Novel carrier based vaccine delivery systems for mucosal immune responses for HPV L1 capsid protein administered subcutaneously and intra nasally

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ABSTRACT

Human papillomavirus (HPV) vaccines based on L1 capsid protein can prevent genital HPV infection and associated complications after three intramuscular injections. The currently available vaccines for HPV infection are based on capsid protein L1 virus like particles (VLPs) and they are formulated using aluminium adjuvants. The aluminium based vaccines always possess some constrains as they cannot induce the cell mediated immune response and the complete protection is based on the humoral antibodies. Here we have experimented subcutaneous and intranasal immunization of HPV L1 antigen formulated in Niosomes in order to induce both Humoral and Cell Mediated Immune (CMI) response. The Niosomes were formulated with the HPV antigen, with or without Cholera Toxin B (CTB) and immunized in mice. The immune response was studied in both systemic and as well as in mucosal surfaces. The subcutaneous injection elicited an acceptable level of systemic immune response, but failed to produce significant response in mucosal surfaces. The intranasal immunization was effective in eliciting both systemic and mucosal immune responses as tested from serum and vaginal fluids respectively when compared with subcutaneous injection.

Keywords: HPV L1; Intranasal & Subcutaneous immunization; Niosomes; Serum; vaginal fluid

INTRODUCTION

A high percentage of human cervical tumors contain human papillomavirus (HPV) DNA sequences, mainly HPV type 16 (HPV-16), HPV-18 (3, 9, 20, 30, and 34). The natural history of HPV infection is rather short in the majority of subjects, since clearance of the virus or of low grade cytological and histological lesions occurs in the majority of cases in few months to 1-3 years. However, cervical cancer development is a long process (usually lasting 15 – 20 years). It implies the persistence of infection with a high-risk HPV type in a majority of infected women, leading to pre-cancerous lesions in the minority of infected women, leading to pre-cancerous lesions in the middle term (3-5 years), and eventually to the development of invasive cancer in the long term (>10 years). Although widespread implementation of cytological screening is available in many countries, cervical cancer represents a major cause of morbidity and mortality. Worldwide, cervical cancer is the second most common cause of cancer death in women worldwide with about 80% cases occurring in developing countries (Parkin M et al., 2006).

The development of HPV vaccine is a landmark in the history of immunization, since this is a vaccine primarily directed and considered as an anti-cancer vaccine. The two presently available vaccines in the market prove to be efficacious in the prevention of pre-cancerous lesions. However, they need to extend their protective efficacy for many years, if a substantial impact on HPV-related diseases has to be achieved. With the increasing use of highly purified synthetic peptides in modern vaccinology, appropriate adjuvants are often required to enhance the immunogenicity of these peptides which are generally poorly immunogenic. The development of novel adjuvants is especially important since alum has been reported to be a relatively weak adjuvant for induction of CMI. Concerns about potential toxicity have restricted the widespread use of adjuvants in man since aluminium was first introduced more than 50 years ago which demonstrated better safety & efficacy and approved by USFDA and WHO. However, some adjuvants developed in the recent decades were found to be toxic for clinical use.

The currently available prophylactic vaccines based on non-infectious L1 VLPs have proven to be well tolerated, highly immunogenic and efficient in preventing type specific cervical HPV persistent infection and associated neoplasia. However, these vaccines require multiple intramuscular doses administered over 6 months to be efficient and the primary target groups being pre-adolescent girls, and may decrease the accessibility of the vaccines in developing countries. The
use of non-invasive routes of immunization may thus be an advantage towards more widespread implementation of HPV vaccines. Mucosal administration of VLPs also has the potential to induce locally produced secretory IgA (sIgA) in all mucosal sites, which may in theory compensate for the decrease of VLP-specific IgG during ovulation.

