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ASSESSMENT OF CELL MEDIATE IMMUNE RESPONSES AFTER ADMINISTRATION OF LIPOSOMAL STRONG IMMUNOMODULATORY ANTIGEN OF *B. MALAYI*

Neeraj Jain*, Neelam Jain[1], Jagat Pal Yadav[2], Shubhrat Maheshwari[3], Lakshmi Mishra[4]

*Teerthankar Mahaveer College of Pharmacy, TMU, Moradabad- 244001, U.P., India.

[1]Faculty of Pharmacy, Oriental University, Indore-453555, Madhya Pradesh, India.

[2]Department of Pharmacology, Kamla Nehru Institute of Management and Technology, Faridipur, Sultanpur-228118, U.P., India

[3]Rajarshi Rananjay Sinh College of Pharmacy, Amethi-227405, U.P., India.

[4]Faculty of Pharmaceutical Sciences, Rama University, Kanpur, 209217, U.P., India.

***Corresponding author name:** Neeraj Jain

Address: Teerthankar Mahaveer College of Pharmacy, TMU, Moradabad- 244001, U.P., India.

Email id: jneerajdops@gmail.com

Contact no: +91 8462933618

Abstract

Objective: The present study was aimed on developing and characterizing Liposomal Delivery System loaded with antigen of filaria parasite *B. malayi* extracted protein for assessment of Cell Mediate Immune responses of antigen.

Methods: Liposomes were prepared by Reverse Phase Evaporation (REV) method (Szoka and Papahadjopolus; 1978) with slight modification using molar ratio of Soya PC:PE:Cholesterol in different molar concentration.

Results: NO release from peritoneal macrophages of the animals (Gr.I, II, III, IV and V) was increased by exposure to LPS or no exposure to any stimulants in-vitro as compared to cells of non-immunized animals (Gr.V). In summary, F6 was able to induce greater NO production. The TNF-α release in cells of F6 immunized animals was elevated in response to F6, LPS or no stimulation in-vitro over non-immunized ones. The IFN-γ release in cells of F6 immunized animals was elevated in response to F6 or without any stimulation in-vitro in comparison to non-immunized ones. Up-regulation in Th-I responses and down-regulation in Th-II responses show that the immunological cytokines were in function and cause triggers to body immunity to destroy the parasite, the cytokines production checked at mRNA transcription level using RT-PCR.

Conclusion: These results suggest that the liposomal antigen delivery system shows Th-1/Th-2 promising responses towards vaccine development.

Keywords: Th-1/Th-2 responses, cytokinens, IFN –γ, BmAFII, , B. malayi, TNF-α, RTPCR liposomes.

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1. INTRODUCTION

The World Health Organization (WHO) ranks it as the second most common cause of long-term disability and estimated that over 1.25 billion people are at risk of the infection in 83 countries and territories (WHO, 2006a). Approximately 125 million already have been infected with LF, and over 40 million (WHO, 2004a) are seriously incapacitated and disfigured by the disease

The methods to control and prevent the filarial infection include administration of antifilarials alone or combination of diethylcarbamazine (DEC)/ivermectin and albendazole and exposure control programs. In recent years, identification of several filarial antigens/proteins or molecules raised hopes for developing vaccines (Gregory et al., 2000; Ramachandran et al., 2004; Krithika et al., 2005; Babayan et al., 2006; Vedi et al., 2008; Sahoo et al., 2009; Shakya et al., 2009; Joseph et al., 2012) against lymphatic filariasis.

Novel adjuvants have been developed for enhancing antigen delivery and reducing the vaccine delivery to a single injection. For future human use, it is however necessary to use an adjuvant that is safe, biodegradable and which does not require repeated administration to produce the desired result.

Studies conducted in laboratory revealed that of the two major Sephadex G-200 eluted fractions of *B. malayi* adult worm extract, BmAFII is protective *in-vivo* and stimulates predominantly pro-inflammatory cytokines to both adult worms and L3 while BmAFI facilitates parasite survival and stimulates predominantly IL-10 release (Dixit et al., 2004; Dixit et al., 2006). Further, to narrow down to molecular entities that have cytokine release stimulating potential, *B. malayi* adult worm extract was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved fractions were blotted onto NCP and screened for the cytokine release potential. Some NCP-bound molecules corresponding to the molecular weight range of BmAFI and BmAFII, were found to possess proinflammatory and mixed pro- and anti-inflammatory cytokine stimulating potential *in-vitro* and provided a starting point for precisely identifying the functional molecules/proteins of interest (Dixit et al., 2004).

Based on the above findings 6 molecular fractions (F1, F2, F5, F6, F10 and F14) were studied and F6 was selected for the present study to find out which of these fractions have the molecules potentially relevant to host protection or filarial pathology. The present work was aimed at characterizing parasite molecules with respect to their immunogenicity, involvement in the pathology of filariasis and to check it’s potency via novel carrier delivery system.

Liposomes have been used in drug delivery for many years. Liposomes, discovered in 1965 by Bangham and his colleagues are spherical colloidal particles containing an aqueous core surrounded by phospholipid bilayer which replicates cell membrane 10. Liposomes are microscopic lamellar structures formed on the admixture of soya lecithin with subsequent hydration in aqueous media. Liposomes have been widely evaluated for controlled and targeted drug delivery for treatment cancer, viral infections and other microbial diseases.

Liposome vaccines have several advantages, including sustained release, prevention of local irritation, reduced toxicity, improved stability in the large aqueous core, and the possibility to manipulate release and targeting by altering the bilayer constituents and changing the preparation technique. The drug

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carrying capacity, release rate, and deposition of liposomes is dependent on the lipid composition, size, charge, drug/lipid ratio, and method of delivery. Conventional liposomes are composed of neutral or anionic lipids (natural or synthetic). The most commonly used are the lecithins (phosphatidylcholines), phosphatidylethanolamines (PE), sphingomyelins, phosphatidylserines, phosphatidylglycerols (PG), and phosphatidylinositols (PI). Dry powder liposomes have been produced by lyophilization followed by milling, or by spray-drying.

There is an immediate need for the development of new and improved adjuvant and delivery system, which are potent, safe and can be used as a new generation vaccine. In the present study it is thought worthwhile to prepare liposomal-system having the potential benefits of reducing the number of dosages for primary immunization, reducing the total antigen dose required for effective immunization, enhancing both humoral and cell-mediated immune responses over a longer period of time, enabling combined vaccine administration and permitting effective primary or booster immunization. In addition, the integrity of the antigen is maintained by avoiding the use of organic solvents and a pH changes, preparation process is simple and easy to scale up for chemical studies and eventual manufacture.

