(\beta\)-D-galactoside as the hapten coupled to liposome. A feeble inhibition was noticed with melibiose in contrast to lactose suggesting the preference of antibody for \(\beta\)-anomeric specificity.

Wood and Kabat conjugated isomaltosyl oligosaccharides to stearylamine by reductive amination and incorporated the stearyl-oligosaccharides into sphingomyelin liposomes (243). They did not however observe any adjuvant effect
of these haptenated liposomes. Antibodies were elicited only with the use of Freund's adjuvant.

Glutaraldehyde is regularly used as a common bifunctional reagent (387). It exists as oligomer at the pH in which the coupling is carried out (pH 7.4). The coupled products which are formed are often complex. Cross linking of proteins by glutaraldehyde often involves a quaternary pyridinium types of intermediate (388). It has been recently shown that the treatment of rabbit and mouse IgG with glutaraldehyde confers new antigenic determinants and the treated IgG's are immunogenic in homologous species (389). In this case, crosslinking was achieved between the primary amino groups of phosphatidylethanolamine in liposomes and \( \text{N}^\varepsilon\text{-aminocaproyl-}]^\text{D}-\text{galactopyranosylamine.} \) Although the spacer arm in galactose coupled liposomes was not characterized chemically it was shown to elicit a hapten specific immune response. This procedure requires the presence of a free-NH\(_2\) group (primary) in carbohydrates in order to couple to the liposomal surface and to produce carbohydrate specific antibodies. It may find applications especially in those cases where the use of toxic adjuvants is to be avoided.