

PHYSIOCHEMICAL EVALUATION OF LOW MOLECULAR WEIGHT PROTAMINE MODIFIED HEPATITIS B SURFACE ANTIGEN NANOPARTICLES FOR MUCOSAL IMMUNIZATION

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ABSTRACT

In this study, low molecular weight heparin modified hepatitis B surface antigen (HBsAg) were prepared to evaluate the effect of coating material for nasal vaccine delivery. The developed formulations were characterized for size, zeta potential, entrapment efficiency, and mucin adsorption ability. Plain HBsAg nanoparticles demonstrated negative zeta potential and LMWP coated nanoparticles showed higher positive zeta potential. When Plain HBsAg and LMWP coated HBsAg were administered intranasally to mice, it was observed that LMWP coated particles showed a markedly increased anti-HBsAg titer as compared to plain HBsAg nanoparticles.

Keywords: Antigen, mucin , mice, nasal vaccine

1. INTRODUCTION

Hepatitis B disease remains a severe worldwide problem, despite the accessibility of a safe and effective injectable vaccine (WHO, 1990). Licensed hepatitis B vaccine consists of recombinant hepatitis B surface antigen (HBsAg) adsorbed to aluminium adjuvant is both safe and effective with about 90% vaccines being protected upon completion of full three intramuscular doses vaccination regimen over the course of six months. However following drawbacks are reported with presently available vaccine: 10% to 15% non-responders rate, ineffective for limiting HBV replication in chronic carriers, poor compliance due to three intramuscular dose regimens, high cost due to aseptic production and maintenance of sterility, requirements of sterile needles and technical personnel for immunization. These disadvantages associated with currently available hepatitis B vaccine hamper the success of large scale immunization schedule (Pandey and Dixit, 2010).

Alum as an adjuvant present in current hepatitis B vaccine although is well tolerated yet few drawbacks are reported such as formation of granuloma at site of injection and induction of poor cellular immunity (Ma et al., 2007). Since antibody mediated (including mucosal antibody) and cellular immunity both are essential to an efficient response for elimination of viral pathogens, it would be enviable to develop anti-viral vaccine(s) capable of inducing balanced, robust and durable cell-mediated immunity together with strong antibody response. Single or multiple dosage of mucosal intranasal vaccine could significantly improve patient compliance, and this could in turn have a greater impact on global immunization programs.

However, development of mucosal vaccines remains limited by lack of effective mucosal adjuvants (Kido et al., 2008). This underlines the need of new nontoxic potent mucosal adjuvants for Hepatitis B.

Low molecular weight protamine (LMWP), which possess high arginine content and carry significant potency in mediating cellular translocation of the attached cargos. In addition, unlike other cationic proteins/peptides, LMWPs were neither antigenic nor mutagenic, and exhibited a much reduced toxicity and thus an improved safety profile over protamine. Besides these advantages, LMWP can be produced in mass quantities direct from native protamine with limited processing time and cost. Therefore, LMWP could be practically employed as an effective, safe and economical carrier for drug delivery. Indeed, it has been reported that LMWP has been utilized in facilitating anticancer and percutaneous drug delivery.

Therefore, in the present study we investigated whether LMWP coating on hepatitis B surface antigen (HBsAg), are suitable antigen carrier systems for nasal administration. We found that, if optimal formulation parameters are used, LMWP modified HBsAg nanoparticles could greatly enhance systemic immune response.

2. MATERIALS AND METHODS:

2.1 Materials

HBsAg (MW ~24 kDa) (from genetically modified yeast *Pichia pastoris*) was gift sample (1.32 mg/ml in PBS pH 7.0) from Shantha Biotechnics Ltd (Hyderabad, India). LMWP was prepared by method reported by Lee et al., (2001) from Protamine sulphate procured from Sigma Aldrich, Bangalore, India.

2.2. Preparation of LMWP modified HBsAg nanoparticles and alum adsorbed HB

HBsAg nanoparticles surface modified with LMWP were prepared by adding equal volume of 0.5% w/v LMWP solution to HBsAg dispersion (1.25 mg/ml) and gently mixing for about 25 s. In the formulation steps the 1:20 diluted dispersion of HBsAg was used. Alum adjuvanted HBsAg was prepared by emulsification of 1 mg HBsAg with 2.5 mg alum as previously reported (Abraham et al., 1999).