The surface antigen(s) have important role in generation of protective immunity. Consequently, characterization of protective responses generated by surface antigen(s) that can be used as vaccine is worth considerable. A few body wall antigens have earlier protective and are potential vaccine candidates against filarial infections. The purified native protein or recombinant filarial protein might be more useful for achieving the desired immunity.

Therefore, the present study was aimed to isolate the purified native protein of parasite and to prepare novel liposomal system in optimized ratio which would enhance desired immune response with minimum toxicity and characterize the prepared dosage form in terms of size, shape and adsorption capacity to determine optimum dose and also access immunoadjuvancity of prepared dosage form in antigen dose reduction by using different immunization protocols.

Materials and methods

PC:PE:CH were generous gift from Lipoid, Germany. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were Sigma, USA. All other chemicals and reagents are available at in-house facility of CDRI, Lucknow.

Antigen isolation

Parasite

For laboratory experimental purpose, Brugia malayi, having many of the biological aspects similar to Wuchereria bancrofti, was used in the study. It is a sub periodic strain of human filarial infection and has successfully been transmitted to various vertebrate hosts including monkeys, cats and rodents. The infection is transmitted through black eyed susceptible strain of Aedes aegypti mosquitoes developed by McDonald (Liverpool School of Tropical Medicine and Hygiene, U. K.).

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Animal models

Rodents are preferred models for laboratory studies throughout the world. 'GRA' strain (Germany) of Mastomys coucha (belonging to family Muridae) as shown in Figure 1 is susceptible to B. malayi and is being maintained in the animal house of Central Drug Research Institute, Lucknow, India since last 35 years. It is a multimammate, prolific breeder with average litter size of 8 - 10 babies. The female may have her young at any time of the year and if conditions are right, may do so regularly at intervals of 33 days (Wilson and Reeder, 1993). M. coucha model is found amenable to perform chemotherapeutic and immunobiological investigations in experimental filariasis (Mukhopadhyay et al., 1996; Tyagi et al., 1998). Keeping in view their similarity of immune responses to human, this animal is used as model for experimental purpose (Dixit et al., 2004; Gaur et al., 2007; Khan et al., 2004). Another rodent, the Mongolian gerbil (Meriones unguiculatus, family Gerbillinae) as shown in Figure 1 has proven to be an excellent permissive rodent model for the study of lymphatic filariasis using B. pahangi or B. malayi (McVay et al., 1990). The animal (called 'jird') is used for the propagation of B. malayi parasites (McCall et al., 1973). All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. The animals are kept in plastic cages and were housed in animal quarters under controlled climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light-dark cycles). They were fed with standard rodent diet pellets and had free access to drinking water.

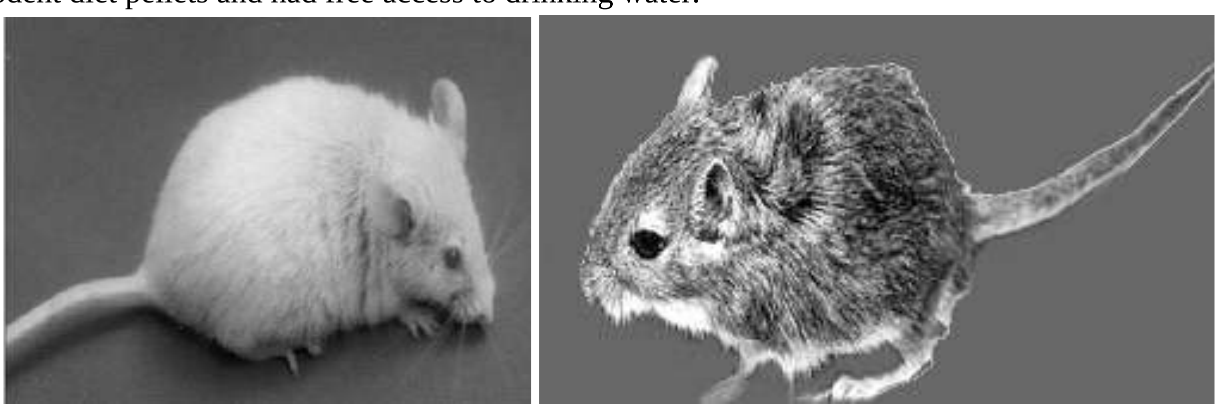

Fig. 3.1: Experimental Filarial models – *Mastomys coucha* **&** *Meriones unguiculatus*.

Maintenance of *B. malayi* infection

Rearing and breeding of A. aegypti colony

In the laboratory the mosquitoes were reared and bred in an insectarium maintained at controlled temperature (26 ± 1°C) and humidity (80 ± 5%). The adult mosquitoes were kept in nylon mesh cages and provided 10% glucose solution with vitamin B supplement, soaked in cotton for feeding. From time to time female mosquitoes were fed on normal *M. coucha* blood to promote egg laying. A beaker containing water was kept in the cage for egg laying. The eggs layed after about 40 hrs blood feeding were filter separated and stored after drying at same temperature. Eggs can be preserved under such condition for 3 - 4 months. For maintenance of mosquito life cycle the eggs were transferred to enamel bowl containing tap water. The larvae hatch out in the water within 24 hrs and these were provided

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with feed containing dog bix and yeast powder. The larvae usually took about 8-10 days to become pupae, which ultimately developed into adult mosquitoes within 48 hours.

Feeding of mosquitoes on mf positive M. coucha

B. malayi infected *M. coucha* showing 100-200 mf/10 µl of blood were used as donors. The feeding of mosquitoes on donors was carried out between 12 noon and 1:00 PM (peak microfilaraemia time). Mosquitoes starved for 2-3 hrs were allowed to feed on the donors, which was kept inside the mosquito cage in a wire netting immobilized cage. After 1 hr of feeding the donor animal was removed and mosquitoes were provided with glucose solution as mentioned above. In 9 - 10 days time the mf in the mosquitoes developed into L_3.

Isolation of L_3 from mosquitoes

On day 9 or 10 post feeding, the mosquitoes were paralyzed and crushed gently in 4-5 ml of 0.6% insect saline (IS) and transferred to Baerman's apparatus which consisted of glass funnel, muslin cloth and transparent rubber tubing with a pinch cock. The funnel was filled with lukewarm IS. Crushed mosquitoes were then put onto muslin cloth and allowed to stand for half an hour with light provided from top by a table lamp. The L_3 released from the mosquitoes move away from light, traverse through the muslin cloth and settled down at the bottom of the tube. These were collected by opening the pinchcock and washed with IS several times to remove the mosquito debris. The larvae were counted and used immediately for exposure to animals.

Inoculation of L_3 to M. coucha or jird

For infection purpose 6-8 weeks old male *M. coucha* were inoculated with active and motile L_3 (100 per animal) subcutaneously. Establishment of successful infection was examined in blood smear after day 90 post larval inoculation and thereafter monitored at regular intervals. Animals showing desired levels of infection were used for transmission to healthy animals. Thus, the cycle was continued. Similarly in jirds of the same age group about 200 L_3 were inoculated intraperitoneally. The larvae develop into adult worms in about three months and can be harvested thereafter when required.

