PUBLICATIONS
Rabies DNA vaccine encoding lysosome targeted glycoprotein supplemented with EMULSIGEN®D confers complete protection in pre- and post-exposure studies in BALB/c mice

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Abstract

The world-wide incidence of rabies and the inability of currently employed vaccination strategies to provide highly potent and cost-effective therapy indicate the need of an improved rabies vaccine. In this regard, DNA vaccine based on lysosome targeted glycoprotein of rabies virus was evaluated in BALB/c mice. However, it imparted partial protection (60%) against challenge with 20 LD₅₀ of rabies challenge virus standard (CVS). To improve outcome of vaccination, to ultimately enhance the immune response, we investigated different routes for DNA vaccine delivery, varied doses of DNA and influence of adjuvant supplementation. Highest immune response pertaining to IgG antibody titer, with a predominantly IgG1/IgG2a subclass distribution, effective cellular immunity and high level of rabies virus neutralizing antibodies (RVNA) were attained by the optimized DNA vaccine formulation comprising of intramuscular administration of 100 μg of DNA vaccine supplemented with EMULSIGEN®D. In pre-exposure prophylaxis, three dose regimen of this formulation generated high RVNA titer (32 IU/ml) and conferred complete protection against challenge with 20 LD₅₀ of CVS. For post-exposure efficacy analysis, rabies was experimentally induced with 50 LD₅₀ of CVS. Subsequent therapy with five doses of the formulation completely prevented rabies in BALB/c mice, which maintained protective RVNA titers of 4 IU/ml. WHO recommends Rabies protective titer threshold as 0.5 IU/ml. Thus, this optimized DNA vaccine formulation provides an avenue for preventing and controlling rabies.

Keywords

Virus neutralizing antibodies, survival, prophylactic, therapeutic.

Introduction

Rabies; progressive fatal encephalitis, infects all mammals. WHO estimates 40,000 to 70,000 rabies deaths every year. However, rabies can be entirely prevented by vaccination. The WHO recommends a prophylactic or pre-exposure scheme in humans and animals that are at risk and post exposure prophylaxis (PEP) in humans who have been attacked by rabid animals [1]. Vaccination if initiated timely after suspect contact can prevent the onset of rabies in virtually 100% of exposures. More than 10 million PEPs are given annually [2]. The cell culture derived rabies vaccines were developed to replace the conventional nerve tissue vaccines (NTV). Although they are safer and more efficacious than the unpurified and reactogenic NTVs, they are highly expensive pertaining to cell line maintenance, extensive purification, cold storage requisite, multiple boosters and therefore not affordable for many developing countries with limited budgets for healthcare [3].

In this view, DNA vaccines have come a long way since the concept of "DNA immunization" was introduced by Wolff in 1990 [4]. They have proved their potency in eliciting cellular and humoral immune responses against a variety of infectious (viruses, bacteria, parasites) and non-infectious (allergies, tumors) agents. This combined with the inherent advantages provided by DNA vaccine models, such as low cost, simple design and administration, and relatively fast production, has created much excitement for this potential vaccination platform.

The last significant hurdle in implementing DNA vaccines as therapeutics or prophylactics has been the difficulty of translating small animal success to larger models including in clinical studies. This requires introspection of various considerations. Even though immune response elicited upon DNA vaccination is highly dependent upon the inherent immunogenicity of the protein antigen encoded by the DNA vaccine [5, 6]; enhanced immune responses may be achieved in large mammals by optimizing immune-parameters like the form in which antigen is expressed (soluble, intracellular, membrane anchored), dosage of vaccine, delivery methods employed and usage of adjuvants [7, 8, 9].

Our group investigated inter- and intra-cellular targeting strategies for immune enhancement against rabies DNA vaccine. We have previously shown that vaccination of mice with targeted rabies virus–glycoprotein (RV-G) plasmid DNA constructs elicited higher anti-RV-G antibody levels than the unmodified (native) one. They also generated enhanced rabies virus neutralizing antibody (RVNA) response. The highest RVNA titer was observed for pgp.LAMP-1. Further, it conferred 60% protection against lethal intracerebral rabies challenge [6]. In this study, we attempted enhancement of immune response by optimization of immune-parameters like delivery route, dose of DNA vaccine and adjuvant supplementation.

2. Materials and Methods

2.1 Cells and Virus

Baby hamster kidney (BHK-21) cells were procured from National Centre for Cell Science (NCCS), Pune, India. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biological Industries) and 100 U/ml Penicillin (Amersham) and 100 µg/ml Streptomycin (Amersham), in a humidified 5% CO₂ incubator at 37°C. Pitman-Moore (PV-11) strain of Rabies virus was propagated on BHK-21 cells. The virus was then purified and inactivated with beta-propiolactone (BPL). This preparation was employed for in vitro re-stimulation assay. The Challenge Virus Standard (CVS-11) strain was propagated in mice brain and then titrated. CVS was employed for Rapid fluorescence focus inhibition test (RFFIT) and intracerebral rabies virus challenge.

2.2 DNA vaccine

pgp.LAMP-1 (GenBank Accession Number EU215588), a eukaryotic expression plasmid has been described earlier [6]. Briefly, the glycoprotein gene derived from rabies virus (ERA strain) was cloned in eukaryotic plasmids bearing the C-terminal LAMP-1 signal sequence.

2.3 Immune potential of plasmid DNA vaccine in mice

A. Mice

All animal experiments were conducted in compliance with the animal ethics committee. Four to six weeks old female BALB/c mice were employed in the study. They were purchased from National Institute of Nutrition (NIN), Hyderabad; and maintained in pathogen free environment at the Animal House Facility. Each group comprised of ten mice.

B. Immunizations

B.1 Route of administration

For intramuscular (i.m.) route, mice were vaccinated with 100 µg Endotoxin-free plasmid DNA in 200 µl PBS/animal in anterior quadriceps muscle, in the individual groups (DNA vaccine or vector control), thrice at three-week intervals. Control mice were immunized with only PBS.

For gene gun delivery of plasmid DNA, pgp.LAMP-1 plasmid DNA/empty vector was precipitated onto gold microcarriers as per the instructions for usage of Helios gene gun system (Bio-Rad). Briefly, 25 mg of 1 µm gold microcarriers was resuspended by sonication in 100 µl of 0.05 M spermidine. 125 µg of DNA was then added and sonicated; 100 µl of 1 M CaCl₂ was subsequently added drop-wise. This
gold-DNA mixture was allowed to stand for 10 min before being washed 3 times in 250 µl of 100 % ethanol. After the final wash, the pellet was resuspended in 200 µl of 0.025 mg/ml polyvinylpyrrolidone (PVP) in 100 % ethanol, transferred to a 15 ml tube, and made up to 1 ml with PVP/ethanol. This resulted in a DNA loading ratio (DLR) of 5 µg/mg gold and microcarrier loading quantity (MLQ) of 0.2 mg of gold per shot, which results in the delivery of 1 µg of DNA per shot. The abdominal fur was removed using commercial depilatory to expose the epidermis, the shaved area was cleaned with 70% ethanol and DNA-coated gold particles were delivered into the skin with the Helios gun at a helium pressure setting of 400 lb/in².

For administration, via oral route, two groups of BALB/c mice were inoculated orally (by gavage) with pgp.LAMP-1 or vector DNA. The DNA dose administered was approximately 100 µg per mouse.

B.2 Dose of DNA vaccine
For determining the optimum dose of DNA vaccine, mice were vaccinated with 50, 100 or 200 µg Endotoxin-free plasmid DNA in 200 µl PBS/animal thrice at three-week intervals. Control mice were immunized with vector DNA or PBS.

B.3 Adjuvant supplementation
For investigating the immune enhancement potential of adjuvants- EMULSIGEN® and EMULSIGEN®-D (MVP Laboratories, Inc., USA); 100 µg of plasmid DNA was mixed with adjuvant at 20% and volume was then made up to 200 µl with PBS. The mixture was administered intramuscularly thrice at three-week intervals. Control mice were immunized with only plasmid DNA (DNA vaccine or vector) or PBS.

C. Immunological assays
C.1 Determination of anti-glycoprotein antibody and its isotypes
The mice from each group were bled at respective days; sera were prepared and stored at -80 °C. Antigen-specific antibody (IgG total) and isotypes (IgG1, IgG2a) levels were determined by ELISA in the serum from immunized mice as described earlier [6]. The antibody response generated in a group of vaccinated mice was represented as the geometric mean of the absorbance obtained by pooled serum samples of the animals; the reaction being carried out in triplicates.

C.2 Virus Neutralizing Antibodies (VNA) assay
Mouse sera were tested for the presence of virus neutralizing antibodies by rapid fluorescent focus inhibition test (RFFIT) as described previously [10]. Briefly, sera were heat inactivated at 56 °C for 30 min. 100 µl of sera dilutions were mixed with 100 µl of the 50 FFD50 CVS-11 and added in 96-well tissue culture plate and incubated at 37 °C, 5 % CO₂ for 90 min. After the incubation period, BHK-21 cells (1×10³) were added to each well and the plates were incubated for 40 h. Following this incubation, cells were fixed with chilled acetone and stained with FITC-conjugated anti-Rabies monoclonal antibody (VMRD, USA) for 45 min. The wells were washed thrice with PBS, mounted in glycerol: PBS (1:1), and visualized under fluorescence microscope (Nikon, Japan). Data were expressed as the neutralizing antibody titer that is the mean of the serum resulting in a 50 % reduction in the number of the virus-infected cell foci in the presence of the test serum. Rabies Reference antiserum of known international units (IU/ml) of RVNA was included as positive control in the assay.

C.3 T-cell restimulation assay
Mice were sacrificed and spleens were dissected out. Splenic cells were prepared by grinding spleens between frosted slides. Erythrocytes were lysed with 0.1 M ammonium chloride. The spleen cells were then washed twice with DMEM medium and afterwards suspended in complete DMEM medium (supplemented with 10 % heat inactivated fetal bovine serum) and 10⁻⁶ M β-mercaptoethanol. Viability was determined by Trypan blue exclusion test. Splenocytes were cultured in triplicates (1X10⁶ cells/well) in a 24-well culture plate (Costar) and stimulated without antigen, with 5 µg/ml antigen (BPL inactivated PV-11 virus) or 1 µg/ml concanavalin A (ConA, Sigma), and incubated at 37 °C under 5 % CO₂ and 95 % humidity. Supernatants were harvested after 24, 48 and 72 h and the levels of cytokines were determined.

