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Research Article

EVALUATING THE EFFICACY OF ALUMINUM PHOSPHATE FORMULATED L2 BASED HUMAN PAPILLOMA VIRUS VACCINE

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ABSTRACT

Objective: Human papilloma virus (HPV) caused cervical cancer the second most common cancer among women worldwide and the most common cancer in developing countries like India. Though, currently type specific prophylactic vaccine has been developed, there is a need for cross-protective virus neutralizing vaccine. In this study, we have tried to show the multi-epitope vaccine and check the final aluminum adjuvant formulated vaccine antibody titer.

Methods: Our study was targeted to analyze the *in vivo* vaccine efficacy of the aluminum adjuvant formulated recombinant multi-epitope antigen with two different grades of aluminum phosphate (pH 5.5 and 6.4). Neutralizing antibody titers against the major neutralizing epitope 17-36 a region of the N-terminal domain.

Results: The results of this study showed that the final aluminum adjuvant recombinant L2 based multi-epitope vaccine produced antibody against 17-36 peptide one of the proven major virus neutralizing epitopes.

Conclusion: L2 based multi-epitope recombinant antigen formulated with aluminum adjuvant can be a low cost, broadly protective HPV vaccine.

Keywords: Human papilloma virus L2, Multi-epitope recombinant vaccine, Aluminum adjuvant.

INTRODUCTION

Cervical cancer is one of the most common cancers in women worldwide. Clinical and molecular studies have shown that certain types of human papilloma virus (HPV), referred to as high-risk types (e.g. HPV 16, 18, 45, and 31), are the etiological agents of this disease [1]. Two prophylactic anti-HPV vaccines targeting HPV 6, 11, 16, and 18 have been licensed recently by Merck (Gardasil™) and GlaxoSmithKline (Cervarix™), respectively [2,3]. An icosahedral HPV capsid is composed of major capsid protein L1 and minor capsid protein L2. Since it is difficult to obtain a large amount of HPV particles by using conventional cell cultures, surrogate systems capable of expressing L1 and L2 have been developed to obtain HPV capsids for structural and immunological analysis [4]. The limited cross-protective capacity of L1-based vaccines, which is the main reason for a continuing effort toward the development of improved vaccination strategies, likely, reflects the HPV type specificity of L1 neutralizing epitopes [5].

Minor capsid protein L2 is a possible alternative to the highly multivalent L1 virus-like particles (VLP) vaccines to obtain broad protection against oncogenic HPVs [1]. Vaccination with L2 as a full-length protein or as polypeptides protects animals against homologous-type viral challenges at both cutaneous and mucosal sites [6-8]. Protection is not mediated by cellular immunity, suggesting the importance of neutralizing antibodies [7,9] L2 is subdominant in the context of L1/L2 VLPs [10], but antibodies elicited by recombinant L2 immunogens are able to neutralize a remarkably broad range of HPV genotypes [11]. This suggests that neutralizing epitopes of L2 may be conserved across HPV types due to some critical viral function [12]. Furthermore, it raises the possibility that a single L2 protein or peptide based vaccine might provide comprehensive protection against the HPV types causing genital cancer and genital warts and possibly even those associated with cutaneous warts and epidermodysplasia verruciformis.

Here, we report on the use of this novel recombinant multi-type L2 protein comprising tandem repeats of 11-88 amino acid regions from dissimilar therapeutically significant HPV types expressed in *Escherichia coli*. Our studies were targeted to study the vaccine efficacy of the formulated multi-epitope antigen and to check the level of neutralizing antibody titers against the major neutralizing epitope region aa 17-36 of the N-terminal domain.

METHODS

Cloning and expression of multi-epitope-L2 gene

A chimeric construct containing conserved nucleic acid regions coding for of HPV Type 6B, 16, 18, 31, and 39 cloned in pET28a vector was obtained from National Institute of Health, USA. The vector was transformed into an expression host *E. coli* (BL21 (DE 3) pLysS.

Purification with Ion exchange chromatography

About 1% overnight culture was inoculated into a fresh 500 ml LB broth for scale-up and incubated at 37°C till the culture reaches 0.6 OD at A_{600}. The culture was induced with 1 mM IPTG at 37°C for 6 hrs and cells were pelleted and lysed using lysis buffer (Glycine phosphate buffer, 0.5M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.5). Inclusion bodies (IB) were subjected to solubilization with solubilization buffer (50 mM Tris, 150 mM NaCl, 8 M urea, 10 mM DTT, 5 mM EDTA, and pH 8) at 1:6 ratio under stirring condition at 4°C±2°C for 6-16 hrs. The IB solubilized product was purified through ion exchange chromatography and eluted with buffer containing 20 mM sodium acetate, 8 M Urea and 5 mM EDTA, 75 mM NaCl, and pH 4.