Hence the need to develop newer and effective adjuvants is of utmost importance. Non-ionic surfactant vesicles (NISVs) have clearly demonstrated their ability to function as adjuvants following parenteral administration with a number of different antigens and peptides such as OVA (Brewer, Roberts et al. 1996), BSA (Brewer and Alexander 1992), a soluble T. gondii antigen (Roberts, Brewer et al. 1994), a synthetic measles peptide (Brewer, Roberts et al. 1996) and HSV-1 (Hasan, Brewer et al. 1996) in addition to other adjuvant systems such as microspheres, nanospheres and dendrimers. Results from these studies have suggested that the immune responses preferentially activated by NISV are of the Th1-type, and this is particularly useful for vaccines directed against intracellular pathogens which generally require cell mediated immune responses. Also NISV have been reported to possess extremely low toxicity (Brewer, Roberts et al. 1994). Indeed recent studies comparing NISV and Alum, demonstrated that unlike Alum, NISV did not induce chronic granuloma formation at the injection site.


MATERIALS AND METHODS

Materials

The surfactants (Spans 40, 60, 80, Tween 20, 40, 60), cholesterol and Cholera Toxin B (CTB) were procured from Sigma Chemical Co. (St Louis, MO, USA). The aluminium phosphate was procured from Brenntag, Denmark. The HPV L1 protein was obtained from Dept. of Microbiology, PSGCAS, Coimbatore, TN, India. The ELISA plates were procured from NUNC. All other chemicals and reagents were of analytical grade and purchased from local suppliers.

Formulation of HPV with aluminium adjuvants

Formulation was done using aluminium phosphate (pH 5.9) & aluminium Hydroxide adjuvant (pH 6.4). The formulation of HPV protein with aluminium adjuvants was performed under aseptic conditions. Sterile formulation buffer containing Sodium chloride, Sodium dihydrogen phosphate was taken and required volume of aluminium adjuvant was added. The calculated volume of HPV antigen was added to ensure that the final concentration is 100 µg/mL. The final volume is made up with sterile buffer.

Formulation and optimization of HPV with Niosomes:

Non-ionic surfactant vesicles (Niosomes) were prepared according to the thin film hydration method: the surfactants (Spans 40, 60, 80, Tween 20, 40, 60) and cholesterol (at a ratio of 1:1 surfactant/cholesterol) were completely dissolved in 10 mL of chloroform in a round bottom flask, and the solvent was removed under vacuum in a rotary vacuum evaporator on a water bath at 60°C to form a thin film on the wall of the flask. Residual chloroform was evaporated in a vacuum oven for 4–8 h at room temperature. The film was then hydrated with distilled water under mechanical stirring at 60–70°C. After 12 h, the dispersion was sonicated in an ultrasound bath at 60°C for 30 min. HPV protein was added to the Niosomes at various concentrations (0.1–0.75 mg/ml) together with surfactants and cholesterol. The untrapped HPV antigen was removed by ultracentrifugation (Beckman Coulter, CA, USA) at 105,000 xg at 4°C for 1 h. The niosomes were optimized based on entrapment efficiency as part of pre-formulation studies using hepatitis B surface antigen as a model antigen.

Four formulations were done using Niosomes viz., i) Niosomes with 0.5 mg/mL of HPV antigen (subcutaneous immunization) ii) Niosomes with 0.75 mg/mL of HPV antigen (subcutaneous immunization) iii) Niosomes with 0.5 mg/mL of antigen with CTB, subcutaneous & intranasal immunization.

In vivo immunogenicity studies

The vaccine preparation was injected into female Balb/c mice weighing 14–17 g, using sterile disposable syringes into one group of 20 (n=20) mice each. The other group of 4 mice was injected with placebo (without HPV antigen) on the same day, which served as control. All the animals used were approved by Institutional Animal Ethics Committee. The immunization dose was 0.5mL, in the subcutaneous route. A booster dose was given on day 21 and day 42 for both test mice and control group. Nasal dosing was performed by inserting a small piece of polyethylene tubing (sterile), attached to a Hamilton syringe , 0.2 cm into the nostril (50 µl of formulation/nostril) of the non-anesthetized animal (supine position) and ejecting into the nasal cavity[K.S. Jaganathan, Suresh P. Vyas , 2006]. The formulations of HPV protein with aluminium adjuvant and Niosomes, with and without CTB were studied for the in-vivo response in both serum & vaginal fluid samples. The immunizations were done by subcutaneous route and intranasal route. The formulations of Niosomes with CTB and aluminium containing HPV antigen were immunized with and without booster doses in intranasal and subcutaneous routes respectively.
Sample collection

Blood was collected from immunized mice using hæmatocrit capillary tube. The tube was inserted midway between the medial and lateral canthi of the eye. As the tube was entering the orbital sinus, it was rotated between the thumb and forefinger to pierce the periorbital vein and blood is collected.

