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Research Article

IMMUNOGENICITY OF pALPHA RECOMBINANT PLASMID ENCODING RABIES VIRULENT VIRUS GLYCOPROTEIN GENE

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ABSTRACT

Virus neutralization test performed on serum of mice vaccinated with pAlpha-rvvg revealed that antibody titer was 64, SI was 3.1 at 28 days post immunization, the groups maintained as vector control and healthy showed very low antibody titer 8 and 4 and SI 1.15 against rabies virus. The protection test showed that the recombinant plasmid induced 93.33% protection while vector alone produced 13.33% protection and no protection in healthy control group. It may be a good candidate vaccine for rabies control.

Key Words: pAlpha vector, rabies glycoprotein gene, DNA vaccine, immunogenicity, replicase gene.

INTRODUCTION

The discovery of Wolff *et al* (1990) that naked DNA injection into the muscle of mice expressed the encoded protein, many researchers successfully used this technique to develop DNA vaccine against infectious pathogens in different animal models (Ulmer *et al*., 1993; Major *et al*., 1995; Michel *et al*., 1995; Gupta *et al*., 2001). With the help of recombinant DNA technology, plasmid expression vector carrying gene of interest is delivered and expressed in animal muscle. The DNA as a vaccine assumes great significance since it can not only offer protection against dreadful infectious diseases in developing countries, it is temperature stable and affordable for the poor when compared to recombinant/ cell culture vaccines. In vivo synthesis of antigen structurally identical to those produced during active viral infection and induction of strong humoral as well as cell mediated immune responses renders DNA vaccine advantageous over conventional vaccines. In addition, DNA vaccines are non-infectious, economical to produce in large amount and easy to purify using simple and inexpensive techniques. Further, DNA vaccines do not require cold chain, which occupies about 80% of the cost of vaccination in developing countries. Plasmids encoding multiple antigens of same pathogen or different pathogens can be constructed. DNA vaccines can be given at younger age without the risk of neutralization by maternal

antibodies. Moreover, booster doses can be given without risk of vector immunity. G protein of rabies virus CVS strain was earlier cloned and shown to protect mice (Rai *et al*., 2002).

It is desirable that a vaccine should function in low dose with simultaneous induction and maintenance of strong immunity. The replicase based DNA vaccines fulfil the stringent criteria for all DNA vaccines in general (Hariharan *et al*., 1998) and vaccines for breaking immunological tolerance (Leitner *et al*., 2000; Leitner *et al*.; 2003; Leitner *et al*., 2004).

MATERIALS AND METHODS

Recombinant plasmid

It was constructed as described earlier (Kanojia and Rai, 2016).

Plasmid DNA isolation

Plasmid DNA isolation was done using TELT method (Ausubel *et al*, 1990; He *et al*, 1990).

Immunization of mice

Healthy Swiss albino mice were immunized with recombinant plasmid DNA intramuscularly in quadriceps muscle in the hind leg, each with 10 εg DNA. The mice were grouped, 15 mice in each group as shown in Table 1.

Table 1. Different groups for immunization in mice

Groups	No. of Mice	Amount of DNA
pAlpha-rvvg	15	10 εg
Vector Alone	15	10 εg
Rabies vaccine (Rabipur)	15	0.5 ml
Healthy control	15	nil

Serum Neutralization Test (SNT)

Serum neutralization test was performed using sera of mice (WHO, 1996; Gupta *et al*., 2005). Briefly, sera were inactivated at 56^0C for 30 min and two-fold dilutions were prepared 1:4, 1:8, 1:16 and 1:32 and1:64 . Assays were performed in 96 well micotitre plates by mixing 0.05 ml of serial two- fold dilution of sera in PBS with 0.05 ml of rabies virus suspension containing $10LD_{50}$ virus. The serum virus mixture was then incubated for 2 h at 37^0C after which 0.03 ml was injected via master muscle of mice. The neutralizing antibody titre was calculated as the reciprocal of the highest dilution that neutralized the virus.

Lymphocyte proliferation assay

Lymphocytes were collected from blood in heparin (20 IU/ml of blood) as per protocol of Boyum (1976) .Equal volume of BSS was added. Withdrew 3 ml of Histopaque 1.077 density gradient medium (Sigma) from the bottle by a syringe and placed it in a centrifuge tube. Carefully layered the diluted blood on top of the Histoaque. Centrifuged at 400 g for 30 min. Discarded the upper layer of plasma using a pasteur pipette. Removed the lymphocytes collected at the interface with a clean pasteur pipette into a centrifuge tube, washed in RPMI-1640 supplemented with 10% FBS and resuspended in RPMI-1640 supplemented medium (Boyum, 1976). For proliferation assay, 100µl lymphocytes at a