2.3.1. Photon correlation spectroscopy (PCS)

Size, polydispersity and zeta-potential of HBsAg formulations were measured by Photon correlation spectroscopy (PCS) using Zeta Nano ZS 90 (Malvern, UK) following dilution (1:100) with PBS pH 7.0 prior to analysis.

2.3.2. Transmission electron microscopy (TEM)

Particle morphology of formulations was examined by transmission electron microscopy (TEM) (Phillips Morgagni D-268, Netherlands) of negatively stained samples using 2% phosphotungstic acid, pH 5.2 as a contrasting agent.

3. In vivo evaluation

3.1. Immunization

Female Balb/c mice (age 7–9 weeks; weight 15–20 g) were used in all experiments. Animals were housed at the animal house facility of the SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur, India in group of six (n=6) and kept for acclimatization one week before the experiments started. They were withdrawn of any food intake 3 h before immunization. The study protocol including handling, care and immunization were approved by Institutional Animal Ethics Committee of Guru Ghasidas Vishwavidyalaya, India. The studies were carried out as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

To evoke an immune response, 10 µg HBsAg in saline, LMWP- HBsAg were inoculated in small drops (in volume of 20–25 µl) intranasally with the help of micropipette (0.5–10 µl, Microlit, India) in divided dose of 5 µl in 5 minute intervals into nares of mild ether anesthetized mice (supine position). Booster nasal immunizations were performed

after 3 and 6 weeks with the same dose of formulations. (Table 1). Blood was collected from the saphenous vein of mice (under mild ether anesthesia) at 0, 21, and 42 days of immunization approximately 3 h before priming and each boost. Serum was separated by centrifugation (1500 X g for 5 minutes) from collected blood samples after allowing coagulation for 30 minutes at room temperature. Serum samples were stored at -22 °C until analyzed for antibody. The anti-HBsAg antibodies in blood samples were determined by enzyme linked immunoassay kit (AUSAB®, Abbott Laboratories, USA).

4. Statistical analysis

Data from in vivo studies were compared using ANOVA followed by the Tukey's post-test to assess statistical significance. Results were considered to be statistically significant if $p \geq 0.05$. All calculations were performed using GraphPad® Prism® version 5.03 for Windows, GraphPad Software, San Diego, California, USA.

5. RESULTS AND DISCUSSION

5.1. Formulation and characterization of LMWP modified HBsAg nanoparticles

LMWP modified HBsAg were prepared by adding an equal volume of LMWP solution to a HBsAg dispersion in PBS buffer pH 7.4. Plain HBsAg dispersed in PBS pH 7.4 had average mean size of 37.2 ± 13.2 nm, narrowest size distribution (PDI) and the surface charge -17.4 ± 1.2 mV. LMWP HBsAg prepared showed comparable size and zeta potential being 100 nm and

$+8.4 \pm 3.6$ respectively (Student's t test, $P > 0.05$) (Table 1). The size of the protamine-coated particles was larger than that of the non-coated particles the zeta potential of the protamine-coated nanoparticles was, as expected, positive ($+8.4 \pm 3.6$), which is due to the polycationic charge of protamine as previously reported (Gómez et al., 2008).; on the contrary, the non-coated particles had negative zeta potential (-17.2 ± 2.4)

5.2. In vivo evaluation

The resulting antibody responses after nasal immunizations of HBsAg formulations are shown in Fig. 1. Surface coating of LMWP to HBsAg induced significantly higher IgG titers ($P < 0.001$) compared to plain HBsAg but lower than subcutaneously delivered HBsAg vaccine. Polycationic charge of protamine enhances the nasal residence time of nanoparticles thus improve uptake by nasal mucosa (Gómez et al., 2008). The enhanced immunogenicity of the protamine-coated particles was mediated through their preferential interaction with immune competent cells. Positively charged particles are more likely to bind to the negatively charged cell surfaces, and thereby facilitate uptake and stimulation of immune responses. The mechanism suggested for the enhanced uptake in those studies is the electrostatic attraction between the positively charged nanoparticles and the negatively charged cell surface mediating binding and subsequent internalisation. In addition to its positive charge at physiological pH, protamine specifically contains arginine-rich sequences, which share structural similarity with certain viral proteins and can act as an efficient membrane-translocating peptide (Park et al., 2005). Data from this study reveal that LMWP HBsAg can act novel mucosal vaccine against hepatitis B.