Protein characterization

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was performed to check the purity and measure the approximate molecular weight of the protein. The purified protein was resolved in 12% SDS-PAGE and the bands were developed by Coomassie staining along with standard protein molecular weight marker (Sigma, USA). The proteins were separated on SDS-PAGE and then transferred to

nitrocellulose membrane. The membrane was blocked with skim milk to remove non-specific protein. A primary antibody RG1 monoclonal (1:5000 dilutions) was added to the solution which was able to bind to its specific target protein followed by a secondary antibody with goat anti-mouse alkaline phosphatase (Sigma Aldrich, USA) at 1:10,000 dilution for detection. Finally, the commercially available substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich, USA) was added.

Protein estimation

The total protein content in native protein was estimated by Lowry *et al*. [13].

Animal studies

Female BALB/c mice were obtained in the animal house of Shantha Biotech, Hyderabad, India. The animals were kept in polypropylene cages, under controlled temperature, humidity, and 12/12/light/dark cycles. The animals were fed a pellet diet and water *ad libitum*. This study was carried out with prior approval from the Institutional Animal Ethical Committee (181/199/CPCSEA).

Experimental design

The mice were randomly divided into three groups (n=5) as given in Table 1:

Group I: Antigen formulated with aluminum hydroxide gel 1.

Group II: Antigen formulated with aluminum hydroxide gel 2.

Group III: Antigen formulated with freund's adjuvant.

L2 immunogen enzyme-linked immunosorbent assay (ELISA) to determine total serum antibody

ELISA plates were coated with the multi-type L2 protein (50 ng/well) by incubating it for 14-16 hrs at 4°C. The wells were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) and incubated for 1 hr at 37°C. After washing with PBS containing 0.5% Tween 20, 100 µl of the serum sample collected from both groups was diluted (1:200 to 1:204,800) and added to the well and incubated for 1 hr at room temperature. The secondary antibody, anti-mouse IgG goat serum conjugated with horseradish peroxidase (Sigma-Aldrich, USA), was added in 1:3000 dilutions with PBS. The plate was developed using a mixture of H_2O_2 and 3,3',5,5'-Tetramethylbenzidine (TMB) in citrate buffer (pH 4.7) as a substrate. The enzyme-substrate reaction was stopped after 15 minutes with 1 N sulfuric acid, and the absorbance was measured at 450 nm using ELISA plate reader (Molecular Devices, USA), and data analysis was done using the Soft Max Pro Software.

17-36 amino acid peptide ELISA

Biotinylated 20-mer peptides covering the N-terminal 17-36 amino acid region of L2 were chemically synthesized from JPT Peptide Technologies, Germany. Peptide ELISA using test sera was carried out by binding 31.25 ng per well of the Biotinylated peptides onto a Streptavidin coated microtitre plate (Nunc, Denmark). The plates were incubated overnight at 4°C, blocked with 5% skimmed milk and incubated at 37°C for 1 hr. After incubation the plates were washed with PBST thrice. The test serum samples were diluted 1:200 to 1:204,800 in a two-fold serial dilution and 100 were added to the plate and incubated at 37°C for 1 hr. After completion of incubation, the plates were washed and 100 µl/well of HRP-conjugated goat anti-mouse IgG polyclonal antibody (1:3000 dilution; Sigma-Aldrich, USA) were added and incubated for 1 hr at 37°C. The plate was developed using a mixture of H_2O_2 and TMB in citrate buffer (pH 4.7) as a substrate. The enzyme-substrate reaction was stopped after 15 minutes with 1 N sulfuric acid, and the absorbance was measured at 450 nm using ELISA plate reader (Molecular Devices, USA), and data analysis was done using using graphpad prism.

RESULTS

Expression and purification of multi-epitope-L2 gene

The HPV type 6b, 16, 18, 31, and 39 synthesized by codon optimization were cloned in the pET28a vector. The plasmid vector was transformed into BL21 DE3 *E. coli* cells. The protein was purified from the bacterial lysate using the ion-exchange chromatographic method. The eluted recombinant protein was confirmed (change the word) in SDS-PAGE and confirmed in western blot using the RG1 Mab (Fig. 1a and b). The purified, concentrated protein content was found in the range of 750 µg/ml.

Animal studies

To check the efficacy of the multi-epitope recombinant L2-antigen, it is provided with two aluminum adjuvants and freund's adjuvants maintained as a control.

L2 Immunogen ELISA to determine total serum antibody

Female 4-5 weeks old BALB/c mice were selected for the study. The vaccine was administrated subcutaneously as a three dose regime at a 15 days interval. Each dose contained 25 µg of antigen and 125 µg of aluminum phosphate. Immunogenicity of the vaccine is primarily determined by the potential of the vaccine to elicit an antibody response against the vaccine antigen. L2 multi-epitope antigen ELISA has been used to assess the immunogenicity of the final formulated product (Fig. 2).

17-36 aa peptide ELISA

Similarly to the level of neutralizing antibody titer was checked against the 17-36 aa epitope using peptide ELISA showed a significant level of antibody titer in both formulations compared with control group (Fig. 3).