Preparation and fractionation of antigen

The worms were washed several times and crushed in phosphate buffered saline (PBS) in cold followed by sonication (Soniprep 150) for 10 cycles of 30 seconds each at 10 micron amplitude with intermittent gap of 1 minute. The total homogenate was mixed with equal amount of 2X sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (0.125 M Tris-HCl, pH 6.8; 4% SDS, 5% β-mercapto ethanol, 20% glycerol and 0.01% bromophenol blue) and boiled in water bath for 5 min. The protein samples thus prepared were centrifuged to remove any particulate residue before loading to gel.

Sodium dodecyl sulphate -polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used analytical method to resolve separate components of a protein mixture. SDS-PAGE simultaneously exploits differences in molecular size to resolve proteins differing by as little as 1% in their

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electrophoretic mobility through the gel matrix. In order to isolate dominating fractions which have been identified to be stimulators of pro- and mixed pro- and anti-inflammatory cytokine release (Dixit *et al*. 2004; Table 3.1) extract of adult worms was used. The extract was resolved in 10% gels (Laemmli, 1970). Preparative (13.8×13 cm slab) gels were run in gel with a dual-gel electrophoresis chamber (AE-6220, Atto Japan). Resolved fractions (six) of interest were cut with the help of pre-stained molecular weight markers run along the side. These were designated as F1, F2, F5, F6, F10 and F14. The fractions in gel strips were stored as such in gel at -10^{0}C till elution.

Table 3.1: Cytokine-release stimulating potential of NCP-bound molecules of adult *Brugia malayi* soluble extract in THP-1 cell systems

Fraction (MW kDa)	**Cytokine**
F1 (>180)	TNF-α (++), IL-10 (++)
F2 (169-180)	TNF-α (+++), IL-10 (+++)
F5 (67.8-84.3)	IL-1β (+++)
F6 (54.3-67.8)*	IL-1β (+++), IL-6 (+++), TNF- α (+)
F10 (38.44-41.84)	IL-1β (++)
F14 (17.0-22.5)	IL-10 (+)

+, ++ & +++ indicate ascending grade of predominance.

* selected for present study.

Electro-elution of proteins from gel

Proteins from gel strips were electro-eluted by micro electro eluter (Millipore, USA) as per method described by manufacturer. Briefly, about 75 % of the perforated tube was filled with SDS PAGE gel strips in small pieces. The tube carrying gel was fitted into centricon™ tubes having a membrane filter of required cut off limit. After filling both upper and lower chambers of the microeluter with Tris-glycine buffer, electricity (~200V) was applied for 2 to 4 hrs depending on the size-based mobility of the fraction ensuring near complete elution from the gels. After elution is over the centricon tube was disassembled from the slot and the gel carrying tube was removed. Protein solution retained in the centricon was centrifuged at 2000g in cold (4 °C) in an angular rotor till the volume reached to required level.

The eluted fractions were run in SDS-PAGE to confirm their molecular weight. Finally, the protein solution thus obtained was filter sterilized with 0.22µ membrane filter and stored at -20°C until used.

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ANTIGEN SELECTED FOR STUDY

Bovine serum albumin (BSA) as a model antigen

BSA is a white to light tan colored powder that contains not more than 3.0% w/w of water, containing about 96 % protein. It has a molecular weight of 67KDa. It consists of a carbohydrate free polypeptide chain connecting four globular segments of unequal size. It must be protected from light and moisture and store at temperature between 2° C and 25° C.

Brugia malayi adult worm protein extract (F6) as a candidate antigen

F6 is a sephadex G-200 eluted fraction of B. malayi adult worm extract. It has a molecular weight of 54.3-67.8 KDa. It has five proteins namely heat shock protein (HSP60), NAD dependent epimerase/dehydratase, intermediate filament (IF), elongation factor 2 (EF2), hypothetical protein CBG00623. It must be protected from light and moisture and store at temperature -20ºC.

PREPARATION OF LIPOSOMES

Liposomes were prepared by reverse phase evaporation (REV) method (Szoka and Papahadjopolus; 1978) with slight modification using different molar ratio of Soya PC: PE: Cholesterol. PC: PE: CH was dissolved in 5ml of diethyl ether. To the above solution, 3ml of phosphate buffer saline (PBS) pH 4.2 was added. The mixture was sonicated for 1.0 minute at 4ºC. The mixture was kept in a rotary vaccum evaporator; the organic solvent was removed under vacuum (260 to 400 mm Hg) at 37±1ºC until it became thick. The gel was then subjected to vigorous mechanical agitation on a vortex mixture to form a suspension of liposomes. Various liposomal formulations with increasing concentration of PE were prepared.

Table 1. Composition for liposomes.

S. No.	Formulation Code	Ratio (PC: PE: Chol)
1	CL1	8:0:2
2	CL2	7.2:0.5:2
3	CL3	**7.0:1.0:2**
5	CL4	6.6:1.4:2
6	CL5	6.2:1.8:2

Evaluation of liposomes

Antigen entrapment efficiency (% EE)

The proportion of entrapped antigen was obtained by ultracentrifugating 1 ml of the liposomal suspension at 15,000 rpm for 1 hr using a cooling centrifuge at 4^0C (Remi C-24, Mumbai, India). The liposomes were separated from the supernatant and were washed twice, each time with 1 ml distilled water, and recentrifuged again for 1 hr. The amount of entrapped antigen was determined by lysis of the separated vesicles with Triton X-100. 0.2 ml of the liposomal formulation was taken and about 0.2 ml of 1% Triton X-100 was placed into it. This was kept for 5 minutes for disrupting the vesicles. Then vesicle was centrifuged for 5 minutes at 2000 rpm. The supernatant was collected and used for

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the quantization of antigen entrapped by BCA method using U. V. spectrophotometer (UV 1700 Pharm Spec, Shimadzu, Japan) at 562 nm.

Table 5.1: **Optimization of PC: PE: Cholesterol ratio wrt size and % entrapment**

S.No.	Code	PC:PE:Chol (molar ratio)	Average size (µm)	% antigen entrapped
1	CL1	8:0:2	1.55±0.19	44.23±0.8
2	CL2	7.2:0.5:2	1.75±1.04	52.89±0.67
3	**CL3***	**7.0:1.0:2**	**2.52.±0.54**	**60.10±1.45**
5	CL4	6.6:1.4:2	1.98±0.38	54.28±0.64
6	CL5	6.2:1.8:2	1.59±0.49	49.39±0.52

CHARACTERIZATION OF LIPOSOMES

Vesicle shape

The prepared liposomal formulations were characterized for their shape using transmission electron microscopy (TEM). (Guo et al.; 2000).