Collection of vaginal fluid: A vaginal wash was obtained according to the method reported by Debin et al. (2002). Briefly, 50 µl of PBS containing 1% (w/v) bovine serum albumin (1% BSA–PBS at pH 7.4) was introduced into the vaginal tract of non-anesthetized mice using a Gilson pipette. These 50 µl aliquots were withdrawn and reintroduced nine times.

Measurement of serum and vaginal fluid antibody response

The immune response in terms of OD was determined from the collected blood for Anti-HPV L1 antibody by using a solid phase Enzyme Linked Immuno Assay. HPV antigen was diluted at a concentration of 0.5 µg/mL in sterile phosphate buffer saline (PBS) and 100 µl of the diluted antigen was added to the 96 well ELISA plate. It was incubated overnight at 2 to 8°C. After the incubation, the plate was washed with wash buffer three times and the remaining wash buffer was removed and added with 300 µl of blocking buffer (5% skimmed milk) and incubated for one hour. Test samples were diluted 1:200 and control was added and incubated at 37°C for one hour. After washing, 100 µl of secondary antibody (Goat Anti Mouse HRP conjugated secondary antibody in PBS diluted 1:1500) was added to the wells and incubated at 37°C for one hour. After the incubation, the wells were washed and 100 µl of freshly prepared TMB substrate was added and incubated at 37°C for 10 minutes. The reaction was stopped by addition of 1N HCl and the OD was immediately measured at 450 nm/570 nm in a micro plate reader.

Secretory antibody (IgA) level in vaginal fluid was determined by ELISA using slightly modified method of Debin et al. (2002). Briefly, microtiter plates (Nunc-Immune Plate® Fb96 Maxisorb,Nunc, India) were coated with a solution of HPV L1 at 0.5µg/mL in sterile phosphate buffer saline (PBS) for overnight at 2 to 8°C. Wells were blocked with PBS–BSA (3% (w/v)) for 1 h. The plates were washed three times with 300 µl of PBS containing 0.05% w/v Tween 20. 1: 100 diluted vaginal fluid, diluted using PBS–BSA (0.1% (w/v) was added and the plates were held at room temperature for 2 hours followed by washing and addition of horseradish peroxidase-conjugated goat anti-mouse IgA (Sigma, USA). IG A antibodies present in mucosal samples were analyzed using 1/10 dilution as the first dilution of the sample. After 1 h incubation and washing, 100 µl of o-phenylenediamine dichloride (OPD; Sigma, USA) in phosphate-citrate buffer pH 5.5 and H2O2 was added as a substrate. Colour development was stopped after 30 min via the addition of 50 µl of 1N H2SO4 and the absorbance was measured at 450 nm.

Statistical analysis

Table 1: Optimization of Entrapment Efficiency

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Surfactants</th>
<th>Entrapment Efficiency (%)</th>
<th>Hydrodynamic diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-I</td>
<td>Span 40 + Chol (1:1)</td>
<td>12.6 ± 1.0</td>
<td>355.5 ± 31.0</td>
</tr>
<tr>
<td>F-II</td>
<td>Span 60 + Chol (1:1)</td>
<td>23.8 ± 1.8</td>
<td>431.0 ± 27.4</td>
</tr>
<tr>
<td>F-III</td>
<td>Span 80 + Chol (1:1)</td>
<td>31.0 ± 1.9</td>
<td>331.2 ± 21.2</td>
</tr>
<tr>
<td>F-IV</td>
<td>Tween 20 + Chol (1:1)</td>
<td>9.4 ± 0.7</td>
<td>435.2 ± 31.6</td>
</tr>
<tr>
<td>F-V</td>
<td>Tween 40 + Chol (1:1)</td>
<td>34.2 ± 2.4</td>
<td>351.4 ± 28.0</td>
</tr>
<tr>
<td>F-VI</td>
<td>Tween 60 + Chol (1:1)</td>
<td>50.8 ± 3.7</td>
<td>372.0 ± 31.5</td>
</tr>
</tbody>
</table>

Note: 0.5mg/mL concentration of Antigen was used in all formulation. The formulation Tween 60 + Chol(1:1) was the optimum one with better entrapment efficiency, used in all Niosomal formulation.
Statistical analysis was performed on the data obtained in the in vitro and in vivo studies by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post test using GraphPad Ver 5.04™ software (GraphPad Software Inc., San Diego, California). Throughout, the level of significance was chosen as less than 0.05 (i.e., p < 0.05).