C.4 Evaluation of cytokine levels by ELISA
Levels of IL-4 and IFN-γ were determined using BD Opt EIA™ kits according to manufacturer's
protocol (Pharmingen). Briefly, 96 well microtiter ELISA plate was coated with capture antibody of the respective cytokines and incubated overnight at 4 °C. Wells were aspirated and washed thrice and subsequently blocked with 200 µl of 2 % BSA for 2 h at 37 °C. After the incubation period, wells were aspirated, washed thrice and then incubated with the harvested supernatants for 2 h at RT. The wells were then aspirated and washed five times; incubated with Detector (Anti-mouse IgG-HRP) for 1 h at RT. Following this, wells were aspirated and washed 7 times and incubated with 100 µl substrate solution for 30 min in dark at RT. Reaction was stopped by adding 50 µl stop solution (1M H$_3$PO$_4$) to each well. The absorbance was read at 450 nm using a Microplate Reader (Bio Rad) within 30 min of stopping the reaction. The concentrations of cytokines in the culture supernatants were calculated using a linear regression equation obtained from the absorbance values of the standards provided by the manufacturer.

C.5 Protective efficacy against intracerebral Rabies virus challenge
For challenge studies, immunized mice were intracerebrally inoculated with 20 LD$_{50}$ of rabies virus CVS strain 21 days after the last immunization. The challenged mice were observed for eighteen days for symptoms indicative of rabies virus infection. Mice that developed complete bilateral hind leg paralysis, characteristic of the terminal stage of rabies, were euthanized for humanitarian reasons. Surviving mice were kept and observed for an additional two to three weeks to ensure that they survived the infection. Survivorship rates obtained with the different vaccine constructs were compared and Kaplan-Meir curves for survival were plotted.

D. PEP
Mice were placed in eight groups of 10 mice each. The dose of the lethal challenge was 50 LD$_{50}$ of challenge virus standard in 0.03 ml volume. In all the groups, CVS was intramuscularly administered, 6 h later, the first dose of PEP was applied. Group 1 received 100 µg of DNA vaccine supplemented with E$p$, diluted in 200 µl of PBS by IM route. The next doses were applied on days 2 and 4 after the challenge. Group 2 similarly received the vaccine formulation, but the next doses were applied on days 0, 2, 4, 6 and 8 after the challenge. Groups 3 and 4 received only DNA vaccine on days 0, 2, 4 and 0, 2, 4, 6, 8 respectively. Groups 5 and 6 were immunized with vector, with three and five dose respectively. Group 7 was administered Rabipur – the conventional vaccine for humans by IM route (5 doses, one each on day 0, 2, 4, 6, 8). Group 8 was immunized with PBS. Blood samples were collected on days 7, 14, 21, 28 and 35. The sera were prepared and assessed for generation of RVNAs as described above. All the groups were also observed for development of rabies-specific paralytic symptoms or death and percent survival was calculated.

2.4 Immune potential of plasmid DNA vaccine in dogs
A. Dogs
Two to three months old dogs were used to verify the immunogenicity of pgp.LAMP-1. Dogs were maintained in pathogen free environment at the Animal House Facility at IVRI, Izatnagar.

B. Immunization
100 µg of DNA vaccine or empty vector was intramuscularly injected in to dogs. Boosters were given at days 21 and 42. The negative control group received only PBS.

C. Immunological assays
The serum from each dog was collected on day 62 post-immunization and analyzed for rabies-specific antibodies in virus neutralization test, as described above.

2.5 Statistical analysis
The experimental data were analyzed by Sigma Plot 10.1 and were expressed as means ± standard deviations (SD). Comparisons between individual data points were made using a Student’s t-test and levels of significance ($p$ value) were determined. $P$ value < 0.05 was considered statistically significant.

Results
Effect of route of administration on immune response
The present study was performed to analyze the effect of DNA vaccine following different protocols of vaccine administration. Figure 1A shows the result of an immunization experiment comparing geometric mean of antibody titers of mice that were vaccinated with plasmid DNA in
three ways: i) via gene gun (GG) with 1 μg DNA, ii) intramuscularly (IM) with 100 μg DNA and iii) orally (ORAL) administered with 100 μg DNA. The dose range was based on the optimal doses for delivery via respective routes, this was done to ensure that immune responses will not be misjudged due to inadequate dose of DNA vaccines employed in the study. Detectable levels of neutralizing antibody were observed in mice after priming via gene gun and intramuscular route (Fig. 1A). However, there wasn’t any significant antibody response subsequent to priming or booster administration via the oral route. With regard to the serum IgG subclasses, IM immunization resulted in an increase in both IgG1 and IgG2a titers, whereas GG immunization had a propensity to raise the IgG1 levels (Fig. 1B). There was no significant IgG subclass induction via the oral route. Splenocytes from the IM immunized mice stimulated with BPL-PV11 produced significantly high levels of both IL-4 and IFN-γ as compared to the control (PBS immunized) group (Fig. 1C). In contrast, GG immunized group mounted elevated levels of IL-4, indicating a typical Th2 skewed phenotype was induced in these mice. RVNA titer profile was assessed on day 62, post primary immunization. Mice immunized both IM and via GG elicited RVNA. IM route was found to be more potent in the same, with 2 fold enhancement in RVNA titer with respect to the GG route (Fig. 1D). Oral route didn’t mount neutralizing antibody response. 63 days post-primary vaccination, the mice were challenged intracerebrally with 20 LD50 of CVS of rabies virus. GG immunization protected 50% of the immunized mice. In comparison, IM immunized mice exhibited a survival percentage of 62.5% (P value < 0.05). All the mice immunized with pDNA via the oral route, vector DNA or with PBS didn’t survive the challenge (Fig. 1E).

Effect of dose of administration on immune response
The study compared 50, 100 or 200 μg dose levels of pDNA for induction of optimum immune responses. The effect of varying the antigen dose on serum antibody titer was compared over a period of 62 days for each of the three formulations (Fig. 2A). It was observed that group of mice receiving 50 μg pDNA had a lower antigen-specific titer than those groups receiving 100 or 200 μg pDNA. Thus, at lower levels, on increasing the dose of antigen administered, the immune response elicited also increased. However, when the dose administered was further increased to 200 μg, there was no relationship between an increase in antigen dose and immune response. Serum specific antibody titers of IgG1 and IgG2a were measured by ELISA with secondary antibodies specific for the homologous sub-class and of equivalent sensitivity of detection. IgG1 titers were consistently higher than IgG2a titers in all groups; indicative of Th2 type response (Fig. 2B). The pDNA vaccine immunized groups also demonstrated a significant increase in T cell response of group receiving 200 μg was not significantly different from that of group receiving 100 μg. RVNA titers were assessed by RFFIT; three weeks post the last immunization corresponding to the time of lethal challenge. The RVNA titer in all the groups of immunized mice was > 0.5 IU/ml; the minimum titer against Rabies as recommended by WHO. As shown in Fig. 2D, groups immunized with 100 or 200 μg of DNA elicited equivalent RVNA tiers of 16 IU/ml, while the group immunized with 50 μg exhibited lower titers of 4 IU/ml. The ability of these groups to confer protection upon challenge also mirrored these observations, whereby the 50 μg immunized group showed 42.85 percent survival in comparison to 57.14 percent survival in 100 or 200 μg immunized groups (Fig. 2E, P value < 0.05). Thus, overall, there appeared to be no advantage in administering more than 100 μg dose of pDNA by the intramuscular route.

Effect of adjuvant supplementation on immune response
We assessed the ability of EMULSIGEN adjuvants to enhance the immunogenicity and protective efficacy of a pDNA vaccine encoding glycoprotein. Groups receiving formulations of pDNA emulsified with E5 produced higher specific antibody titers to glycoprotein than groups receiving only pDNA or that emulsified with E (Fig. 3A). Further, IgG1 titers were significantly enhanced in the E5 supplemented group (Fig. 3B). On assessment of induction of effective cellular immunity, it was observed that, E5 supplemented group generated high IL-4 and IFN-γ titers, in comparison to the only naked
DNA vaccine or that E supplemented (Fig. 3C). The $E^D$ supplemented group also substantially increased anti-rabies virus neutralizing antibody titer to 32 IU/ml ($p$ value); 64 times the minimum level of RVNA as recommended by WHO (Fig. 3D). Additionally, it conferred complete protection against lethal intracerebral rabies virus challenge (Fig. 3E, $P$ value < 0.005). Thus, the efficacy of rabies DNA vaccine can be radically improved by including a potent formulation agent such as EMULSIGEND.

**Therapeutic potential of rabies DNA vaccine**

We also investigated the capacity of pDNA to confer protection in BALB/c mice experimentally inoculated with rabies virus challenge strain. The post-exposure therapy was then initiated with pDNA vaccine with/without $E^D$ as 3 or 5 dose regimens. Another group of mice received cell-culture derived vaccine Rabipur® as per its recommended five injection regimen as the post-exposure therapy. Control groups were immunized with vector backbone or PBS. The kinetics of rabies virus-neutralizing antibodies in mice that received post-exposure therapy, were investigated by RFFIT. PBS and vector immunized groups produced very low level of neutralizing antibodies (0.125 IU/ml) at day 7 post-challenge. Beyond this, neutralizing antibodies couldn’t be detected in these groups. The other groups had a consistent increase in neutralizing antibody production till day 21, after which they plateaued. In contrast, in mice vaccinated with pDNA supplemented with $E^D$, antibodies continued to rise till day 28, post-exposure, after which they plateaued (Fig. 4A). In the group, immunized with 5 doses of pDNA supplemented with $E^D$, significant RVNA titer of 4 IU/ml was attained ($P$ value < 0.05). As shown in Figure 4B, 100% of vector backbone or PBS vaccinated mice developed fatal rabies. On the contrary, when post-exposure therapy was carried out using three/five injections of the pDNA encoding the rabies glycoprotein, 60 % of mice survived the rabies exposure. Additionally, three injections of the pDNA supplemented with $E^D$ and five injections of Rabipur® conferred equivalent partial protection of 80%. Further, five injections of the pDNA supplemented with $E^D$ conferred 100% protection in the post exposure efficacy analysis ($P$ value < 0.005). Therefore, considering the present 5 dose regimen for post-exposure prophylaxis, the optimized DNA vaccine formulation is superior to the currently employed Rabipur®.

**Prophylactic potential of rabies DNA vaccine in dogs**

The dogs immunized with rabies DNA vaccine induced virus neutralizing antibodies of 8 IU/ml, assayed at day 62 post-immunization, indicating protective status against rabies as recommended by WHO. The vector and PBS immunized dogs had VN titer of <0.5 IU/ml (Fig. 5). The dogs could not be challenged with rabies challenge virus because of biosafety and bioethics considerations.

**Discussion**

The prime issue in developing an effective DNA vaccine is to design protocols that can be translated from murine models to large animal models and eventually clinical human trials without affecting their potency [11; 12]. Although DNA vaccines have been shown to elicit potent immune responses in animal models, initial clinical trials in humans have been disappointing, highlighting a need to optimize their immunogenicity.

In the present study, we investigated whether the delivery route and dose of rabies pDNA vaccine and chemical adjuvants can modulate the outcome of immune response elicited against it. We found that the immune response against optimized vaccine formulation correlated well with both its prophylactic and therapeutic potential against rabies.

The quality of the immune response elicited by a DNA vaccine is dependent upon the DNA delivery that influences the mechanisms of DNA uptake, transgene expression, and transgene product processing [13]. Several studies have demonstrated the efficacy of various modes of DNA immunization in animal models and highlighted that diverse protocol of DNA immunization result in the activation of different immune responses with respect to magnitude and duration and thus, exert different protective effects [14-21].