Vesicle size and Size Distribution

The size and size distribution of vesicles was determined by particle size analyzer (Cilas, 1064 L, France).

Vesicle Count

Liposomal preparations were diluted 10 times with 0.9% w/v sodium chloride solution and no. of vesicle /mm^3 were counted by optical microscopic method using hemocytometer. The liposome in 80 small squares counted and total no of vesicle/mm^3 were calculated using following formula.

$$\text{Total no. of vesicles/mm}^3 = \frac{\text{Total no. of vesicles} \times 4000 \times \text{Dilution factor}}{\text{Total no. of squares counted}}$$

The observed values were recorded in table 5.3 and presented graphically in figure 5.3.

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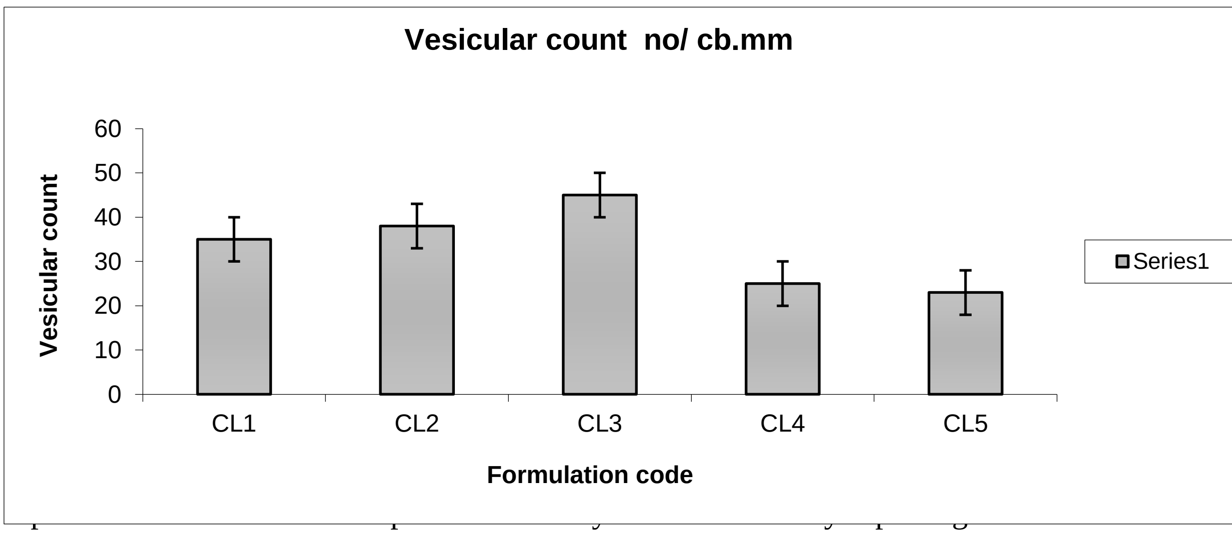
Table 5.3: Vesicle count of PC:PE:CH liposome

S. No.	**Formulation code**	Vesicle count/ mm3
1.	CL1	**35±2**
2.	CL2	**38±3**
3.	CL3	**45±4**
4.	CL4	**25±2**
5.	CL5	**23±3**

Figure 5.3: Vesicle count of PC:PE:CH liposome



(1978) mn of on X - 100 (1.0%).

Preparation of Minicolumn

Column of sephadex G-50 gel was prepared as described by Fry et al. (1978) by slight modification.

Methods:

1. Sephadex G-50 was accurately weighted 1g and swollen in 10ml of 0.9% NaCl solution for 12 hrs at room temperature with occasional shaking.
2. The barrel was plugged with whatman filter paper pad.
3. The prepared gel was filled to the top in the barrel of 1ml disposable syringe.
4. The barrel was then placed in the centrifuge tube.
5. The tubes were centrifuged at 3000rpm for 3minutes to remove excess saline.

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Purification of liposome

Method:

1. Prepared minicolumn was taken and then 0.2ml of liposomal preparation was placed on the sephadex bed.
2. The minicolumn was then spine for 3 minutes at 1000 rpm to expel the liposomal material from the column into the test tube.
3. Non-Encapsulated solute from the column was removed by washing with buffer and eluted by centrifugation at 3000 rpm.

Surface Properties of Liposomes

The change in surface property of the liposomes were evaluated by measuring the zeta potential of the prepared liposomal formulation with a zeta meter (Zetasizer, Malvern. UK) (Tatiana et. al.;2002). The observed values were presented in table-5.4 and graphically in figure 5.4.

Table 5.4: Zeta potential of different formulation of CL liposome

S. No.	Formulation code	Zeta potential(mV)
1.	CL1	-9.64±0.53
2.	CL2	-12.54±0.72
3.	CL3*	-15.04±0.47
4.	CL4	-19.32±0.34
5.	CL5	-22.21±1.28

Figure 5.4: Zeta potential of Optimized formulations

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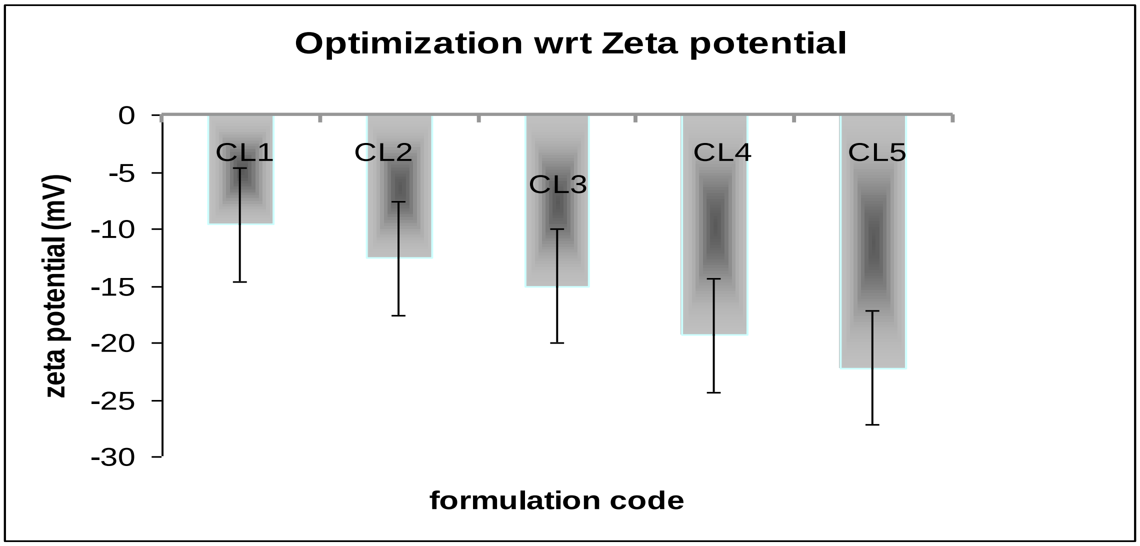

In-vivo studies

The work was carried out with an aim to develop delivery system that can deliver parenterally administered antigen in its immunologically active form to their target site to elicit immune response. In-vivo experiments were performed to evaluate the therapeutic and prophylactic effectiveness of the antigen as well as drug delivery system.