Table 1. Characterization of HBsAg formulations (n=3).

S. No.	Parameters	HBsAg	LMWH HBsAg
1.	Mean particle diameter (nm)	37.2± 13.2	117±14.2
2.	Polydispersity Index	0.203 ± 0.04	0.192 ± 0.04
3.	Zeta potential	-17.2±2.4	+8.4±3.6
4.	Appearance*	Spherical and discrete	Spherical with aggregation

* By transmission electron microscopy.

Table 2. Immunization Protocol for HBsAg formulations (n=6)

Pulmonary immunization*		
Group 1	10 µg HBsAg in PBS pH 7.4	Priming (Day 0, 1 and 2) and one booster doses after 3 weeks
Group 2	LMWP- HBsAg formulation**	
Subcutaneous immunization		
Group 3	2 µg alum adsorbed HBsAg formulation (Positive control)	Priming (Day 0)and one booster dose after 3 weeks

*Total volume of formulation was kept constant to 50 µl. Mice were tilted to 45°C tilted position during dose installation and kept in the same position for 2 min. Following vaccine administration, 200 µl of air bolus was blown into the nostril of mice.

** Equivalent dose of 10 µg of HBsAg

6. CONCLUSION

LMWP HbsAg has great potential as nasal vaccines although high and frequent doses of ISCOMs might be needed to produce protective and high immune response. Advantages such as non invasive administration with nasal delivery of vaccines warrant future research in this area.

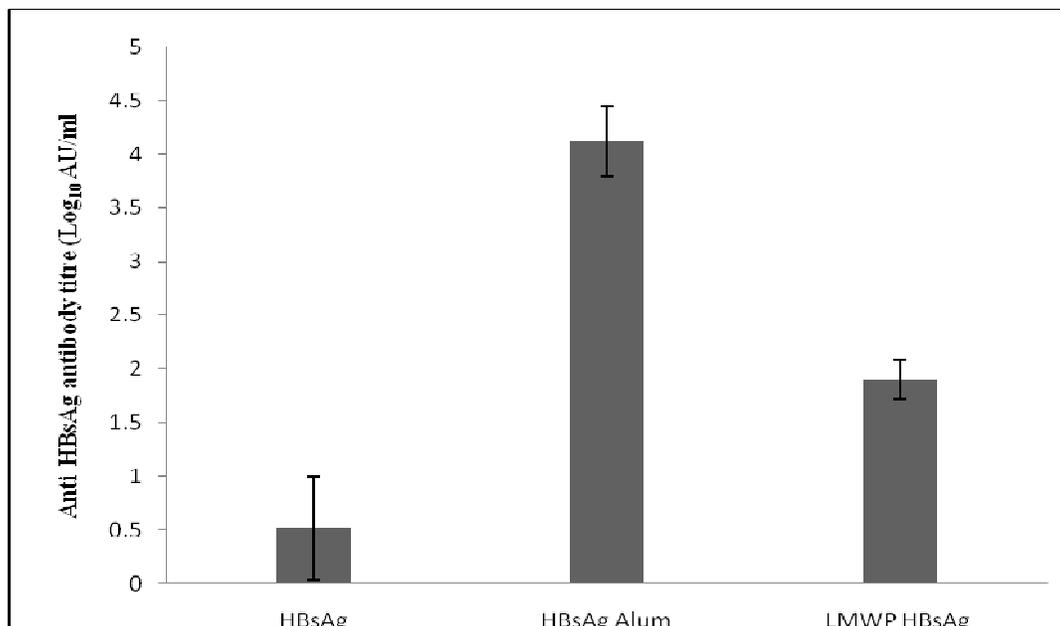


Figure 1. Serum anti-HBsAg IgG titre following mice immunized nasally with 10 µg HBsAg and, 10 µg LMWP HBsAg in PBS, and 2 µg HBsAg- Alum vaccine injected subcutaneously. Analysis of sera collected at 8 weeks after primary immunization. Values are expressed as mean ± SD (n = 6).

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