DISCUSSION

The minor L2 capsid was identified as an alternate to the highly multivalent L1 VLP vaccines to obtain broad protection most of the oncogenic HPV types [14]. The minor capsid protein L2 is a promising candidate for the construction of an anti-HPV broadly protective vaccine

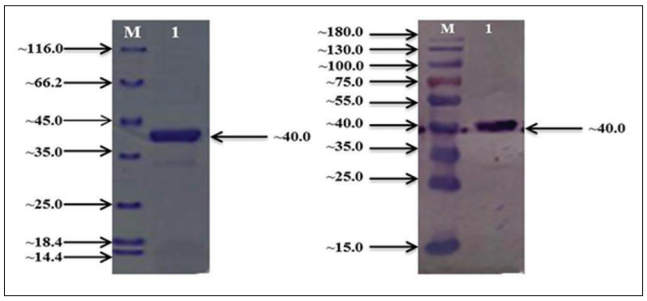

Fig. 1: Analysis purified multi-epitope recombinant L2-antigen, (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with coomassie brilliant blue, (b) Western blot with 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. M: Molecular weight marker, Lane 1: Purified, L2: Antigen

Table 1: Experimental design for *in vivo* studies, recombinant multi-epitope antigen formulated with aluminum phosphate adjuvant

Antigen	Concentration	Formulation B.No.	BALB/c	Injection volume (µl)
(11-88)×5 finished Ag=100 µg/ml	Ag=25 µg AP=125 µg	Group I (pH 5.5)	5	250
Adjuvant=0.5 mg/ml		Group II (pH 6.4)	5	250
Bulk=200 µg/ml	Bulk=25 µg FA=125 µl	Group III	5	125 bulk+125 FA

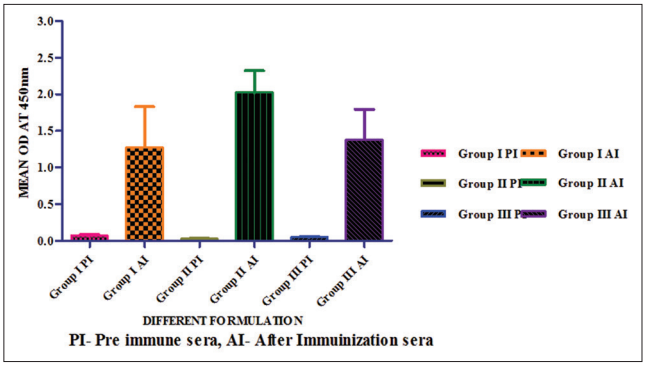

Fig. 2: Recombinant multi-epitope L2 enzyme-linked immunosorbent assay showing the level of immune response in BALB/c mice vaccinated with multi-epitope recombinant L2-antigen

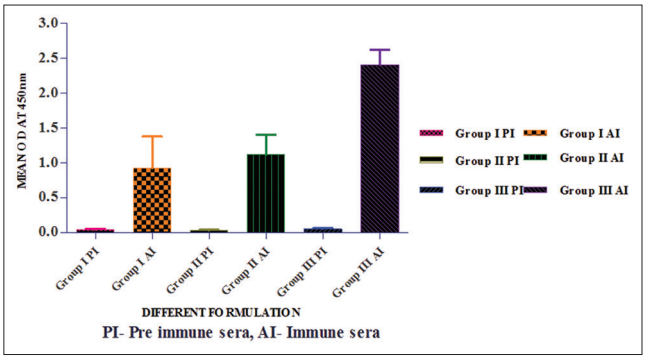

Fig. 3: Peptide (17-36) Neutralizing epitope enzyme-linked immunosorbent assay showing the level of immune response in BALB/c mice vaccinated with multi-epitope recombinant L2-antigen

for the prophylaxis of cervical cancer. However, L2-derived peptides are usually poorly immunogenic and extensive knowledge of the most relevant (cross) neutralizing epitope(s) is still in need [1].

In this study, we have tried to find the level of 17-36 antibody produced against the multimeric antigen prepared so as to prove it to be a potent vaccine candidate. Overexpression of recombinant proteins in bacteria, such as *E. coli*, often results in the formation of IB, which are protein aggregates with non-native conformations. As IB contains relatively pure and intact proteins, protein refolding is an important process to obtain active recombinant proteins from IB [15]. Reported IB refolding processes are poised to play a major role in the production of recombinant proteins.

A monoclonal antibody, RG-1, that binds to highly conserved L2 residues 17-36 and neutralizes HPV16 and HPV18. Passive immunotherapy with RG-1 was protective in mice. Antiserum to the HPV16 L2 peptide comprising residues 17-36 (peptide 17-36) neutralized pseudo viruses HPV5, HPV6, HPV16, HPV 18, HPV31, HPV 45, HPV 52, HPV 58, bovine papillomavirus 1, and HPV11 native virions Alphs *et al.* and Conway *et al.* [16,17] reported similar studies in RG-1 that a cross-neutralizing and protective monoclonal antibody recognizes residues 17-36 of HPV16 minor capsid protein L2. In view of this epitope is highly conserved in divergent HPV types, it was determined that vaccination with HPV16 L2 17-36 peptide was broadly protective.

Besides important insights into the molecular bases of L2-mediated cross-protective immune response and new L2 based vaccine molecule being developed, the results of this study present high resolution analysis with aluminum phosphate formulated multi-epitope protein provides a useful framework for the development of novel L2-based vaccines.

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