Immunization of Animals

Swiss mice (Male, 4–6-week-old and 20–25-gram body weight) were taken and protocol as approved by Institute Animal Ethical Committee of CDRI, Lucknow, India was followed. The studies were carried out as per the guidelines of Council for Purpose of Control and Supervision of Experiment on Animal (CPCEA), Ministry of Social Justice and Empowerment; Government of India. This study included five groups of animals, each group comprising of five animals. The dose administered 250.00µL of formulation administered subcutaneously (s.c.). The protocol followed as given below.

Table 8.1: Immunization protocol

S. No.	Group	Formulation administered	No. of animals
1.	I	Liposomised antigen (Single dose)	5
2.	II	Plain antigen(Single dose)	5
3.	III	Plain antigen(Double dose)	5
4.	IV	Liposomised antigen (Double dose)	5
5.	V	P.B.S.	5

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Sample Collection

The pre immune samples of serum were collected on day 0 before immunization and post immunization samples collected on day 7, 14, 21 and 28th. Blood from each mouse was taken in an eppendrof tube through the retro orbital plexus with the help of glass micro capillaries, allowed to clot and then centrifuged to separate serum. All the collected samples were stored at -20^o C until utilized. On day 28th all the animals were sacrificed and spleen and peritoneal fluid were collected for cell mediate immune response and cytokines determination. The peritoneal macrophages were isolated aseptically for Nitric-Oxide (NO) determination.

Cell Mediated Immune (CMI) response measurement:

Nitric oxide production by macrophages

Peritoneal macrophages were isolated by injecting 4-5 ml of DMEM-EDTA in peritoneum and collecting the lavage in 2-3 minutes, from various groups of animals. Peritoneal cells including macrophages thus obtained were washed thrice with same medium, suspended in complete DMEM containing 10% FBS and dispensed aseptically into 48 well tissue culture plates. After overnight incubation medium was removed along-with non-adherent cells, fresh medium was replenished, and were stimulated with the protein of fraction (F6) or LPS (1µg/ml) for 48 hrs at 37°C in 5% CO_2 atmosphere. The presence of nitrite in the media was quantified using the method based on the chromophore formation from the diazotization of sulphanilamide by acidic nitrite followed by coupling with N-(1-naphthyl) ethylene-diamine dihydrochloride (NEDD), a cyclic amine, and taking absorbance at 510nm (Thomas et al., 1997).

Table 8.3-A: NO assay, nitrate value in µmole nitrate (Mean±SD)(n=5)

Groups/ Stimulants	Formulation Group I		Formulation Group II		Formulation Group III		Formulation Group IV		Formulation Group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPS	22.13	0.25	22.04	0.95	25.01	0.90	76.46	5.95	20.52	1.02
F6	27.26	2.61	25.86	2.27	28.98	2.17	77.38	1.51	24.42	1.86
Unsti.	20.61	0.60	20.43	0.45	20.45	0.39	64.97	1.32	24.88	0.36

Fig 8.3-A: NO assay, nitrate value in µmole nitrate (Mean±SD)(n=5)

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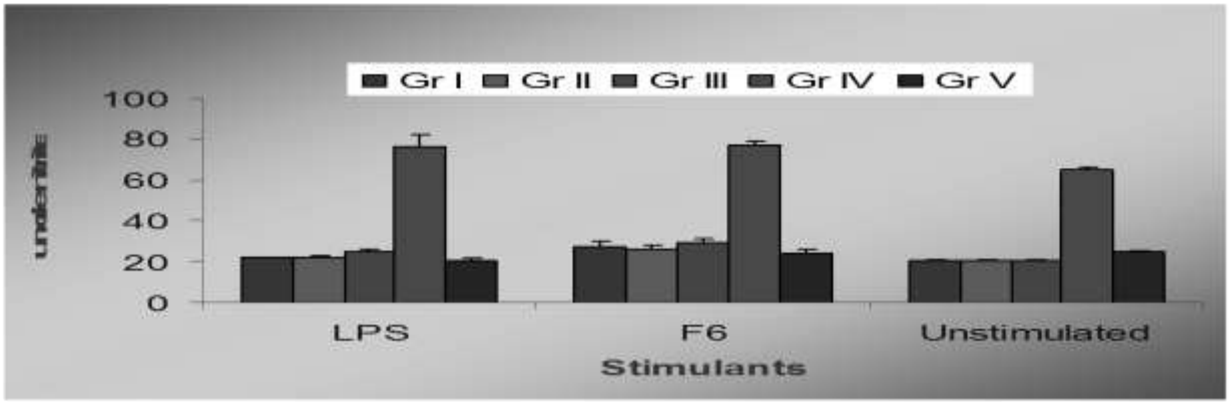

Lymphocyte Transformation Test (LTT)/T-cell proliferation assay:

Isolation of splenocytes

On autopsy, spleen were collected from animals, teased in sterile RPMI-1640 medium containing antibiotics (streptomycin: 100µg/ml and penicillin: 100U/ml) and passed through 70µM cell strainer to get cell suspension. The suspension was hemolyzed osmotically by treating with 0.87% NH4Cl for 1-2 min at 4°C, washed thrice and suspended in complete RPMI supplemented with 10% Fetal bovine serum (FBS). Viability of the cells was checked by 0.1% trypan blue exclusion method, counted using haemocytometer and final cell concentration adjusted to 2 × 106 cells/ml. The splenocytes thus obtained were used for lymphocyte transformation test and cytokines stimulation assay. Lymphocyte transformation test was carried out by using spleen cells (Klei et al., 1990). Cell suspension was dispensed (200 µl per well) in 96 well tissue culture plate (Greiner Bio-one, Germany). Wells received protein fraction (1µg/ml) or concanvalin A (Con A, 10µg/ml) for in-vitro stimulation in triplicate. Unstimulated wells were kept to serve as control. The plate was then kept at 37°C in 5% CO2 atmosphere. After 72 hrs culture 3H-Thymidine (1µci/well) was added to the media followed by 16-18 hr incubation at the same atmosphere. The cells were harvested, suspended in scintillation fluid (Cocktail, Rankem, India) and emission of β-ray was quantified by scintillation counter (LS Analyzer, Beckman Inc.). Results were expressed as counts per minute (CPM) are given in table 8.3B and graphically shown in fig 8.3B.

