Liposomes as adjuvant for combination vaccines

Neeraj Mishra, Prem N Gupta, Sunil Mahor, Kapil Khatri, Amit K Goyal & Suresh P Vyas*

Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences
Dr Harisingh Gour Vishwavidyalaya, Sagar 470 003, India

Received 17 March 2006; revised 18 October 2006

In the present study tetanus toxoid (TT) loaded liposomes and diphtheria toxoid (DT) loaded liposomes were prepared by reverse phase evaporation method and after combining these two vaccines the potential advantages were investigated. Prepared systems were characterized for the size, shape and entrapment efficiency. SDS-PAGE analysis of TT and DT was also performed. The selected liposomal formulations were administered subcutaneously to Balb/c mice and their immune responses were determined using ELISA after 15, 30, 45 days. After boosting the maximum immune response was observed after 45 days and was found to be 0.831 and 0.749 for TT loaded liposome and DT loaded liposomes respectively. When the mice were immunized subcutaneously with the physical mixture of TT loaded liposomes and DT loaded liposomes the immune response for the combination vaccine was found to be 1.44 and 0.741 for the TT and DT respectively. The result showed that the immune response of TT increased when it was combined with DT in liposomes. This confirms adjuvancy of DT vis-a-vis immunogenicity. Thus, carrier mediated cocktail vaccination holds promise for clinical applications.

Keywords: Adjuvants, Combination vaccines, Diphtheria toxoid, Liposomes, Tetanus toxoid

Vaccination is one of major achievement of modern medicine. Vaccination represents one of the most cost-effective preventive measures against illness and death from infectious diseases. As a result of vaccination, disease such as polio, tetanus, diphtheria and measles have been controlled. However, despite these successes, there are still many microbial diseases that cause tremendous suffering because there is no vaccine available or the available vaccines are inadequate. One of the major impediments to ensuring vaccines efficacy and compliance is that of delivery. Presently most vaccines are given by intramuscular administration. Unfortunately this is often traumatic, especially in infants. Thus, it may be beneficial to replace multiple intramuscular immunization by single dose combination vaccine which may improve overall patient compliance. Attention has been given to the development of vaccines that can be used to stimulate immune defenses in-patient after they have been infected/colonized with a pathogen or even after they developed a disease.

An important issue in the immunization of adult against diphtheria is its reactogenicity. It is believed that diphtheria toxoid can induce local and occasionally, general reactions in previously sensitized (immune) individual. This problem may be largely overcome by using mixture of tetanus toxoid (TT) and diphtheria toxoid (DT). Parenteral route is one of the most effective routes for the administration of vaccine as administration via enteric route leads to loss of antigenicity due to gastric microenvironment and large doses are to be administered to bring about protective immunization. Liposome serves as carrier of antigens and adjuvant. Liposome encapsulation could offer two advantages: (i) it reduces local reaction of both antigen and the release of antigen in a sustained manner; (ii) it curtails the necessity of repetitive booster dose administration. The immunological adjuvant properties of liposome extend to a large array of antigens from sources as diverse as bacteria, protozoa, viruses, tumors, spermatozoa and venoms.

Combination vaccine merge into a single product antigens that prevent different disease or that protect against multiple strain of infectious agent causing the same disease. Thus, they reduce the number of injections required to prevent some disease. Combination vaccine may include increasing number

*Correspondent author:
Phone: +917582265525
Fax: +9175822265525
E-mail: vyas_sp@rediffmail.com
of components in different array to protect against other disease. Other potential advantages of combination vaccine include; reduction in the cost of stocking and administering separate vaccine, reduction in the cost for extra health–care visits, facilitating the addition of new vaccine into immunization programmes, safety and efficacy of administering combination vaccine to patient who may already be fully immunized for one or more components. The use of combination vaccine is a practical way to overcome the constrains of multiple injections, especially for starting the immunization series for children. The use of combination vaccine may improve timely vaccination coverage.

The aim of the present work is to develop TT loaded liposomes and DT loaded liposomes and to investigate the potential advantage after combining these two vaccines. The prepared systems are characterized for size, shape and entrapment efficiency. The possible effect of preparation process on the integrity of the antigen is assessed by SDS-PAGE. Immunization of the Balb/c mice was done with various TT and DT loaded formulations and immune response is shown in terms of absorbance at 450 nm.

Materials and Methods

Soya phosphatidylcholine (SPC) and cholesterol (CH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protein estimation kit (by BCA method) and ELISA kit were purchased from Genei, Bangalore, India. All solvents used were of analytical grade. Tetanus toxoid (TT) and diptheria toxoid (DT) was obtained as gift sample from Serum Institute of India, Pune. TT solution contained 3600 LF/ml and DT solution 3500 Lf/ml. All other chemicals were of analytical grade until and used as procured.