Thus, the role of the DNA delivery system on the outcome of the vaccine should be considered in the elaboration of Rabies DNA vaccine. Mostly, rabies DNA vaccines are intramuscularly injection (IM) [22]. In other regimens, cutaneous
delivery, such as intradermal injection [23] or gene gun (GG) delivery [24], has been preferred to intramuscular delivery for DNA plasmids administration with respect to higher efficacies with lower doses of pDNA. Intranasal administration was also effectively employed for rabies prophylaxis [25]. Only few studies directly compared their efficacy for establishment of a generalized regimen for successful immunization [24, 25].

In the present study, we compared three routes of delivery, namely, gene gun immunization, intramuscular injection and oral administration. Oral administration didn’t lead to seroconversion in mice. The gene gun immunization involving 1 μg of DNA was found to be highly efficient in eliciting Th2 type of immune response, which is established to be protective for rabies. It also imparted 50 % protection in the challenge studies conducted in BALB/c mice as against that of 62.5% via intramuscular route employing 100 μg of DNA vaccine. Thus, gene gun immunization is highly potent in generating protective responses with limited amount of plasmid DNA. It is a highly efficient method of achieving antigen presentation; and as a result immunization requires up to 2,500 times less DNA than the standard intramuscular delivery [26]. This is due to the dense network of Langerhans cells that are found in the epidermis, acting as a source of antigen-presenting cells [27]. However, there are some shortfalls in the usage of gene gun; the limitation of amount of DNA that can be delivered requires multiple immunizations in larger animals and the preparation of gold coated particles [28]. Also, until now there is no large scale manufacturing process for plasmid coated gold particles which further adds on to the cost of immunization. A highly skilled person is required for the preparation of coated gold particles and their administration. Therefore, due to cost considerations and practical constraints of gene gun immunization for field applications, we employed intramuscular injection for further studies on assessing generation of sustained and protective immune response against rabies.

The other important consideration for immunization is the dose of administration. In some clinical trials, immune responses measured after DNA immunization was highest corresponding to the highest doses [29; 30]. Thus, the relatively poor immunogenicity of plasmid pDNA in some clinical trials may be in part due to the low doses being used, rather than solely to species specific differences in the response to pDNA. Therefore, we investigated the optimum dose for DNA immunization. To determine the same, we immunized groups of Balb/c mice with varying doses of the pgp.LAMP-1 pDNA formulated in PBS. The dose was varied as 50, 100 and 200 μg/mice. The greatest effect, a 2.2 fold increase in antibody titer was seen at the lower dose of DNA, from 50 to 100μg. This effect of antigen dose dependence may reflect recruitment of more antibody-forming cells persisting through the booster response or dose dependence of immunological memory for local antibody formation. There was marginal increase in antibody titer on further increasing the dose to 200. However, the RVNA and percent protection conferred didn’t exhibit any increment corresponding to the highest DNA dose tested. Thus, a higher pDNA doses (200 μg) had little effect on any of the measured outcomes and 100 μg DNA was adequate to induce protective state against rabies.

DNA vaccines are often unable to provide sufficient protection against challenge infection. Several approaches have been investigated for enhancing the efficacy of DNA vaccines. These mostly relied on usage of genetic or chemical adjuvants. Genetic adjuvants based on cytokines like IL-2, IL-4, IL-12, IL-15, IFN-γ, GM-CSF and CpG oligonucleotides are most commonly employed for enhancing the potency of DNA vaccine. Among various cytokines, DNA vaccines employing IL-2 are most frequently employed for immune enhancement like for HBsAg-specific immune response in hepatitis [31], glycoprotein D antigen based immune response against in HSV1 [32], therapeutic efficacy against HER2/Neu-expressing tumors [33]. CpG oligonucleotides (unmethylated cytidine-phosphate-guanosine) activate B cells and dendritic cells (DCs) and induce pro-inflammatory cytokines [34]. Chemical adjuvants include a range of compounds, aluminium salts, calcium phosphate, DDA, liposomes, polymers, microparticles; they have shown potential in enhancement of expression and immunogenicity of plasmid DNA vaccines in different animal models. Aluminium salts, which constitute one of the few adjuvants approved for humans, were shown to enhance the potency of DNA vaccine up to 100-fold in
mice and guinea pigs, and 5-10-fold in non-human primates by enhancing the immunogenicity of expressed antigen in situ [35]. Another report showed that aluminium phosphate co-administered with a rabies DNA vaccine enhanced both the onset and magnitude of the serological response in ponies; however, DNA vaccine formulation with DMRIE–DOPE generated superior immune response [36].

The experiments carried out here evaluated the immune stimulatory potential of EMULSIGEN® adjuvants- EMULSIGEN® (E) and EMULSIGEN®-D (E'). EMULSIGEN® represents a mineral oil-in-water emulsion containing 1-2 micron size oil droplets; EMULSIGEN®-D further incorporates dimethyldioctadecylammonium bromide (DDA) as an additional immunostimulant in the emulsion. Such oil-in-water emulsions increase the surface area available to antigens. They form a mobile depot of antigen from which antigen is subsequently slowly released. This improves the presentation of antigen thus providing a significant enhancement of the immune response. The antibody profile suggests that E' has the potential to elicit higher levels of humoral antibody and more rapid onset of immunity; thus, it can act as an immunostimulant for the rabies DNA vaccine; however E didn't have any immunostimulatory effect. The SIV vaccine containing E' was also found to produce significantly higher HI titers against both H1N1 and H3N2 [37]. The IgG isotype data obtained in this study indicates that the adjuvant E' has the inclination towards IgG1 response. The profile of the subclasses of IgG antibody induced in mice is believed to reflect the Th1:Th2 response stimulated, and it is generally accepted, that an IgG1 response is induced by help from Th2 cells whereas Th1 activity leads additionally to IgG2a and IgG2b antibody production [38]. The key component of E'; DDA is well established as an immunologic adjuvant for experimental protein vaccines. Subsequently, it was investigated for in vitro and in vivo delivery of polynucleotides [39].

A study conducted in pigs showed that efficacy of a DNA vaccine against pseudorabies virus (PRV) was enhanced in the presence of DDA. Vaccinated pigs were protected against clinical disease and shed significantly less PRV after challenge infection [40]. Therefore, the inclusion of potent adjuvants like EMULSIGEN®-D in vaccines may very well obviate the need of high doses of DNA, multiple boosters and long schedule required for successful vaccination.

Pioneer studies on rabies DNA vaccine reported that it could induce a potent antibody response but with a slow onset [41]. Multiple boosters and long incubation periods, up to ten weeks were required to generate strong immune responses in mice. Under such circumstances, efficacy of DNA vaccine in post-exposure therapy against rabies was questionable. However, a study investigated the immunogenicity of a rabies DNA vaccine and reported it to be superior to the five dose regimen of cell culture-derived vaccine in post-exposure efficacy assessment [42]. Another study showed that four doses of intranasally applied rabies DNA vaccine were superior to intramuscularly administered traditional antirabies vaccine, in both mice and rabbits that received the PEP [43]. In the present study, we also investigated the post exposure efficacy of this vaccine formulation. Here, the standard regimen based on five injections of cell culture derived vaccine was compared with the one receiving rabies DNA vaccine with or without E' after viral challenge in mice. The latter therapies were able to elicit virus neutralizing antibody response as quickly as the former. Within 14 days of challenge, significant levels of neutralizing antibodies (higher than 0.5 IU/ml) were raised. At day 35 (last time point for post exposure efficacy analysis), the RVNA titer in Rabipur immunized group was same as in DNA vaccine group. The RVNA profile in DNA vaccine formulation (DNA + E') was elevated than this. This could be correlated with the subsequent protective effect against the lethal disease. Three doses of intramuscularly applied DNA vaccine formulation gave equivalent protection as the five dose regimen of conventional vaccine (Rabipur) under the same conditions. Five doses of DNA vaccine formulation imparted complete protection against experimental rabies. Therefore, our and previous studies pertaining to rabies DNA vaccination, indicate that this strategy is not only apt for preventive vaccination but also for post-exposure treatment of rabies.

Few studies have been conducted concerning vaccination of dogs with rabies DNA vaccine. One report suggested that the IM route of rabies DNA vaccination is an effective route to vaccinate dogs. All the dogs seroconverted in the
study, with RVNA titers ranging from 1:11 to 1:200 after priming [44]. They concluded that IM route of vaccination results in stronger and more durable virus neutralizing antibody titers than those obtained by intradermal inoculation. Another group reported that only half of dogs receiving a primary IM rabies DNA vaccination seroconverted [22]. Multiple immunizations were required to achieve complete seroconversion. In agreement with this study, Lodmell et al. demonstrated that minimal or undetectable levels of neutralizing antibody were produced in dogs after a single-dose IM vaccination with a rabies DNA vaccine [45]. In accordance with studies of Perrin et al. and Lodmell et al., we also observed minimal neutralizing antibody titers after priming with DNA vaccine (data not shown). Two booster immunizations were given after which all dogs showed seroconversion for rabies and exhibited high rabies virus neutralizing antibody titers (8 IU/ml). The concentration of DNA employed in prior studies did not appear to be as significant factor as site of vaccinations for generating different levels of neutralizing antibody. While administration in the semitendinosus muscle elicits excellent antibody responses, vaccination in the cranial tibial muscle of hind limb and the quadriceps muscle elicits a poor neutralizing antibody response [45]. These results again emphasize on the fact that method of delivery and site of vaccination influence neutralizing antibody responses of dogs immunized with different rabies DNA vaccines. Thus, the immune enhancement studies conducted in murine model of rabies also need to be evaluated in dogs by different methods of delivery, sites of vaccination, dose of vaccination, potent adjuvants; for their ability to elicit neutralizing antibody response and confer protection on rabies challenge before they can be exploited for extensive vaccination drive.

Conclusion
The present study was performed to examine the influence of the administration route and dose of rabies DNA vaccine, and adjuvant supplementation on induction of protective humoral response in murine model of rabies. The results indicate that both GG and IM administration of rabies DNA vaccine per se can build up a strong humoral immune response against experimental rabies in mice. The optimum dosage via intramuscular injection was established as 100 µg. The adjuvant-EMULSIGEN® was found to enhance the magnitude of immune response elicited against the rabies DNA vaccine. Thus, the identification of the best mode of DNA delivery and suitable dosage represents a basic step for the optimization of genetic immunization protocols that can be further improved by the use of immune adjuvant. Such approaches may pave the way towards advancement in vaccination strategies, if they can be well correlated with the induction of protective immune response. To conclude, this approach has great potential for prophylactic and therapeutic applications. Further refinement in the immunization strategy such that a single dose of DNA vaccine formulation can confer complete protection may prove beneficial especially in the developing countries; reducing costs and improving population coverage.