Cytokine Assay

Spleen cell suspensions (500µl per well) from various groups of animals were dispensed in 48 well plates. Protein fraction (1µg/ml) or lipopolysachharide (LPS, 1µg/ml) were used for in-vitro stimulation. Unstimulated wells were kept to serve as control. The plate was then incubated at 37°C in 5% CO2 atmosphere for 48 hours. Various cytokines (TNF-α, IL-1β, IL-6, and IFN-γ) were assayed in the culture supernatant using sandwich ELISA. For the assay mouse monoclonal antibodies of TNF-α, IL-6,(BD Biosciences) IFN-γ and IL-1β (Pierce Endogen, Rockford, USA) were used in a paired antibody sandwich ELISA method following the manufacturer's instructions with some modifications (Murthy et al., 2000). Triplicates of each sample were run separately. The concentration of the cytokines was calculated using O. D. readings for standards (suitable for the paired antibodies

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obtained from the above source).Results are presented in table 8.3 (C1, C2 &C3) and graphically shown in fig 8.3 (C1, C2 &C3).

Table 8.3-B: LTT assay, counts /min of β-rays emission (Mean±SD)(n=5)

Groups/ Stimulants	Formulation Group I		Formulation Group II		Formulation Group III		Formulation Group IV		Formulation Group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Con-A	265310	29539	136147	20289	90852	8282	155567	110 34	42462	7182
F6	64565	42667	57658	19627	21329	9464	51926	242 02	65463	9019
Unsti.	26024	2221	26458	6880	25919	1240 7	25722	118 34	60209	2728 7

Fig 8.3-B: LTT assay, counts /min of β-rays emission (Mean±SD)(n=5)

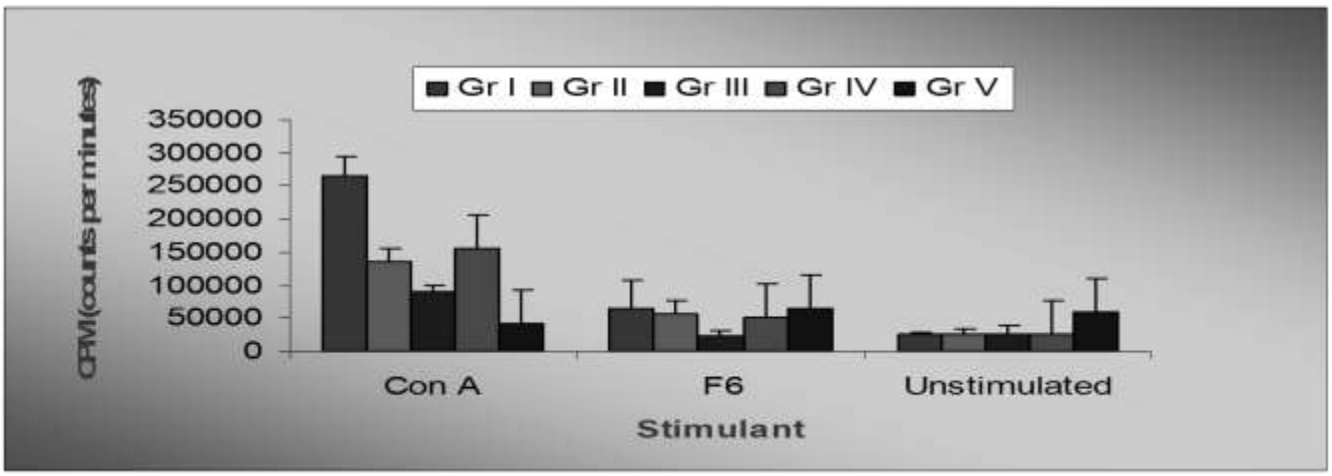

Table 8.3-C1: TNF-α level (pg/mL) in different groups after immunization (Mean±SD)(n=5)

Stimulants/ formulation groups	LPS		F6		Unstimulated	
	Mean	SD	Mean	SD	Mean	SD
Gr. I	679	0.12	428	0.03	138	0.02
Gr. II	1513	0.02	1513	0.05	995	0.17
Gr. III	95.71	0.04	95	0.06	649	0.08
Gr. IV	282	0.10	282	0.05	394	0.11
Gr. V	-352	0.05	-352	0.04	-376	0.06

Fig 8.3-C1: TNF-α level (pg/mL) in different groups after immunization (Mean±SD)(n=5)

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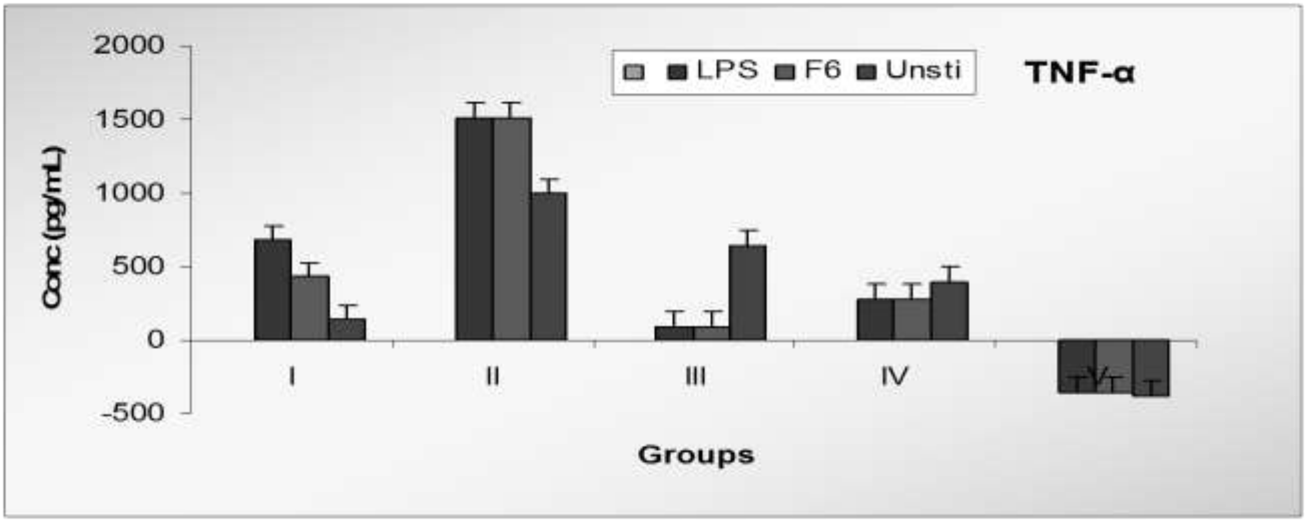