seeding concentration of 2 X 10^6 cells per well were dispensed in 96 well flat bottom tissue culture plate in triplicate. One group of cells were treated with pAlpha-rvvg 10µg/well, second group of cells with Concavalin A (ConA Sigma, stock 5 mg/ml) at a final concentration of 50 µg/well and third group of cells- the negative control contained neither rplasmid nor ConA. The plate was incubated at 37°C in a humidified CO_2 incubator for 72 h. After 72h incubation, 20 µl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, 5 mg/ml) was added to each well. Plates were incubated for another 4 h. Then 150 ul of 10% DMSO was added to each well to dissolve the resultant formazan crystals and A_{570} was measured spectrophotometrically. Background absorbance of multiwall plates was measured at A_{690} and subtracted from 570 nm readings. Blastogenic response for the assay was expressed as stimulation index (SI) calculated by dividing the mean absorbance of stimulated group by mean absorbance of unstimulated group (Bounous *et al.,* 1992; Keck and Bodine 2006).

$SI = A_{\text{stimulated culture}} / A_{\text{unstimulated culture}}$

Challenge test /Protection test in mice

All the immunized mice were challenged with $20LD_{50}$ rabies virus CVS 28 days after immunization using 0.03ml/per mouse through intramasseter muscle route and observed for 14 days post-challenge. Virus end point titers were calculated as per Reed and Muench (1938). Any deaths occurring upto 4 days were considered non-specific and mice showing deaths or rabies specific symptoms like paralysis and death between 5 and 14 day were recorded as positive., and only mice which remained alive at the end of the observation period were considered protected. After 14 days observation period, percent protection was calculated as follows:

$$\% \text{ Protection} = \frac{\text{Number of mice remaining alive X 100}}{\text{Total number of mice in the group}}$$

RESULTS AND DISCUSSION

Immune response of recombinant plasmid in mice

Virus neutralization test performed on serum of mice vaccinated with pAlpha-rvvg was revealed that antibody titer was 64, SI was 3.1 at 28 days post immunization, the groups maintained as vector control and healthy showed very low antibody titer 8 and 4 and SI 1.15 against rabies virus. The protection test showed that the recombinant plasmid induced good protective response and protection obtained was 93.33% while in vector alone it was 13.33 and no protection i healthy control group. The results obtained with each group are shown in Table 2 &3.

Table 2. Immune response and challenge test of mice vaccinated with pAlpha-rvvg*.

Group	No. of mice	DNA/ vaccine	SNab#	Alive	% protection
pAlpha-rvvg	15	10 µg	64	14	93.33
Rabipur vaccine	15	0.5 ml	64	14	93.33
pAlpha vector	15	10 µg	8	2	13.33
Healthy control	15	nil	4	0	0

*Mice were challenged with $20LD_{50}$ rabies virus CVS by intra masseter route 28 days after vaccination; # Titers have been shown as reciprocal of maximum dilution of serum neutralizing rabies virus.; ** Stimulation index

Table 3: Mean absorbance (A_{550}) and stimulation index of lymphocytes.

Groups	ConA	virus	unstimualted	SI*
Rabipur vaccine	0.62	0.63	0.24	2.625
pTargeT.rvv.g	0.635	0.62	0.20	3.1
pTargeT Vector	0.60	0.23	0.20	1.15
Healthy control	0.655	0.15	0.10	1.5

*dpi = days post immunization, *SI = Mean absorbance (A_{550}) with virus}/ Mean absorbance (A_{550}) of unstimulated

In our study, we recorded 93.33% protection in immunized mice. Previous studies have shown that DNA-based immunization with plasmids encoding the rabies virus glycoprotein (CVS, ERA and PV strains) protects mice against rabies (Bahloul *et al.*, 1998; Jallet *et al.*, 1999; Lodmell *et al.*, 1998; Xiang *et al.*, 1994). Among other workers, Rai *et al.* (2002) reported 88.88% protection against rabies using a monovalent rabies vaccine. Saxena (2011) recorded 95 % protection in mice immunized with the r-plasmid DNA vaccine, whereas 85% protection was observed in the group receiving commercial cell culture vaccine. DNA-based immunization with plasmids encoding rabies virus G gene induce rabies antibodies in dogs (Perrin *et al.*, 1999; Lodmell *et al.*, 2003; Osorio *et al.*, 1999; Rai *et al.*, 2002). Neutralizing antibody titre, as recorded in mouse neutralization test also showed sero-conversion on primary immunization. Previous studies have reported presence of detectable level of neutralizing antibodies as early as 7 days post-inoculation of rabies DNA vaccine (Bahloul *et al.*, 2003). DNA vaccines are particularly useful in situation where cytotoxic T cell mediated immune responses are essential for protective immunity. It mimics the live replicating agents because of endogenous production of cell associated antigens and their association with MHC class I molecules often result in CTL responses. Sometimes when live vaccines are contraindicated such as in immunologically compromised or suppressed hosts, DNA vaccines are particularly useful. DNA vaccine selectively induces type 1 T cells whereas protein vaccines induce Th2 or mixed Th1/Th2 responses (Patricia *et al.*, 2000).