Acknowledgments
Inactivated PV viral antigen and Rabies Reference Antiserum were kindly provided by Dr. V. Srinivasan, Indian Immunological Ltd. We are grateful to Anuj Kumar Sharma, School of Biotechnology, JNU; for his help in manuscript preparation. We acknowledge the financial support from the Department of Biotechnology, Government of India for carrying out this work. Manpreet Kaur is recipient of Senior Research Fellowship from CSIR, Government of India.

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and cats with a DNA vaccine against rabies virus. Vaccine 17 (9-10), 1109–16.

**Figure legends**

Fig. 1A: Humoral immune response in mice vaccinated with pgp.LAMP-1. Female BALB/c mice were immunized via different means: gene gun (GG), intramuscularly (IM) or orally (ORAL) with DNA vaccine plasmid, vector DNA or PBS on days 0, 21 and 42. On days 20, 41 and 62, mice were bled, sera were prepared; and subsequently analyzed for anti-RV-G antibodies by ELISA. The figure represents vertical bar charts of mean antibody titers for each group. For clarity, only intramuscularly immunized vector DNA is shown in the figure.

Fig. 1B: The isotype profile of the RV-G-specific IgG1 (a) and IgG2a (b) titers in mice immunized via different routes. Mice were bled at three weeks interval and glycoprotein-specific IgG1 and IgG2a titers were detected by ELISA. Data shown represents geometric mean titers and standard deviations (SD) for each group of animals.

Fig. 1C: Concentrations of cytokines in cell-culture supernatants of BALB/c mouse splenocytes. Splenocytes (5 X 10^5 cells/ml) were stimulated with 5 μg/ml of BPL-inactivated PV-11 virus. 24, 48 and 72 h later, culture supernatants were collected and analyzed by a capture ELISA for IL-4 and IFN-γ. Splenocytes from two mice immunized with DNA vaccine via different routes were included in each experiment. Data are expressed as mean values ± SD of triplicates.

Fig. 1D: Rabies virus neutralizing antibody (RVNA) titers following immunization via different routes in mice. Sera were obtained from mice at day 62 and assayed for the presence of RVNA response. RVNA titers are presented as the mean from each group. Rabies protective titer threshold at 0.5 IU/ml is recommended by WHO.

Fig. 1E: Survival percentage of mice immunized with rabies DNA vaccine via different routes. All mice were challenged intracerebrally with 20 LD_{50} of CVS strain of Rabies virus on day 62 and observed for 18 days for rabies specific symptoms or death.

Fig. 2A: Humoral immune response in mice vaccinated with different concentrations of pgp.LAMP-1 DNA vaccine or empty vector. For comparison purpose, only the group immunized with 100 μg of empty vector is represented in the figure. On indicated time periods, mice were bled, sera were prepared; and subsequently analyzed for anti-RV-G antibodies by ELISA. The figure represents vertical bar charts of mean antibody titers for each group.

Fig. 2B: Effect of dose of pDNA vaccine on IgG isotypes of anti-GP antibodies. Groups of mice were immunized intramuscularly with 50, 100 or 200 μg of plasmid DNA. Data represent the geometric mean RV-G-specific IgG1 (a) and IgG2a (b) titers and SD for each group of animals.

Fig. 2C: Concentrations of cytokines in cell-culture supernatants of BALB/c mouse splenocytes. Splenocytes (5 X 10^5 cells/ml) were stimulated with 5 μg/ml of BPL-inactivated PV-11 virus. 24, 48 and 72 h later, culture supernatants were collected and analyzed by a capture ELISA for IL-4 and IFN-γ. Splenocytes from two mice immunized with different dose of DNA vaccine were included in each experiment. Data are expressed as mean values ± SD of triplicates.

Fig. 2D: RVNA titers following intramuscular immunization with varied dose of pDNA vaccine in mice. Sera were obtained from mice at day 62 and assayed for the presence of RVNAs. RVNA titers are presented as the mean from each group. 0.5 IU/ml is recommended as the rabies protective threshold titer by WHO.

Fig. 2E: Survival percentage of mice immunized with different concentrations of rabies DNA vaccine. All mice were challenged intracerebrally with 20 LD_{50} of CVS strain of Rabies virus on day 62 and observed for 18 days for rabies specific symptoms or death.

Fig. 3A: Humoral immune response in mice vaccinated with pgp.LAMP-1 potentiated with
EMULSIGEN adjuvants (E, E°). Periodically, mice were bled, sera were prepared; and subsequently analyzed for anti-RV-G antibodies by ELISA. The figure represents vertical bar charts of mean antibody titers and SD for each group.

Fig. 3B: Effect of adjuvant supplementation of pDNA on IgG isotypes of anti-GP antibodies. Glycoprotein-specific IgG1 (a) and IgG2a (b) titers were detected by ELISA. Data represent the geometric mean RV-G-specific IgG1 and IgG2a titers and SD for each group of animals.

Fig. 3C: Concentrations of cytokines in cell-culture supernatants of BALB/c mouse splenocytes. Splenocytes (5 X10^5 cells/ml) were stimulated with 5 µg/ml of BPL-inactivated PV-11 virus. 24, 48 and 72 h later, culture supernatants were collected and analyzed by a capture ELISA for IL-4 and IFN-γ. Splenocytes from two mice immunized with DNA vaccine with/without EMULSIGEN adjuvants were included in each experiment. Data are expressed as mean values ± SD of triplicates.

Fig. 3D: RVNA titers following intramuscular immunization of rabies DNA vaccine with/without adjuvant in BALB/c mice. Sera were obtained from mice at day 62 and assayed for the presence of neutralizing antibodies. RVNA titer equivalent to 0.5 IU/ml is the minimum adequate titer against rabies as recommended by WHO.

Fig. 3E: Survival percentage of mice immunized with rabies DNA vaccine with/without EMULSIGEN adjuvant. All mice were challenged intracerebrally with 20 LD₅₀ of CVS strain of Rabies virus on day 62 and observed for 18 days for rabies specific symptoms or death. pgp.LAMP-1 DNA vaccine supplemented with EMULSIGEN° conferred complete protection in the efficacy assessment.

Fig. 4A: RVNA titers in BALB/c mice after post-exposure treatment with either DNA vaccine or cell culture-derived vaccine (Rabipur). Mice were challenged at day 0 with rabies virus and treated as detailed in methods. Sera were obtained at days 7, 14, 21, 28 and 35 and assayed for the presence of RVNA response. Results correspond to the mean RFFIT antibody titers of each group expressed in IU/ml.

Fig. 4B: Percent survival of BALB/c mice challenged with rabies virus and treated with a post-exposure vaccination using either DNA vaccine or cell culture-derived vaccine (Rabipur). Mice were challenged intramuscularly at day 0 with 50 LD₅₀ a rabies challenge virus. Six hours later, post exposure treatment was initiated as described in the methodology. The results represent the percent survival of mice monitored up to 35 days after the viral challenge. pgp.LAMP-1 DNA vaccine supplemented with EMULSIGEN° conferred complete protection in the post-exposure efficacy assessment.

Fig. 5: RVNA titers following intramuscular immunization of rabies DNA vaccine in dogs. Sera were obtained from each dog at day 62 and assayed for the presence of neutralizing antibodies. RVNA titers are presented as the mean from each group.
Fig. 1D

Fig. 1E
Fig. 2A

Fig. 2B

Fig. 2C
Fig. 3D

![Graph showing RVNA Titer (IU/ml) vs. Days post-challenge for pDNA, pDNA-E, pDNA-ED, VECTOR, and PBS.]

Fig. 3E

![Graph showing Percent survival vs. Days post-challenge for pDNA, pDNA-E, pDNA-ED, VECTOR, and PBS.]
Fig. 5

![Graph showing RVNA Titer (IU/ml) vs. Days, post-challenge for pDNA, VECTOR, and PBS.](image)
Rabies DNA vaccine: No impact of MHC Class I and Class II targeting sequences on immune response and protection against lethal challenge

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ARTICLE INFO

Article history:
Received 14 November 2008
Received in revised form 24 January 2009
Accepted 29 January 2009
Available online 6 February 2009

Keywords:
Targeting sequence
Rabies virus-neutralizing antibody (RVNA)
Survival

ABSTRACT

Rabies is progressive fatal encephalitis. WHO estimates 55,000 rabies deaths and more than 10 million PEPP every year world-wide. A variety of cell-culture derived vaccines are available for prophylaxis against rabies. However, their high cost restricts their usage in developing countries, where such cases are most often encountered. This is driving the quest for newer vaccine formulations; DNA vaccines being most promising amongst them. Here, we explored strategies of antigen trafficking to various cellular compartments aiming at improving both humoral and cellular immunity. These strategies include use of signal sequences namely Tissue Plasminogen Activator (TPA), Ubiquitin (UQ) and Lysosomal-Associated Membrane Protein-1 (LAMP-1). TPA, LAMP-1 and their combination were aimed at enhancing the CD4+ T cell and antibody response. In contrast, the UQ tag was utilized for enhancing CD8+ response. The potency of modified DNA vaccines assessed by total antibody response, antibody isotypes, cytokine profile, neutralizing antibody titer and protection conferred against in vivo challenge; was enhanced in comparison to native unmodified vaccine, but the response elicited did not pertain to the type of target sequence and the directed arm of immunity. Interestingly, the DNA vaccines that had been designed to generate different types of immune responses yielded in effect similar response. In conclusion, our data indicate that the directing target sequence is not the exclusive deciding factor for type and extent of immune response elicited and emphasizes on the antigen dependence of immune enhancement strategies.

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and tuberculosis [4-6]. In addition, human clinical trials have established their safety and potency, further encouraging studies in this direction [6-10].

In this regard, various DNA vaccination strategies have shown to provide protection against lethal rabies virus challenge. These strategies relied on the usage of adjuvants like cationic-lipids [11,12], intradermal injection using gene gun [13-15], repeated DNA vaccination [16,17] or DNA vaccine in association with a single dose of anti-rabies immune serum [18] for immune enhancement. Prophylactic immunization was found to be effective in preventing canine rabies [17,19,20]. Rabies DNA vaccine has also been found to be highly efficient in large size mammals [21]. For post-exposure treatment, single dose of Rabies DNA vaccine was found to be as potent as 5 dose regimen of cell-culture vaccine in BALB/c mice [22].

Considering these studies, we attempted further improvement in humoral and cell-mediated immune response elicited by DNA vaccination by antigen trafficking to various cellular compartments. Efficient delivery of antigens to both MHC Class I and Class II processing and presentation pathways is required for generating an ideal immune response comprising of both cos+ and cos- lines of anti-rabies immune serum [18] for immune enhancement. Accordingly, this study investigates strategies for targeting glycoprotein antigen to MHC Class I and Class II pathways for improving its antigenicity, immunogenicity and protective efficacy.