Table 8.3-C2: IL-6 level (pg/mL) in different groups after immunization (Mean±SD)(n=5)

Stimulants/ formulation groups	LPS		F6		Unstimulated	
	Mean	SD	Mean	SD	Mean	SD
Gr. I	1027	0.06	753	0.03	577	0.04
Gr. II	963	0.05	661	0.02	612	0.03
Gr. III	992	0.02	772	0.05	00	0.01
Gr. IV	523	0.03	287	0.02	2.29	0.01
Gr. V	403	0.01	203	0.01	151.61	0.01

Fig 8.3-C2: IL-6 level (pg/mL) in different groups after immunization (Mean±SD)(n=5)

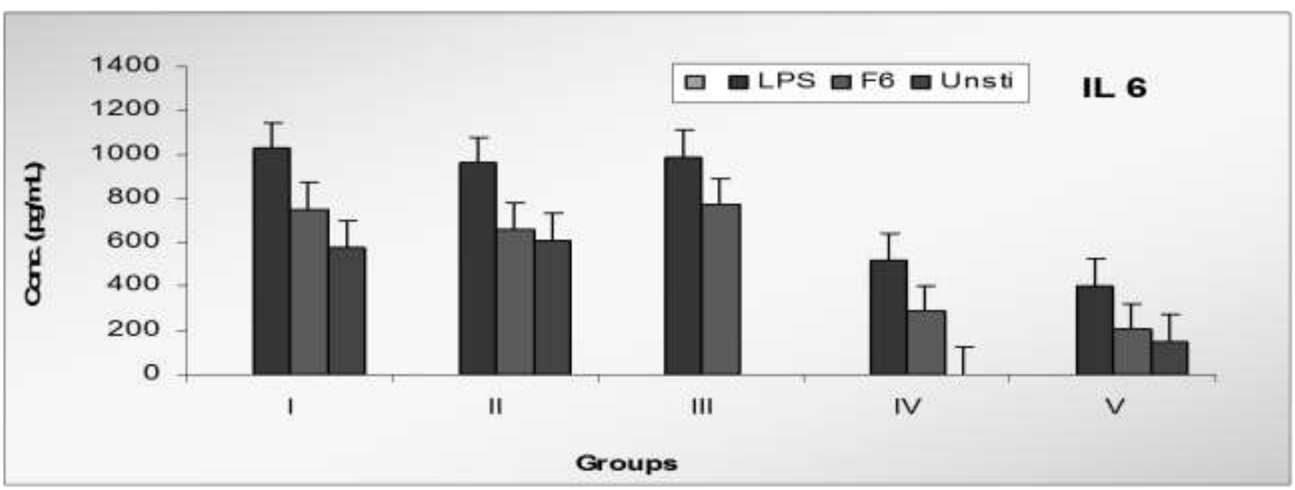

Table 8.3-C3: IFN-γ level (pg/mL) in different groups after immunization (Mean±SD)(n=5)

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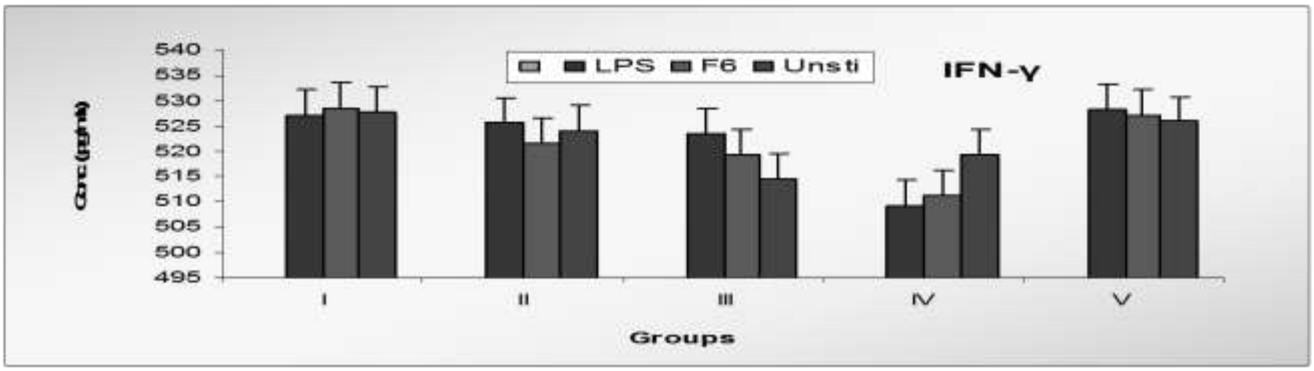
Stimulants/ formulation groups	LPS		F6		Unstimulated	
	Mean	SD	Mean	SD	Mean	SD
Gr. I	527	0.02	529	0.01	528	0.01
Gr. II	526	0.01	522	0.01	524	0.01
Gr. III	524	0.01	519	0.06	514	0.03
Gr. IV	509	0.01	511	0.10	519	0.08
Gr. V	528	0.02	527	0.01	526	0.01

Fig 8.3-C3: IFN-γ level (pg/mL) in different groups after immunization (Mean±SD)(n=5)



Real Time Polymerase Chain Reaction (RT-PCR)

RNA Preparation

Lymph nodes from each group of animals were aseptically separated, explants cells were harvested and RNA were prepared using RNAeasy mini-kit (Qiagen, Valencia, CA). RNA from cells of animals of five groups were pooled to make cDNA for real time RT-PCR.

cDNA Preparation

Pre-developed cDNA synthesis kit (TAKARA, Bio.,USA) was used to make cDNA of each group. Protocol and reagents were of (TAKARA, Bio., USA) and Instrument was DNA Engine (BioRad, Inst.,USA).

Real Time PCR

Predevloped protocol of Sybrgreen assay was followed (Sybrgreen, Qiagen, CA). Real time PCR was done to detect STAT* genes activities. Reagents and protocol were of (Sybrgreen, Qiagen; CA) and instrument was of (BioRad, Inst.; USA).