RESULTS AND DISCUSSION

Optimization of Niosomes: Entrapment efficiency

The ability of different niosomal preparations was studied, obtained by using different non-ionic surfactants together with cholesterol at a ratio 1:1, to trap (0.5 mg/ml) shown in Table 1.

In-vivo studies

Formulation with aluminium adjuvants

The antibody response of HPV antigen formulated in aluminium phosphate (pH 5.9) & aluminium hydroxide adjuvant (pH 6.5) was studied. It was clearly demonstrated that aluminium phosphate elicited a better immune response than aluminium Hydroxide (p < 0.05). It may be due to its adsorption mechanism since both aluminium phosphate and aluminium hydroxide has different antigen adsorption mechanism and adsorption capacity. The immune response with aluminium phosphate formulation was significant after the first booster dose given on 21st day. However, the response between day 42 & day 60 was not significantly different. The formulation at pH 5.9 elicited a better immune response at day 20 and improved with the subsequent booster doses (Fig 1). However, there was no antibody response in the vaginal fluid in both the formulations.

Formulation with Niosomes

Niosomes based different formulations were prepared to evaluate the immune response in the systemic and mucosal. The niosome formulations with 0.5 mg/ml formulation elicited a better immune response. The OD values obtained at all the three time points (day 20, 42 & 60) are comparable with aluminium phosphate (pH 5.9) formulation. The response at day 60 was marginally better than the day 42 sample (p< 0.05). The Niosome formulation with 0.75 mg/mL antigen payload elicited an immune response comparable to 0.5 mg/mL formulation. In both the formulations, the vaginal fluid had demonstrated a significant immune response as compared with aluminium adjuvant (Fig 2). The response in the vaginal fluid was comparable with the systemic response obtained with aluminium adjuvant.

Niosomes with CTB

The Niosome formulations containing CTB were immunized in both subcutaneous route and intranasal route as CTB is a co-mucosal adjuvant. The systemic immune response was comparable with the other formulations. The mucosal immune response of this formulation in both subcutaneous route and intranasal route was significantly higher than the mucosal response obtained without CTB and also formulations containing aluminium phosphate (p<0.05). However, there was no significant difference in the mucosal immune response elicited by niosome formulations containing CTB and niosomes without CTB, immunized through intranasal route (Fig 3).

The vaginal antibody response in Niosome formulation containing 0.5 mg/mL HPV protein + CTB with and without booster is given in Fig 4. The niosome formulations with booster doses elicited significantly better response (p<0.05) than the formulations given without booster doses.

DISCUSSION

The aluminium based adjuvants for recombinant proteins using aluminium phosphate or aluminium Hydroxide have limitations in the induction of immune response in lacking the capability to induce the mucosal antibodies. Non-ionic surfactant vesicles (Niosomes) have clearly demonstrated their ability to function as adjuvants following parenteral administration with a number of different antigens and peptides for eg. a synthetic measles peptide (Brewer, Roberts et al. 1996) and HSV-1 (Hassan, Brewer et al. 1996). Also NISV have been reported to possess extremely low toxicity (Brewer, Roberts et al. 1994).

The aim of the present study was to study the immune response of HPV L1 antigen formulated in aluminium phosphate adjuvants and comparing the performance of Niosomes in eliciting a response, both systemic and mucosal, which could qualify them as potential alternative adjuvant systems for the alum based adjuvants. The antibody response from each immunized group was studied using ELISA from serum and vaginal fluid samples collected from the animals at different intervals after immunization, with and without booster doses. The response was evaluated based on the Optical Density at 450 nm (OD 450) of test samples against a control sample collected from unimmunized animal.