For targeting MHC Class II pathway, we utilized Tissue Plasminogen Activator (TPA) and human Lysosomal-Associated Membrane Protein-1 (LAMP-1) signal sequences. TPA-fused antigens are highly expressed secreted proteins with elevated uptake by antigen-presenting cells; and thus, bring about a more generalized activation of the immune system. They have been shown to induce significant humoral and cell-mediated responses [23]. LAMP-1 is a type of transmembrane protein localized predominantly to lysosomes and late endosomes. Antigen trafficking of LAMP-1-fused antigens to the cellular site of MHC Class II processing and presentation pathway could enhance its presentation to MHC Class II restricted CD4+ T cells [24] and thus augment the humoral response. On the other hand, for directing the antigen to MHC Class I, signal sequence of Ubiquitin A-76 (UQ) was employed. UQ-conjugated antigens are trafficked through the proteasome, an organelle that generates short peptides for presentation via the MHC Class I pathway [25]. Such UQ-conjugated antigens are expected to enhance the cellular immune response. UQ-conjugated proteins have been shown to generally undergo rapid intracellular degradation and can elicit cytokine responses in the absence of specific antibody production [26]. Thereby, we explored the potential of targeted DNA vaccine encoding the glycoprotein antigen fused to TPA, LAMP-1 or UQ to elicit superior immune response in comparison to the unmodified (without target sequence) DNA vaccine.

2. Materials and methods

2.1. Cells

Baby hamster kidney (BHK)-21 cells; procured from National Centre for Cell Science (NCCS), Pune, India were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biological Industries) and 100 U/ml Penicillin (Amersham) and 100 μg/ml Streptomycin (Amersham), in a humidified 5% CO₂ incubator at 37°C.

2.2. Virus

Virus Pitman-Moore (PV-11) strain of rabies virus was propagated on BHK-21 cells. Virus was purified, inactivated with beta-propionolactone (BPL) and used for in vitro re-stimulation assay. The Challenge Virus Standard (CVS-11) strain was propagated and maintained in mice brain. It was titrated on BHK-21 cells to determine the optimal dose for rapid fluorescence focus inhibition test (RFFIT) to determine virus neutralization antibodies and for intracerebral rabies virus challenge to determine protection conferred.

2.3. Cloning of glycoprotein in mammalian expression vectors

Plasmid pTarget-Rab-G [27] was used as the parental plasmid for construction of all the clones used in this study. The glycoprotein (1.57 kb) gene was amplified by PCR from pTarget-rabgp plasmid using sequence-specific primers (Supplementary Table 1) and cloned in eukaryotic plasmids bearing address tags; pDNAVACC vectors (Nature Technology Corporation, Nebraska). The sequences of clones bearing the address tags, ppg-Native, pTPA-gp.LAMP-1 (GenBank Accession Number EU715587), pTPA gp (GenBank Accession Number EU715587), pLAMP1 (GenBank Accession Number EU715588) were confirmed by sequencing using ABI PRISM, Model 3730, Version 3.0 (Sequencing primers listed in Supplementary Table 1) and designated as represented in the Fig. 1. The respective constructs were processed for the purification of plasmid DNA using the Endofree plasmid isolation maxi kit (Qiagen) according to the manufacturer’s instructions. The purified plasmid DNA (1–2 mg/ml) was dissolved in autoclaved Milli Quartz (MQ) water and stored at -20°C, until further use. The glycoprotein gene was similarly PCR amplified (see Supplementary Table 1 for primer sequences) using pTarget-rabgp as template and cloned in pQE30 expression vector (T5 expression system). rGP was expressed as a fusion protein with 6x histidine tag in E. coli SG (pREP-4) strain and was purified on a Ni²⁺-NTA column to more than 95% homogeneity under native conditions. The rGP was dialyzed against 10 mM HEPES overnight and stored in aliquots at -80°C.

2.4. In vitro expression of candidate DNA vaccines

The ability of vaccine constructs to express glycoprotein antigen was studied in vitro in a mammalian cell-culture system. Briefly, BHK-21 cells were cultured and seeded at a density of 1 x 10⁶ cells/ml in a 24-well tissue culture plate, a day prior to transfection. BHK-21 cells were subsequently transfected with 800 ng DNA complexed with 2 μl of Lipofectamine (Invitrogen) and 8 μl of Plus Buffer (Invitrogen). The analysis of expression and localization was carried out 40 h post-transfection. For assessing expression, flow cytometric analysis was carried out, in which BHK-21 cells were transfected with various DNA vaccine combinations. Transfected cells were fixed with 2% paraformaldehyde (PFA) and then permeabilized in 0.1% Triton X-100 in PBS. The cells were then probed with mouse anti- rabies polyclonal sera, diluted in PBS containing 0.5% BSA; followed by staining with Alexa Fluor 488 labeled secondary antibody (Molecular Probes). Fixed BHK-21 cells were then analyzed. Ten thousand cells per sample were analyzed using FL1 filter (525 nm) and percent green fluorescent cells were recorded using Quanta SC MPL Analysis Software Version 1.0 (Beckman Coulter Inc.).

For immunoblotting, total cell lysate was prepared. Transfected cells were solubilized using radioimmunoprecipitation assay (RIPA) buffer (50 mM tris(hydroxymethyl)aminomethane (tris), pH 7.4, 150 mM sodium chloride (NaCl), 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenyl methyl sulphonyl fluoride (PMSF)) supplemented with protease...
inhibitor cocktail (Sigma). Lysosomal fraction was extracted using lysosome enrichment kit for tissue and cultured cells (Pierce) according to the manufacturer's protocol. The presence of lysosomes in different fractions was determined by analyzing the activity of β-hexosaminidase [28]. Cell membrane protein fraction was prepared by QProteome membrane protein kit (Qiagen) according to the manufacturer's protocol. The presence of cell membrane in the fractions was determined by the associated NADH oxidase activity [29]. Culture supernatant proteins were precipitated by ice-cold acetone. Solubilized proteins from the total cell lysate, lysosomes, membranes and cell-culture supernatants were subjected to 12% SDS-PAGE, blotted on to nitrocellulose membrane and probed with mouse anti-rabies polyclonal sera, followed by incubation with goat anti-mouse immunoglobulins conjugated with alkaline phosphatase (Sigma) and visualized with NBT/BCIP substrate (Sigma).

2.5. Immunization of mice

All animal experiments were conducted in compliance with the animal ethics committee. Four to six weeks old female BALB/c mice were used to verify the immunogenicity of the constructs. Mice were purchased from NIN, Hyderabad; and maintained in pathogen free environment at the Animal House Facility. Each group comprised of ten mice. Mice were vaccinated intramuscularly (i.m.) with 100 μg endotoxin-free plasmid DNA in 200 μL PBS/animal in the individual groups (DNA vaccine or vector control), thrice at 20-day intervals. Control mice were immunized with only PBS. The mice from each group were bled at days, 20, 41 and 62; sera were prepared and stored at –80°C.

2.6. Determination of anti-glycoprotein antibody and its isotypes

Antigen-specific Antibody (IgG total) and isotypes (IgG1, IgG2a) levels were determined by ELISA in the serum from the immunized mice. Recombinant glycoprotein, expressed in bacterial system (500 ng/well) in 100 μl of 0.1 M PBS was coated overnight at 4°C [30]. Plates were then blocked with 2% BSA in PBS for 2 h at 37°C followed by three washings with PBS-Tween (0.05%). This was followed by incubation with sera samples for 2 h at 37°C and washing with PBS-T. Secondary antibodies, anti-mouse IgG or its isotypes conjugated with hors eradish peroxidase; raised in sheep (Santa-Cruz) were incubated for 1 h at 37°C. Estimation of the enzymatic activity was carried out using TMB as the substrate. The reaction was stopped with 50 μl of 1 M H3PO4 and the absorbance was measured at 450 nm, with 630 nm as the reference filter using Microplate Reader (Bio Rad). The antibody response generated in a group of vaccinated mice was represented as the geometric mean of the absorbance obtained by pooled serum samples of the animals; the reaction being carried out in triplicates.

2.7. Virus-neutralizing antibodies (VNA) assay

Mouse sera were tested in vitro for the presence of virus-neutralizing antibodies with RFFIT, as described previously [31]. Briefly, sera from mice were heat inactivated at 56°C for 30 min. 100 μl of various sera dilutions were mixed with 100 μl of the CVS-11 strain of rabies virus (containing 50 FID50) in 96-well tissue culture plate and incubated at 37°C, 5% CO2 for 90 min. After the incubation period, BHK-21 cells (1 x 105) were added to each well and the plates were incubated for 40 h, following which they were fixed with chilled acetone and stained with FITC-conjugated anti-rabies monoclonal antibody (VMRD, USA) for 45 min. The wells were washed thrice with PBS, mounted in glycerol: PBS (1:1), and visualized under fluorescence microscope (Nikon, Japan). Data were expressed as the neutralizing antibody titer that is the mean of the serum resulting in a 50% reduction in the number of the virus-infected cell foci in the presence of the test serum. Rabies Reference antisera of known international units (IU/ml) of rabies virus-neutralizing antibody was included as positive control in the assay.

2.8. T-cell re-stimulation assay

Spleenic cells were prepared by grinding spleens between frosted slides. Erythrocytes were lysed with 0.1 M ammonium chloride. Remaining spleen cells were washed twice with DMEM medium and then were suspended in complete DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 10−6 M 2-mercaptoethanol. Viability was determined by Trypan blue exclusion test. Splenocytes were cultured in triplicate (1 x 105 cells/well) in a 24-well culture plate (Costar), stimulated without antigen or with 5 μg/ml of BPL inactivated PV-11 virus or concanavalin A (ConA) (1 μg/ml; Sigma), and incubated at 37°C under 5% CO2 and 95% humidity. Supernatants were harvested after 24, 48 and 72 h and the levels of cytokines were determined.

2.9. Evaluation of cytokine levels by ELISA

Levels of IL-2, IL-4, IL-12, and IFN-γ were determined using BD Opt EIA™ kits according to manufacturer's protocol (Pharmingen). Briefly, 96-well microtiter ELISA plate was coated with capture antibody of the respective cytokines and incubated overnight at 4°C. Plate was aspirated and washed thrice and blocked with 200 μl
Fig. 2. (a) Flow cytometric analysis of cells expressing rabies glycoprotein. BHK-21 cells transfected with various plasmid DNA constructs were stained with anti-rabies hyperimmune sera as the primary antibody. The number of cells showing fluorescence, after staining with Alexa Fluor 488 labeled secondary antibody were analyzed using FL1 and displayed as histograms, which are means ± S.D. were obtained from duplicate cultures. Actin was used as positive control. (b) The address tags efficiently target glycoprotein to various subcellular locations. Cell lysates, lysosomal fractions, concentrated culture supernatant and membrane fractions were prepared 40 h post-transfection. Subsequently, the protein samples were resolved on 12% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. Presence of rabies glycoprotein was detected using mouse polyclonal anti-rabies hyperimmune sera followed by alkaline phosphatase-conjugated anti-mouse IgG. The blot was developed using BCIP/NBT as substrate.
of 2% BSA for 2h at 37°C. After the incubation period, plate was aspirated and washed thrice and incubated with the harvested supernatants for 2h at RT. The plate was then aspirated and washed five times; plate was incubated with Detector (Anti-mouse IgG-HRP) for 1h at RT. Following this, plate was aspirated and washed 7 times and incubated with 100 μl substrate Solution for 30 min in dark at RT. Reaction was stopped by adding 50 μl Stop Solution to each well. The absorbance was read at 450 nm using a Microplate Reader (Bio Rad) within 30 min of stopping the reaction. The concentrations of cytokines in the culture supernatants were calculated using a linear regression equation obtained from the absorbance values of the standards provided by the manufacturer.