Preparation of antigen loaded liposomes—Liposomes were prepared by reverse phase evaporation technique as reported by Sazoka and Papahadjopoulos, with slight modifications. Briefly, SPC and CH (7:3%, w/w) were dissolved in 5ml diethyl ether to which 2 ml of aqueous phase, i.e., phosphate buffer saline containing TT was added. The mixture was sonicated (Soniweld, India) for 5 min at 4°C over ice bath. A thick emulsion was formed, which was then kept over a vortex mixer in order to remove any residual ether. To this emulsion 3 ml PBS was added in order to hydrate the vesicles. Similarly, DT loaded liposomes were prepared by the same method. TT and DT encapsulated liposomes were mixed in equimolar proportions to yield a physical mixture of liposomes encapsulating TT and DT.

Morphology and size determination—Prepared vesicular systems were characterized for shape using optical microscope and transmission electron microscope (JEM-200 CX, JEOL, Tokyo, Japan). One percent phosphotungstic acid (PTA) was used as negative stain. The particle size of the prepared vesicular formulations was measured by photocorrelation spectroscopy with an Autosizer IIC apparatus (Malvern Instruments, UK).

Entrapment efficiency—Prepared vesicular formulations were separated from the free (unentrapped) antigen by using a Sephadex G-150 minicolumn using centrifugation technique as described by Fry et al. The method was repeated thrice with a fresh syringe packed with gel each time. The fraction was finally collected which was free from unentrapped antigen. The vesicular fraction was added with minimum amount of triton ×100 (0.1%, v/v) to disrupt the vesicles and the liberated antigen was estimated using a bicinchoninic acid (BCA) protein assay kit and percent antigen entrapment was calculated.

Gel electrophoresis—Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the stability and integrity of antigen during the preparation of liposomal systems. Antigen was extracted by dissolving the liposomal systems in 2 ml of triton ×100 (0.1% v/v). The extracted antigen was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 10% separating gel at 200 V (BioRad, Hercules, CA) until the coomassie dye stained protein band reached the gel bottom.

In vivo animal studies—Balb/c mice, aged 6-8 weeks, weighing 15-20 g were used for in vivo studies. Animals were housed in 7 groups, each group consisting of 6 mice, with free access to food and water. The Institutional Animals Ethical Committee of Dr Harisingh Gour University approved the study. The studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Each mouse was immunized, SC, with an equivalent dose of 5 LF TT, DT and mixture of both followed by booster dose after days 15 with the same formulations.
**Determination of IgG titre by ELISA**—Specific anti-IgG antibody level in the serum was determined by ELISA as described previously\(^\text{14,15}\). Tetanus toxoid (100 µl; 10 µg/ml in phosphate buffer, pH 7.4) was coated to each well of Nunc-Immuno plate. The plate was incubated at 4°C overnight. Plate was then washed thrice with PBS tween-buffer. BSA (100 µl; 2%) was added in each well and plate was incubated for 2 h at room temperature and washed thrice with PBS-tween. Diluted serum sample (100 µl) was added to each well and incubated for 2 h at room temperature. Plate was washed three times with PBS-Tween (PBS-T) buffer. Diluted horse-radish-peroxidase-conjugated antoglobulin specific anti-rat IgG (100 µl) was added to each well and incubated for 2 h. Plate was again washed three times with PBS tween buffer and then 100 µl of substrate solution 3,3’, 5,5’-tetramethyl benzidine containing hydrogen peroxide was added to each well. Plate was incubated in darkness at room temperature for 15 min. The reaction was stopped by adding 50 µl of 2M H\(_2\)SO\(_4\) to each well. The absorbance was measured at 450 nm using a microplate ELISA reader (Lab System Multiscan, Finland). Immune response was shown in terms of absorbance at 450 nm.

**Statistical analysis**—The results were expressed as mean±SD. Statistical analysis was carried out by using Student’s \(t\)-test and statistical significance was designated as \(P <0.05\).

**Results and Discussion**

Reverse phase evaporation method developed by Szoka and Paphajopoulos,\(^\text{11}\) is simple and convenient way to make multilamellar vesicles (MLV) with fairly good entrapment efficiency. Liposomes were characterized for their shape using transmission electron microscopy (Fig. 1) and optical microscopy (Figs 2 and 3). The morphological characteristics of TT loaded liposomes, DT loaded liposomes and mixture of TT and DT encapsulating liposomes were similar as studied by optical microscopy. Prepared liposomes were optimized for soya PC: cholesterol ratio in term of entrapment efficiency (Table 1 and 2). The optimum ratio was found to be 7:3. Maximum entrapment efficiency for TT and DT loaded liposomes was found to be 38.7 ± 2.3 and 39.8 ± 2.3% respectively.