*STAT (Signal Transducer and Activator of Transcription) regulates many aspects of cell growth, survival and differentiations involved in development and functions of immune system and play a role in maintaining immune tolerance and tumor surveillance. These types of STAT genes were studied which are given below-

STAT 1 - for IFN-γ stimulation

STAT 2 – for IFN-α and it's complex stimulation

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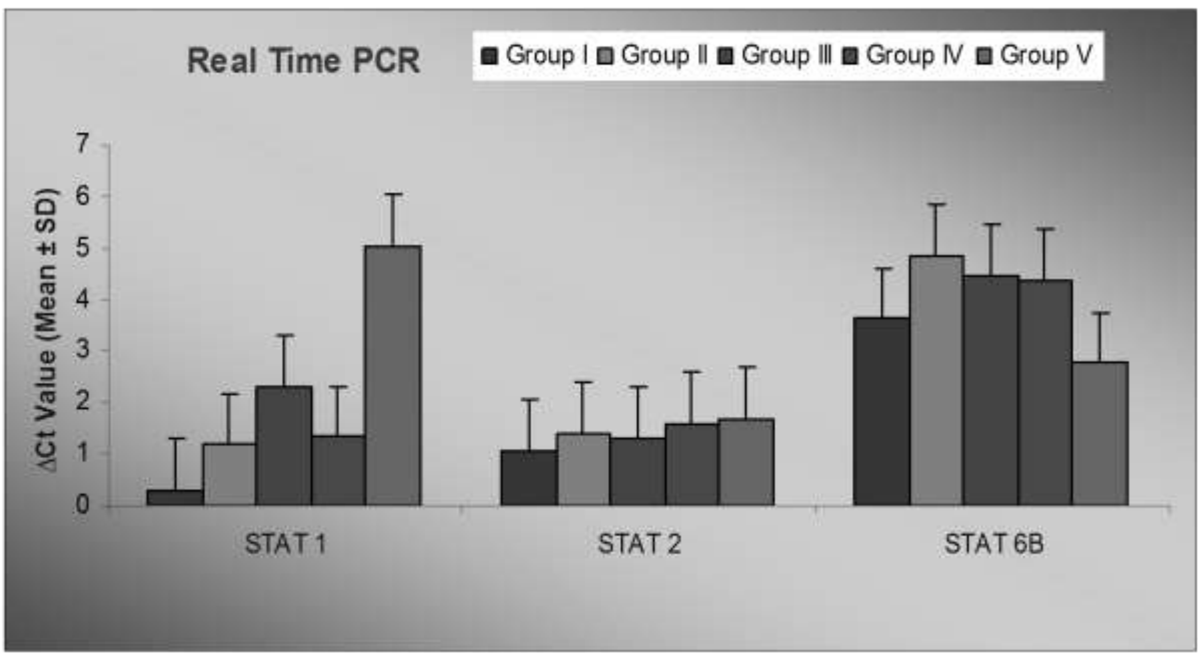
STAT 6 – for IL-4 and Th-II differentiation.
HGPRT – for reference.

Table8.4: ΔCt Value (Mean ± SD) with STAT genes.

STAT gene	Group I		Group II		Group III		Group IV		Group V	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
STAT 1	0.291	0.32	1.178	1.14	2.316	0.12	1.321	0.46	5.021	1.21
STAT 2	1.059	0.90	1.379	0.09	1.300	1.74	1.567	0.53	1.667	1.00
STAT 6	3.619	0.09	4.858	0.33	4.481	0.17	4.366	0.99	2.763	1.06

Fig 8.4: ΔCt Value (Mean ± SD) with STAT genes.



RESULTS & DISCUSSION

BSA loaded liposomes of Soya PC:PE:CH were prepared by reverse phase evaporation method. Reverse phase evaporation was selected because it was reported that higher encapsulation efficiency of macromolecule (protein and peptide) can be achieved by this method, moreover cast film method is

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associated with relatively mild processing conditions (no exposure to organic solvent, sonication etc.) and help in the maintenance of three-dimensional structural integrity of protein. The result revealed that the liposomes can be prepared by any of the method described for the preparation of liposome i.e. entrapment efficiency was found to be more in the case of vesicle prepared by reverse phase evaporation method.

The Soya PC:PE:CH liposomes were prepared with 7:1:2, PC:PE and cholesterol molar ratio and optimized. Entrapment efficiency of conventional Soya PC:PE:CH liposomes prepared by reduced phase evaporation was found to be 60.10±1.45%. The Soya PC:PE:CH liposomes prepared by reverse phase evaporation method were selected for further study for immunomodulatory effect through in vivo and animal studies.

Transmission electron microscopy and light microscopy were used to study shape and lamellarity of the vesicles which revealed unilamellar and spherical shape of the conventional Soya PC:CH liposomes. The particle size of liposomes decrease with increasing sonication time the particle size was measured to be 2.52±0.12µm for formulation CL-1, 2.02±0.21µm for formulation CL-2, 0.89±0.29 µm for formulation CL-3, 0.56±0.45 µm for formulation CL-4, 0.33±0.15 µm for formulation CL-5. Zeta potential of optimized Soya PC:PE:CH liposomes was found to be -15.04±0.45mV which is significantly low indicates that PE might have responsible for the lowering of zeta potential.

NO release from peritoneal macrophages of the animals (Gr.I, II, III, IV and V) was increased by exposure to LPS or no exposure to any stimulants in-vitro as compared to cells of non immunized animals (Gr.V). In summary, F6 was able to induce greater NO production.

Experiments were carried out to see the effect of immunization with F6 plain antigen and liposomised antigen on the proliferative responses of splenocytes of the animals to in-vitro stimulation with antigen and correlate with plain and liposomised antigen delivery. As expected cells from Gr.I and IV proliferated several folds by Con A compared to unexposed cells. In summary liposomised F6 upregulated cells proliferation as showed below.

Immunization with F6 plain and liposomised cause release of cytokines from cells in response to stimulation with F6 or LPS in-vitro as compared to non-immunized ones the same trend was observed in unstimulated group.

The TNF-α release in cells of F6 immunized animals was elevated in response to F6, LPS or no stimulation in-vitro over non-immunized ones.

The IL-6 release in F6 immunized animals was enhanced to F6, LPS or no stimulation in-vitro compared to non-immunized ones . No change in IL-1β release was observed in cells of immunized animals to any of the stimulants in vitro.

The IFN-γ release in cells of F6 immunized animals was elevated in response to F6 or without any stimulation in-vitro in comparison to non-immunized ones.

The genes involved in the inflammatory responses shows the Th-I and Th-II mediate responses. The Th-I responses (STAT-1 and STAT-2) for IFN –γ and it's family cytokines for inflammation and Th-II responses (STAT-6) for antiinflammation. The regulation of these cytokines was confirmed using real-

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time quantitative RT-PCR. As seen, mRNA expression of these genes was increased 3-5 times in STAT-1 and in STAT-2 but decreased in STAT-6.

Up-regulation in Th-I responses and down-regulation in Th-II responses show that the immunological cytokines were in function and cause triggers to body immunity to destroy the parasite, the cytokines production checked at mRNA transcription level using RT-PCR.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This protocol was approved by the Institutional Animal Ethical committee of CSIR-CDRI which implements the national guidelines of CPCSEA for use and handling of animals.

HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human subjects performed by any of the authors; all institutional and national guidelines for the care and use of laboratory animals were followed.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

Not applicable

INFORMED CONSENT STATEMENT

Not applicable

AUTHOR CONTRIBUTIONS

The corresponding author designed and performed the experiments. The co-authors supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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