2.10. Protective efficacy against intracerebral rabies virus challenge

Each vaccine construct was tested in two independent experiments. For challenge, immunized mice were inoculated with 20 LD₅₀ of rabies virus CVS-11 strain intracerebrally 21 days post-immunization. The challenged mice were observed for 18 days for symptoms indicative of rabies virus infection. Mice that developed complete bilateral hind leg paralysis, characteristic of the terminal stage of Rabies, were euthanized for humanitarian reasons. Upon challenge, PBS or vector vaccinated mice died within 6–13 days. Surviving mice were kept and observed for an additional two to three weeks to ensure that they survived the infection. Survivorship rates obtained with the different vaccine constructs were compared.

2.11. Statistical analysis

The experimental data were analyzed by Sigma Plot 10.1 and were expressed as means± standard deviations (S.D.). Comparisons between individual data points were made using a Student’s t-test and levels of significance (P value) were determined. P value <0.05 was considered statistically significant.

3. Results

3.1. Construction and expression of RV-G DNA vaccine constructs

RV-G based DNA vaccine constructs were made wherein the glycoprotein gene was fused to various signal sequences (Fig. 1) to analyze the influence of signal sequences on immunogenicity and generation of RVNA titers. The sequences of insert in native DNA vaccine construct (pgp) and modified constructs bearing the target sequences, pT.gp.L, pT.gp, pU.gp, pgp.L were confirmed by sequencing.

For assessing the expression of DNA vaccine constructs, transiently transfected BHK-21 cells were subjected to flow cytometric analysis. The percentages of cells stained with the antibody are shown in the figure (Fig. 2a). The transfected cells were expressing the rabies glycoprotein as indicated by fluorescence recorded as 69.33%, 55.28%, 74.43%, 69.41% and 74.85% for pgp, pT.gp, pT.gp.L, pU.gp and pgp.L respectively; whereas the control cells revealed a low fluorescence signal (8.39%) (Fig. 2a). As the majority of cells showed expression of the rabies glycoprotein, it can be inferred that all the constructs were capable of expressing the protein efficiently in transfected cells.

For assessing the localization of DNA vaccine constructs, transiently transfected BHK-21 cells were subjected to subcellular fractionation and subsequently visualization by western blotting. For the same, total cell lysate, lysosomal fraction, membrane fraction and cell-culture supernatant of transfected cells were resolved on SDS-PAGE followed by probing with hyperimmune polyclonal serum from mice immunized with rabies virus. Prominent immunoreactive protein bands were observed on the blot corresponding to cell lysate of all the DNA vaccine constructs (Fig. 2b, topmost panel). There was no corresponding band in cell lysate from vector-transfected or mock-transfected BHK-21 cells (data not shown). The observed molecular weight of approximately 67 kDa was consistent with the expected sizes of glycosylated glycoprotein. Analysis of subcellular fraction revealed that the pgp.L construct encoded lysosomal form of glycoprotein (second panel). The pT.gp construct encoded secreted form of glycoprotein (third panel). Further, dual tagged construct pT.gp.L expressed both secreted and lysosomal form of glycoprotein (second and third panels). pU.gp construct was exclusively expressed in cell associated form (topmost panel). The native construct encoded membrane associated glycoprotein (fourth panel). Expression of glycoprotein in various subcellular fractions was comparable to that from cell lysate.

3.2. Immune response to plasmid DNA vaccination in mice

To address the issue if these vaccines could induce efficient humoral response was assessed. Groups of 10 BALB/c mice were vaccinated intramuscularly with DNA encoding either the unmodified or modified antigen. Anti-glycoprotein antibody response was estimated by ELISA with recombinant glycoprotein (expressed in bacterial system, unpublished data). All the mice sero-converted after priming, however, maximum titers were obtained after second booster both for unmodified as well as modified DNA vaccine. For clarity, only the means and standard deviations for each group are shown (Fig. 3). All vaccine groups mounted antibody response higher than the unmodified vaccine. The highest antibody response was generated in the pgp.L immunized group (P value <0.005), closely followed by pT.gp.L. There was insignificant antibody response in vector and PBS immunized mice.

3.3. Antibody isotypes

Trafﬁcking of glycoprotein through different pathways may affect the type of immune response elicited against it. Like pT.gp mediated trafﬁcking of glycoprotein from cytoplasm to secretion pathway, which targets molecules through the endoplasmic reticulum and golgi; may lead to higher induction of Th2 type of immune response. Likewise, pgp.L mediated trafﬁcking may drive the glycoprotein through trans-golgi network directly to
endosomes and then to lysosomes, again influencing the Th2 type response. pTgp.L may channelize the glycoprotein through either of the above pathways, to affect the Th2 type of immune response. On the contrary, pLgp is expected to enhance the proteolysis of conjugated glycoprotein mediated by the ubiquitin-proteasome pathway for enhancing the processing and presentation for Th1 type of immune response. To examine such a possibility, serum from mice immunized with pgp, pTgp, pTgp.L, pLgp and pgp.L was assayed by probing with isotype specific secondary antibodies. Immunization with all the constructs led to an IgG1-dominated antibody response, in spite of differential targeting.

3.4. Rabies virus-neutralizing antibody (RVNA) response

Further, we explored the possibility of enhancement in neutralizing antibodies against glycoprotein when modified antigens were employed for the immunization experiments. Rabies virus-neutralizing antibody (RVNA) titers were assessed by RFFIT; three weeks post the last immunization corresponding to the time of lethal challenge. The RVNA titer in all the groups of immunized mice was >0.5 IU/ml; the minimum titer against Rabies as recommended by WHO. As shown in Fig. 5, the highest geometric mean RVNA titer was observed for pgp.L (16 IU/ml, P value <0.005), followed by pTgp.L and pLgp with titer of 8 IU/ml. The neutralizing antibody potential of IA tagged vaccine was found to be the lowest, equivalent to the unmodified antigen based DNA vaccine (4 IU/ml). In comparison, vector or PBS immunized group did not induce significant neutralizing antibodies.

3.5. Cytokine ELISA

T helper cells (Th1/Th2) play an important role in eliciting both humoral and cellular responses via expansion of antigen-stimulated B cells and CD8+ T cells or CTLs respectively. The levels of some cytokines which may play key roles in the induction of protective immune responses against rabies virus were studied as parameters of polarization of immune response. Th1 cytokines (IL-2, IL-12, and IFN-γ) and Th2 cytokine (IL-4) were measured from splenocytes from immunized mice by ELISA at 24, 48 and 72 h after re-stimulation with inactivated PV-11 virus. IL-2 production substantially increased on immunization with pgp.LAMP-1; 28.03 pg/ml i.e., ~ 14 fold higher as compared to the response from control (splenocytes from PBS immunized mice) was observed (P value <0.005). All the constructs exhibited significant IL-4 and IFN-γ production. There was no significant increase in the cytokine levels of mice immunized with vector or PBS. IL-12 production also strongly increased in case of pgp.LAMP-1; ~ 35 fold superior than the control group (P value <0.005). The cytokine profile is summarized in Fig. 6.

3.6. Antiviral protective efficacy

The ability of these DNA vaccines to elicit protective responses in immunized mice was assessed by intracerebral challenge with 20 LD50 of virulent rabies virus CVS strain. For controls, vector and PBS immunized mice were also challenged. The lethality of the challenge was confirmed by death of all the mice in the vector and PBS immunized group within 4–11 days post-challenge. Groups of vaccinated mice that developed significant levels of virus-neutralizing antibodies also survived rabies virus challenge. The protection conferred by DNA vaccines was found to be significant (P value <0.005). All modified antigen groups apart from pTgp conferred higher protection than the unmodified DNA vaccine, with 60% and 40% protection levels conferred respectively. Surviving mice did not show any signs of rabies virus infection. Kaplan–Meier curves for survival of DNA vaccine immunized mice are summarized in Fig. 7.

4. Discussion

A variety of cell-culture derived vaccines are available for prophylaxis against rabies [1]. However, the high cost of the vaccination therapy along with the risk of developing anaphylactic, neuropsychiatric or encephalitic side reactions limit their therapeutic application. These facts indicate the need of more faithful candidates vaccines which must be capable of inducing strong immune response to protect from infection. More importantly, the candidate immunogen must be able to induce a strong Th2 immunity as it has been established that Th2; that is, the humoral immune response plays a predominant role in induction of protective immunity against rabies virus [32–34]. Rabies virus glycoprotein is the main antigen responsible for inducing the production of rabies virus-neutralizing antibodies and for conferring immunity against...

Fig. 4. The isotype profile of the RV-C specific IgG1 (Black bars) and IgG2a (Gray bars) titers in mice immunized by different protocols. Each group of mice (n = 10) was immunized respectively by DNA, vector or PBS. Mice were bled at three weeks after the last immunization and glycoprotein-specific IgG1 and IgG2a titers were detected by ELISA. Optical density was measured at 450 nm. Data shown represent geometric mean titers and standard deviations for each group of animals.

Fig. 5. Rabies virus-neutralizing antibody (RVNA) titers in mice vaccinated with various RV-C plasmid DNA constructs were determined. The bars represent the geometric mean of the RVNA titers obtained with individual serum samples (represented by various symbols) in a group of vaccinated mice. RVNA titer equivalent to 0.5 IU/ml is the minimum adequate titer against rabies as recommended by WHO. The figure represents RVNA titers on day 62.
lethal rabies infection. Out of the various strategies being employed for enhancing the immunoprophylactic potential of vaccination strategy, DNA vaccines have been the most promising.

In an effort to develop an optimal DNA vaccine against rabies virus, this study was aimed at evaluating the immune enhancement potential of different antigen targeting strategies to selectively improve responses mediated by CD8+ and CD4+ T lymphocytes, induced after intramuscular immunization with DNA plasmids. Addition of target sequences like TPA, LAMP-1, UQ have been employed for vaccination against various pathogens including SARS coronavirus [35], Dengue virus [36]; Orthopox virus [37]; Influenza A [38]; Mycobacterium [39]. The signal sequences would target the heterologous protein to different sites targeting the model; for (i) high expression and secretion by fusing with TPA and a more generalized activation of the immune system for induction of significant humoral and cell-mediated responses [23] (ii) lysosomal degradation by fusing with LAMP-1 and Class II presentation [40,41]; (iii) wider and enhanced immune response by fusion with TPA and LAMP-1 and (iv) cytoplasmic degradation by the proteasome by fusing with ubiquitin and Class I presentation [42].