It is evident that our recombinant plasmid which is pAlpha based induced high level of humoral and cell mediated immune responses when given in 10 µg quantity only. It is very well expected since pAlpha vectors produce 1000 to 10,000 times more copies of gene as compared to ordinary plasmid vectors. Kumar *et al* (2009) had also cloned canine distemper virus H gene in a replicase vector and observed high immune responses in a dose of 4 µg plasmid vector. Sandey *et al* (2008) had reported development of a replicase gene based DNA vaccine containing

FAV-4 hexon gene which was highly immunogenic in poultry.

Recent study shows that after intramuscular inoculation muscle cells probably act as a reservoir for the foreign antigen while the bone marrow cells seems to act as the APCs (Iwasaki *et al*., 1997; Torres *et al*., 1997; Ulmer *et al*., 1996). Intravenous route of administration resulted in no detectable antibody response (Smith *et al*., 1998). We used intramuscular route of administration for DNA delivery because it is more convenient and requires no special arrangements. Smith *et al*., (1998) claimed that intramuscular route of administration is the most efficient means of inducing a humoral immune response.

Studies showed that agents that cause muscles necrosis increase immune response to DNA vaccines (Davis *et al*., 1993; Coney *et al*., 1994). In intramuscular injection hydrostatic damage caused by injection of the relatively larger volumes of fluid is also responsible for high immunogenicity of plasmid DNA vaccines in mice (Dupuis *et al*., 2000). pSinCMV serves as negative strand once it enters into the host cell and it is transcribed by host RNA polymerase enzymes using CMV promoter into a full length RNA transcript. This full length transcript then acts as positive sense alpha virus which in turn is translated in cytoplasm to form replicase protein. This replicase protein serves as RNA dependent RNA polymerase enzyme and forms negative sense RNA from positive sense transcript. From this negative sense strand full length as well as smaller fragments from subgenomic promoters are transcribed which in-turn are translated into proteins. Since the cloned insert is downstream to the subgenomic promoter, the translated proteins represent our target proteins. The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it (Kumar *et al*, 2009). Gangwar and Rai (2013) cloned the VP2 gene of a very virulent infectious bursal disease virus in pAlpha vector and demonstrated that it induced high level of humoral and cell mediated immune responses in chicken and gave 90% protection from virulent challenge. Replicase based vectors are superior over other conventional vectors in terms of its lower requirement of dose of immunization (Hariharan *et al*., 1998; Berglund *et al*., 1998; Leitner *et al*., 2000), the mechanism of breaking tolerance against self antigen, the power of inducing apoptosis (Leitner *et al*., 2003) so that transient but robust expression of antigen is achieved in short period without taking risk of integration into host chromosomes (Jolly, 1994; Miller *et al*., 1993) and a broad host range.

In comparative studies of conventional (non-replicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally induces stronger immune responses and at a significantly lower

DNA concentrations than does conventional vectors (Berglund *et al*., 1998 Hariharan *et al*., 1998) It has been shown that innate antiviral pathways implicated in the molecular mechanisms of innate antiviral immunity (double stranded RNA recognition and interferon action) are also one of the mechanism underlying the superior efficacy of replicase based DNA vaccines (Leitner *et al*., 2003). DNA based immunization whereby an eukaryotic expression plasmid carrying a gene of interest is directly administered into the host just by a simple saline intramuscular injection results in vigorous immune responses with both arms of humoral and cell mediated immunity (Calarota *et al*., 1998). Th1 biased immune response induced by DNA based immunization is mediated via action on professional antigen presenting cells to upregulate IL12 production (Asakura *et al*., 1999). Although we kept the dose very minimal upto a maximum of 4 µg, it is of special notice that our vector size is itself large being above 10 kb. Recent experiments utilizing unmethylated CpG motifs indicate that increased amounts of DNA not specifically coding for antigen may have a nonspecific immunostimulatory activity (Krieg , 2000). pSinCMV.cdh being above 10 kb, may have some non- specific immunostimulatory activity. Since most intereference in recombinant DNA vaccine is from circulating antibodies acquired transplacentally or transcolostrally, the intramuscular route of injection overcomes maternal interference to much extent.

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