The initial experiments with aluminium adjuvants demonstrated that aluminium phosphate adjuvant was more effective than aluminium Hydroxide, in eliciting an acceptable level of antibody response in the serum samples. It was also observed that aluminium adjuvants do not induce any mucosal antibodies even after booster doses.

The developed Niosomes based carrier systems containing HPV L1 antigen had shown significant response in both serum and in mucosal surface. The immune response elicited in serum by Niosomes was comparable with the aluminium adjuvants. The study comparing the response of Niosome formulation containing 0.5 mg/mL antigen and 0.75 mg/mL protein demonstrated that increase in the payload did not have any beneficial effect on the in-vivo response. The sample
collected on 60th day without any booster did not show significant level of antibodies. The mucosal immune response as determined in the vaginal fluid in these formulations of Niosomes was moderate.

Induction of antibodies in mucosal secretions is not only dependant on the particular secretion examined, but also on the site of mucosal immunization, i.e., airway, oral, rectal or genital. Hence in this study, we were interested in inducing Anti HPV antibodies mainly in the vaginal secretions, as the cervix is the prevalent site of high-risk types HPV infections. Secretory immunity results from activated B cells arriving from inductive mucosa associated lymphoid tissue [Brandtzaeg P et al, 2007, Neutra MR et al, 2006]. Such mucosa-associated inductive sites not only differ in their location, but also in their ability to efficiently mount specific immune response. The latter being more or less regionalized depending on whether activated B cells may only home back to the mucosa they originated from or mucosa situated at more distant sites [Neutra MR et al, 2006]. For instance the sparse lymphoid aggregates associated to the cervico-vaginal mucosa are less prone to mount efficient immune responses, while nasal associated lymphoid tissues are efficient at inducing antibody responses in the respiratory secretions as well as in the distant genital mucosa [Mestecky J et al, 2005, Balmelli C et al, 2002]. In addition, such properties may differ depending on the antigen and the adjuvant used.

Although sIgA are particularly well suited to protect mucosa through both immune exclusion and intracellular neutralization [Brandtzaeg P et al, 2007], serum derived HPV–L1 specific antibodies induced by intramuscular HPV vaccination, appear to be sufficient to provide close to 100% protection against persistent HPV infection and associated preneoplastic lesions. Hence we wanted to exploit the fact that mucosal vaccination can also induce serum antibodies and is a needle–free administration route that may increase worldwide implementation. Moreover, additional induction of sIgA may still be of benefit for long term protection in ovulating women [Nardelli-Haefliger D et al, 2003]. To demonstrate this, intranasal immunization was done using the Niosome formulation containing CTB. This formulation with the two booster doses, elicited a better immune response in both serum and mucosal surfaces. Also the mucosal response was comparable with the response obtained with the same formulation given subcutaneously. The Niosome formulation with CTB also did not show adequate immune

![Figure 2: Immune response in different niosome formulations](image-url)

F1NI0112 to F1NI00512 – Niosome with 0.5 mg/mL HPV protein
F2NI0112 to F2NI00512 – Niosome with 0.75 mg/mL HPV protein
F1NIC0113 to F1NIC00413 – Niosome with 0.5 mg/mL HPV protein + CTB

![Figure 3: Comparison of immune response in intranasal route](image-url)

F1NIC0113 to F1NIC00413 – Niosome with 0.5 mg/mL HPV protein + CTB - Subcutaneous
F1NIN0113 to F1NIN00313 – Niosome with 0.5 mg/mL HPV protein + CTB - Intranasal
response without booster (F1NINs0113 & F1NINs0213). This demonstrates that intranasal immunization without booster did not produce any sustainable immune response which could be due to early clearance of these Niosome formulated material from the nasal cavity.

In conclusion, Non-ionic Surfactant Vesicles (Niosomes) with or without CTB demonstrated great potential as a drug delivery system (nasal delivery) for vaccines where both systemic & mucosal immune responses are desirable especially in situations after bacterial and viral pathogens invade the host via the mucosal surfaces. Additionally, these formulations demonstrate to be effective in eliciting systemic antibody response in addition to mucosal response, proving to be the most promising alternatives for the currently used Alum adjuvants. This largely supports the fact that these Niosomes and the intranasal route could support the needle less administration for better patient compliance.

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