Classically, the transmembrane (TM) region is excised from the DNA vaccine immunogen, such that it can be secreted into extracellular milieu. Targeted DNA vaccines based on immunogens with deleted TM have been successfully employed for vaccination against tumours [43]. Nevertheless, Wang et al. found that Hemagglutinin (HA) proteins from different serotypes of influenza A virus elicits contrasting response to full length and truncated transmembrane forms [44]. Further, Rath et al. reported that TM domain along with a secretion signal of RV glycoprotein was required for eliciting highest level of neutralizing antibodies. They inferred that TM domain is critical for proper folding of protein otherwise the critical epitopes may get disrupted [30]. Gupta et al. also reported that DNA vaccine encoding rabies virus glycoprotein lacking transmembrane domain though enhances antibody response but does not confer protection [35]. Therefore, we retained the TM domain in our DNA vaccine constructs and utilized full gene for targeting strategies.

Thus, different plasmid DNA constructs were made—ppg, the unmodified constructs and modified constructs including p.gp.L (N terminal TPA and C terminal LAMP-1), pT.gp (N terminal TPA), pU.gp (N terminal UQ), and ppg.L (C terminal LAMP-1). Transient transfection of BHK-21 cells with all the plasmid DNA constructs revealed expression of rabies glycoprotein by flow cytometric analysis. Majority of the cells were found to express glycoprotein as seen by the fluorescence monitored by cell sorter. Thus, DNA vaccine

![Fig. 6. Concentrations of cytokines in cell-culture supernatants of BALB/c mouse splenocytes. Splenocytes (5 x 10^6 cells/ml) were stimulated with 5 μg/ml of BPL-inactivated PV-11 virus. 24, 48 and 72 h later, culture supernatants were collected and analyzed by a capture ELISA for IL-2, IL-4, IL-12 and IFN-γ. Splenocytes from two mice immunized with DNA vaccine constructs were included in each experiment. Data are expressed as mean values ± S.D. of triplicates.](image)

![Fig. 7. Survival percentage of mice immunized with Rabies DNA vaccine. Mice were immunized with the various constructs or empty vector control. All mice were challenged intracerebrally with 20 LD₅₀ of CVS strain of rabies virus on day 21 post-immunization and observed for 18 days for rabies specific symptoms or death.](image)
constructs were capable of efficiently expressing the glycoprotein. Distribution of chimeras was analyzed by subcellular fractionation and immunoblotting. Total cell lysate of transfected BHK-21 cells of all the constructs expressed glycoprotein at approximately 67 kDa. The observed high molecular weight of RV-G expressed in BHK-21 cells could be due to the influence of host factors on glycosylation [45]. Morimoto et al. showed both BHK and Murine Neuroblastoma (MNA) cell lines, transformed with the same retroviral expression vector encoding RV-G cDNA, show different patterns of glycosylation of the expressed RV-G [45], rRV-G expressed by BHK cells was highly glycosylated and sialylated in comparison to MNA expressed rRV-G, indicating that the glycosylation and sialylation of RV G is dependent on the cellular conditions in which RV-G is produced. Analysis of subcellular fractions indicated that glycoprotein along with the targeting sequences was suitably recognized by mammalian cells and directed towards the respective pathway. Flow cytometric and immunoblotting analysis of transfected cells, it can be inferred that there was efficient recognition and expression of DNA vaccine immunogens in the mammalian system. The signal sequences successfully directed the glycoprotein to respective cellular locations, with comparable levels of expression as of total cell lysate.

Vaccination of mice with all RV-G plasmid DNA constructs led to the generation of anti-RV-G antibodies. All the modified vaccines elicited higher anti-RV-G antibody levels than the unmodified one. The highest antibody response was observed with pgp.LAMP-1. The generation of RVNA is the most important adaptive immune system response for conferring protection against rabies. Therefore, to compare the utility of the RV-G plasmid DNA constructs, RVNA response elicited by each construct was determined by RFFIT. The neutralizing antibodies were more than 0.5 IU/ml, which is the minimum titer recommended by WHO. The highest RVNA titer was elicited by pgp.LAMP-1 which is also supported by an enhanced antibody response by ELISA, in comparison to other RV-G constructs.

The effectiveness of the constructs to induce Th1/Th2 type of immune response was indirectly evaluated by determining Th1 (IgG2a) and Th2 (IgG1) antibody isotypes. We found a strong IgG1 response in all the DNA constructs. Even though IgG2a antibodies were produced, the ratios of IgG1/IgG2a were consistently more than one, thus emphasizing on the Th2 bias. Presence of both types of immune responses may be due to the presence of more than one type of antigenic sites in the glycoprotein immunogen. It is worth noting that differential targeting for enhancing Th1 and Th2 responses yielded in effect a similar response.

The increase in antibodies to DNA vaccine may reflect an effect of the antigen on the T helper cell response needed to promote differentiation of naive B cells into antibody secreting plasma cells. This was assessed by cytokine profiling of splenocytes immunized with signal sequence tagged glycoprotein based vaccine or only vector; upon in vitro stimulation with inactivated PV-11 virus. We found that all the cytokines analyzed could be detected from the splenocytes of DNA vaccine immunized mice, with a pronounced enhancement in the level of IL-2 and IL-12 in the pgp.LAMP-1 immunized group. For other cytokines, namely IL-4 and IFN-γ, similar levels of cytokines were observed for all the four groups, with the level being several folds in comparison to the splenocytes from control group. Antigen binding to the T cell receptor (TCR) stimulates the secretion of IL-2, and the expression of IL-2 receptors IL-2R. The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes. IL-2 facilitates production of immunoglobulins made by B cells and induces the differentiation and proliferation of natural killer cells. IL-12, produced mainly by macrophages and dendritic cells, is quickly induced by viral infections or by vaccination stimuli. IL-12 strengthens the non-specific immune responses by activating NK cells to produce IFN-γ and in synergy with IFN-γ, drives the differentiation of CD4+ T cells into Th1 cells, more adapted to the control of viral infections.

Various groups of immunized mice when challenged with CVS virus showed higher protection as compared to a vehicle control. High titers of RVNA and protection conferred in DNA vaccines might be due to the possibility that modified immunogens led to the expression of RV-G with appropriate folding and better accessibility of epitopes to immune system, critical for generating RVNA titers. In spite of similar magnitude of immune response generated, protective efficacies against viral challenge varied. The unmodified and secreted forms of vaccines were found to be inferior in inducing protection against viral challenge. Xiang et al. also reported that secreted form of vaccine did not confer significant protective immunity [46]. Protection against rabies virus is mainly mediated by neutralizing antibodies [47]. subtle differences in the conformation of the secreted protein, not readily detectable by conventional biochemical methods, might select a different repertoire of neutralizing antibodies with lower avidity to the full length G protein present on the surface of viral particles, thus being less able to prevent the spread of virus [48].

Interestingly, pT.gp.L, pT.gp, pUgp, pgp.L DNA vaccine combinations designed to generate different types of immune responses yielded in effect similar data. A probable explanation for this could be that the tagged antigens evoke similar levels of immunity and act to enhance survival via the same primary protective mechanism. We observed that ubiquitination of antigen for MHC Class I targeting also enhanced the IgG1 antibody and CD4+ mediated cytokine response. Thus, we infer that the peptides generated by proteasomal degradation could also be presented by MHC-II. While, there is no specific information of how protein processing in transfected cells occurs in vivo, different mechanisms have been postulated. They include direct priming by somatic cells, direct priming by antigen-presenting cells, or cross priming of antigen-presenting cells. Activation by cross priming appears to be the most probable immune mechanism which occurs following intramuscular vaccination that could be shared by the TPA, LAMP-1 and UQ vaccines [24,49–52]. Cross priming may occur via exit of exogenous antigens from the endocytic compartments and its processing in the cytosol, recycling of MHC-I molecules through endosomal/lysosomal pathway and transfer of processed peptides to the endosomal compartments. It is well known that CD4+ T-cell stimulation can result from endocytosis of exogenous peptides or proteins followed by antigenic processing via MHC Class II pathways [53]. LAMP-1 targeting of antigen has been reported to increase the number of immunogenic peptide epitopes that activate CD4+ T cells, thus inducing a broadened immune response in comparison to untargeted antigen [54]. Recent studies have also demonstrated that exogenous proteins or peptides, possibly complexed to heat shock proteins, can be taken up by antigen processing cells, processed through the MHC Class I pathway, and ultimately stimulate naïve CD8 cells [55,56]. Thus, via cross-priming mechanisms, secreted fusion proteins expressed from TPA plasmids, membrane bound fused proteins expressed from LAMP-1 or peptides released from cells transfected with the UQ constructs could induce both CD4+ and CD8+ T-cell populations.

5. Conclusions

Several researchers have applied targeting strategies and reported conflicting results with different antigens and different infectious systems. Successful targeting was demonstrated for several pathogens including Human papillomavirus [57], Influenza A [38], Mycobacterium [39]; but not for all the constructs tested against malaria [58]. Thus, a tagged DNA vaccine may represent an 'ideal' immunogen for generating protective immune response.
adjuvant supplementation and a greater understanding of the immunity in response to DNA vaccination should facilitate the creation of further improved Rabies DNA vaccination strategies.

Acknowledgments

Inactivated PV viral antigen and Rabies Reference Antiserum were kindly provided by Dr. V. Srinivasan, Indian Immunologicals Ltd. The authors acknowledge Dr. Shardul Solanke (National Biotechnological Centre, IVR, India) for transfection and Anuj Kumar Sharma (School of Biotechnology, JNU, India) for flow cytometry studies. Special thanks are extended to Dr. Subhash Chandra (Cornell University, NY) for viral inputs in the study. This work was supported by Department of Biotechnology, Government of India. Manpreet Kaur is recipient of Senior Research Fellowship from CSIR, Government of India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.01.128.

References

Identification and characterization of immunodominant B-cell epitope of the C-terminus of protective antigen of Bacillus anthracis

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ARTICLE INFO

Article history:
Received 22 October 2008
Received in revised form
26 December 2008
Accepted 30 December 2008
Available online 7 April 2009

Keywords:
B-cell epitope
Immunodominant
Lethal toxin neutralizing antibodies
Protection

ABSTRACT

Bacillus anthracis is the etiological agent of anthrax. Protective antigen (PA) has been established as the key protective immunogen and is the major component of anthrax vaccine. Prior studies have indicated that C-terminus host cell receptor binding region contains dominant protective epitopes of PA. In the present study, we focused our attention on determining B-cell epitopes from this region, which could be employed as a vaccine. Using B-cell epitope prediction systems, three regions were identified; ID-I: 604–622, ID-II: 626–676 and ID-III: 707–723 aa residues. These epitopes elicited potent B-cell response in BALB/c mice. ID-II in particular was found to be highly immunogenic in terms of IgG antibody titre, with a predominantly IgG1/IgG2a subclass distribution indicating Th2 bias and high affinity/avidity index. Effective cellular immunity was additionally generated which also signified its Th2 bias. Further, ID-II induced high level of lethal toxin neutralizing antibodies and robust protective immunity (66%) against in vivo lethal toxin challenge. Thus, ID-II can be classified as an immunodominant B-cell epitope and may prove significant in the development of an effective immunoprophylactic strategy against anthrax.