SDS-PAGE was performed for different liposomal formulation and soluble mixture of antigens to characterize them and to have an insight into the effect of preparation condition on the integrity of the antigen. The vertical gel electrophoresis under denaturing condition (0.1 % SDS), separate proteins based on their molecular weight or size, as they moved towards the anode. Antigen from TT loaded liposomes, DT loaded liposomes, plain TT and plain
DT in PBS, pH 7.4 were resolved using coomassie blue stained band on the 10% SDS-PAGE gel. The integrity of the antigen was maintained during the liposomes preparation as no band was observed for the degradation product of antigen. The bands of the encapsulated antigens were found to be similar to those of the native antigen.

The maximum immune response was observed after 45 days and was found to be 0.376 ± 0.071 and 0.359 ± 0.078 for liposome loaded with TT and DT respectively. When the liposomes bearing TT and DT were physically combined the immune response obtained towards TT and DT was found to be 0.534 ± 0.088 and 0.362 ± 0.029 respectively (Fig. 4). This may be attributed to good immuno-adjuvant action of liposome, which are reported to enhance the immunogenicity of a soluble antigen or acts as a carrier for a hapten. Further, association of poorly immunogenic protein antigens with liposomes can mediate a substantial enhancement of the antibody response in terms of an increased number of antibody forming cells as well as elevated circulating antibody titre. The immune response of liposomal entrapped mixture TT and DT is found to be greater than individual response of TT or DT.

Immune response was boosted on day 15. Boosting with same formulation increase the immune response. High level of antibodies after boosting indicates the presence of memory B and T cells population evoked by primary immunization. After boosting the maximum immune response was observed after 45 days and was found to be 0.831 ± 0.071 and 0.749 ± 0.078 for liposome loaded with TT and DT respectively. When TT loaded liposomes and DT loaded Liposomes were physically mixed, the immune response in the combined vaccine towards TT and DT was found to be 1.144 ± 0.088 and 0.741 respectively (Fig. 5). Immune response was found to be almost sustained after day 30 with very gradual increase in increased response after boosting. The results clearly favor liposomes as good parental antigen carrier in evoking immune responses. Additionally the potential of combined vaccines has been reflected in terms of enhanced immune response for TT and could reduce reactogenicity of DT.

**Conclusion**

The results obtained showed that liposomes are capable of inducing effective immune response

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Soya PC: Cholesterol (mole ratio)</th>
<th>Entrapment efficiency (mean ± SD; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>9:1</td>
<td>34.2 ± 2.4</td>
</tr>
<tr>
<td>T2</td>
<td>8:2</td>
<td>37.5 ± 2.0</td>
</tr>
<tr>
<td>T3</td>
<td>7:3</td>
<td>38.7 ± 2.3</td>
</tr>
<tr>
<td>T4</td>
<td>6:4</td>
<td>36.2 ± 2.2</td>
</tr>
<tr>
<td>T5</td>
<td>5:5</td>
<td>34.5 ± 2.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Soya PC: Cholesterol (mole ratio)</th>
<th>Entrapment efficiency (mean ± SD; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>9:1</td>
<td>33.4 ± 2.7</td>
</tr>
<tr>
<td>D2</td>
<td>8:2</td>
<td>36.5 ± 2.8</td>
</tr>
<tr>
<td>D3</td>
<td>7:3</td>
<td>39.8 ± 2.3</td>
</tr>
<tr>
<td>D4</td>
<td>6:4</td>
<td>36.8 ± 2.7</td>
</tr>
<tr>
<td>D5</td>
<td>5:5</td>
<td>33.5 ± 2.3</td>
</tr>
</tbody>
</table>

**Table 1**—Optimization of liposomal formulation containing TT with different molar ratio of Soya PC and cholesterol

**Table 2**—Optimization of liposomal formulation containing DT with different molar ratio of Soya PC and cholesterol

**Fig. 4**—Immune responses developed in balb/c mice at different time intervals without boosting (n=6)

**Fig. 5**—Immune responses developed in balb/c mice at different time intervals following boosting on day 15 (n=6)
following subcutaneous immunization. The immune response of TT increased when combined DT in liposome. This confirms the adjuvanticity of DT vis a vis immunogenicity. Thus it holds promise for clinical application.

Acknowledgement
SM, PNG and KK are grateful to CSIR, New Delhi, AICTE, New Delhi and LTMT, Mumbai respectively for providing financial assistance.

References
14 Esparza I & Kissel T, Parameter affecting immunogenicity of microencapsulated tetanus toxoid, Vaccine, 10 (1992) 714.