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1. Introduction

Anthrax is an epizootic disease mainly affecting cattle and wild bovidae worldwide (Mock and Fouet, 2001). It is rare in humans but can occasionally be caused through contact with contaminated farm animals and animal products (Leppla et al., 2002). Of potential concern is the use of anthrax spores as biowarfare agents. Although there have been several earlier hoaxes or failed attempts, 2001 saw the first effective anthrax terror attack, infecting 11 and killing 5 people in US (Jernigan et al., 2001). Pathogenesis of Bacillus anthracis is mediated by two plasmids, pX01 and pX02, which encode for primary virulence factors – toxins and capsule, respectively (Brey, 2005). pX01 encodes protective antigen (PA), lethal factor (LF), and edema factor (EF). They act in binary combinations (Stanley and Smith, 1961) to produce exotoxins; Lethal Toxin (LeTx) and Edema Toxin (EdTx) comprising of PA with LF and EF respectively. Prior to exotoxin formation, PA binds to cellular receptors, undergoes proteolytic cleavage and forms heptameric oligomers. The heptamer competitively binds LF and EF, which are then translocated into the cytosol. LF is a zinc dependent protease that cleaves mitogen-activated protein kinases (MAPKs) leading to toxic shock and death (Vitale et al., 1998). EF is an adenylate cyclase converting intracellular ATP into cAMP, therefore provoking a substantial increase in intracellular cAMP levels leading to edema (Leppla, 1982). pX02 encoded capsule enhances virulence in vivo by inhibiting phagocytosis of the organism (Little and Ivins, 1999).

Induction of neutralizing antibodies to PA is considered the key to protection against anthrax (McBride et al., 1998; Brossier et al., 2000). Anthrax lethality is primarily attributed to toxemia (Mock and Fouet, 2001) and PA is essential for host cell intoxication as PA contains the host cell receptor binding site. The cell binding component for both EF and LF (Elliott et al., 2000) and facilitates the entry of the toxin complex into the host cell (Flick-Smith et al., 2002). PA is also the dominant antigen in both natural and vaccine-induced immunity against anthrax (Flick-Smith et al., 2002). However, the extent of immune response and protection conferred by PA-based vaccines against lethal anthrax

Abbreviations: AP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEPES, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) sodium salt; HRP, horse radish peroxidase; ID, immunodominant; LF, lethal factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PA, protective antigen; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

Data deposition footnote: pET-ID-11: GenBank accession number EU828661.

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Induction of cytotoxic T lymphocyte response against Mycobacterial antigen using domain I of anthrax edema factor as antigen delivery system

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Received 26 March 2007
Available online 5 April 2007

Abstract

We have investigated the efficiency of N-terminal 1-260 residues of Edema factor (EFn) as a delivery system for ESAT-6, an antigenic protein of Mycobacterium tuberculosis H37Rv, into the cytosol of mammalian cells. The EFn.ESAT-6 recombinant protein was obtained by genetic fusion of EFn and ESAT-6 DNA. Our data shows that in the presence of PA, EFn.ESAT-6 fusion protein is internalized into the cytosol of antigen presenting cells, and the splenocytes produced both Th1 and Th2 cytokines in vitro. Further, EFn.ESAT-6 elicited effective cytotoxic T lymphocyte (CTL) response in an in vitro CTL assay. This study for the first time demonstrates that EFn can be used as a vehicle to deliver heterologous proteins of therapeutic importance.

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Keywords: Edema factor; ESAT-6; Fusion protein; CTL; Cytokines

Humoral and cellular responses constitute the two main arms of immune system. One of the key functions of cellular immunity is to generate cytotoxic T lymphocytes (CTLs) for destruction of cells expressing intracellularly processed antigens on their surface. The CTLs recognize and kill tumor and other diseased cells, which display non-self peptides on their surface [1,2]. These peptides arise from various sources, such as infectious agents or aberrant expression of self-proteins, and mark defective cells for CTL recognition. Proteins within the cytosol are processed by multi-catalytic proteosome to generate small peptides, which are then displayed by class I major histocompatibility molecules (MHC-I) on the cell surface. Recognition of foreign peptide--MHC-I complexes by CD8+ cells leads to activation of specific CTLs, which clear the defective cells expressing foreign peptides or harboring pathogen [1,2-5]. Activated CTLs lyse infected cell, secrete cytokines, proliferate and differentiate. Vaccines that prime such memory CTLs, provide protection to the host, upon subsequent exposure to similar antigen displaying cells [5].

Development of vaccines with the ability to generate specific CTLs is hindered due to paucity of delivery systems of antigenic CTL epitopes into the cytosol of host cells. Several approaches to this problem have been reported [1] including the use of attenuated viruses, intracellular bacteria, bacterial toxins, naked DNA, electroporation, heat shock protein, polycationic peptides, non-ionic triblock copolymer, and adjuvants [6-11]. Each of these methods have inherent problem of safety and/or efficiency. Non-infectious, non-toxic, modified bacterial toxins for delivery of heterologous proteins have been reported in the past [6,8,12-17].

Bacillus anthracis produces a bipartite exotoxin comprising of two toxins namely edema toxin (ET) and lethal toxin (LT). Both ET and LT contain a protective antigen (PA) component along with either edema factor (EF) or lethal factor (LF), respectively. Individually, PA, EF, and LF are non-toxic. PA mediates entry of EF and LF into the
Evaluation of the ability of N-terminal fragment of lethal factor of Bacillus anthracis for delivery of Mycobacterium T cell antigen ESAT-6 into cytosol of antigen presenting cells to elicit effective cytotoxic T lymphocyte response

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Received 5 October 2006
Available online 30 October 2006

Abstract

We report the ability of N-terminal fragment of lethal factor of Bacillus anthracis to deliver genetically fused ESAT-6 (early secretory antigen target), a potent T cell antigen of Mycobacterium tuberculosis, into cytosol to elicit Cytotoxic T lymphocyte (CTL) response. In vitro Th1 cytokines data and CTL assay proved that efficient delivery of LFn.ESAT-6 occurs in cytosol, in the presence of protective antigen (PA), and leads to generation of effective CTL response. Since CTL response is essential for protection against intracellular pathogens and it is well known that only single T cell epitope or single antigenic protein is not sufficient to elicit protective CTL response due to variation or polymorphism in MHC-I alleles among the individuals, we suggest that as a fusion protein LFn can be used to deliver multiepitopes of T cells or multiproteins which can generate effective CTLs against intracellular pathogens like M. tuberculosis. It can be used to enhance the protective efficacy of BCG vaccine.

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Keywords: Fusion; CTL; Cytokines; Lethal factor

Bacillus anthracis produces a bipartite A/B-type toxin. The B subunit is the 83-kDa protective antigen (PA) receptor-binding moiety (named for its use as a vaccine), and the two catalytic A subunit moieties are edema factor (EF; 89 kDa) and lethal factor (LF; 90 kDa). EF is a Ca²⁺ and calmodulin-dependent adenylate cyclase [1]. LF is a Zn²⁺ protease that cleaves and inactivates mitogen-activated protein kinase kinase-1 and -2 [2]. PA binds to integrin-like I domain of anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8) or the capillary morphogenesis protein2 (CMG2) receptor on the cell surface [3], gets activated by furin family protease to a 63-kDa fragment (PA63). PA63 forms ring-shaped homohexameric prepore (PA63) which can bind up to three molecules of EF or LF or both [4,5] to form (PA63)₃-LF/EF which undergoes clathrin-dependent endocytosis, and LF or EF is released to the cytosol through homohexamer prepore in a pH-dependent manner [4,6] where they exert their cytopathic effects.

N-terminal PA binding region (LFn) of lethal factor has been used to deliver heterologous protein and peptide into cytosol and to process it by MHC class I antigen presentation pathway and to elicit CTL response [7-9]. However, it has been reported that some proteins fused to LFn are not translocated in cytosol via PA [10] and CTLs are very
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**Recent Activity**

- Synthetic construct UQ.GP gene, complete cds
  - EU715587 | 1

- Synthetic construct TPA.GP gene, complete cds
  - EU715586 | 1

**Pick Primers**

Design and test primers for this sequence using Primer-BLAST.

**All links from this record**

- Protein
- Taxonomy
- Related Sequences


3/26/2009
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Write to the Help Desk
NCBI | NLM | NIH
Department of Health & Human Services
Privacy Statement | Freedom of Information Act | Disclaimer
Synthetic construct GP.LAMP-1 gene, complete cds

Features

LOCUS EU715588 1695 bp DNA
linear SYN 01-JUL-2008
DEFINITION Synthetic construct GP.LAMP-1 gene, complete cds.
ACCESSION EU715588
VERSION EU715588.1 GI:193081175
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other sequences; artificial sequences.
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TITLE DNA vaccine against Rabies virus, pgp.LAMP-1
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1695)
AUTHORS Kaur, M. and Bhatnagar, R.
TITLE Direct Submission
JOURNAL Submitted (12-MAY-2008) School of Biotechnology,
Jawaharlal Nehru University, Lab No. 122, New Delhi, Delhi 110067, India
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3/26/2009
Nucleotide - Synthetic construct GP.LAMP-1 gene, complete cds

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3/26/2009
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Three routes of delivery, namely, gene gun immunization, intramuscular injection and oral administration were assessed for conferring potent immune response against rabies. Intramuscular administration of rabies DNA vaccine was found to elicit strongest humoral immune response against rabies.

The optimum dosage via intramuscular injection was established as 100 μg.

Adjuvants - EMULSIGEN® and EMULSIGEN® D (ED) were tested for their immune augmentation potential. The adjuvant ED was found to enhance the magnitude of immune response elicited against the rabies DNA vaccine.

The highest immune response pertaining to IgG antibody titer, with a predominantly IgG1/IgG2a subclass distribution, effective cellular immunity and high level of RVNA titer was attained by the optimized DNA vaccine formulation comprising of intramuscular administration of 100 μg of DNA vaccine supplemented with EMULSIGEN®D.

In pre-exposure prophylaxis, three dose regimen of this formulation generated high RVNA titer (32 IU/ml) and conferred complete protection against challenge with 20 LD₅₀ of CVS.

For post-exposure efficacy analysis, rabies was experimentally induced with 50 LD₅₀ of CVS. Subsequent therapy with five doses of the formulation completely prevented rabies in BALB/c mice, which maintained protective RVNA titers of 4 IU/ml. WHO recommends rabies protective threshold titer as 0.5 IU/ml.

Enhancement of plasmid DNA production was attempted by fed batch culture, whereby 68.79 mg/l of pgp.LAMP-1 plasmid could be attained.

The prime endeavor of this work was to develop a highly potent, safe and cost effective DNA vaccine against rabies which would confer complete protection upon pre- and post-exposure prophylaxis. Thus, this optimized DNA vaccine formulation provides an avenue for preventing and controlling rabies.