DEVELOPMENT OF RABIES DNA VACCINE USING A RECOMBINANT PLASMID

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Summary. – A recombinant plasmid pTargeT.rabgp containing the glycoprotein (G) gene of Rabies virus (RV) was constructed and produced for immunogenicity studies on mice and dogs. The mice immunized twice with 50 μg or 100 μg of pTargeT.rabgp intramuscularly (i.m.), showed a serum antibody titer of 256 or 64, respectively 14 days post immunization (p.i.). When the vaccinated mice were challenged at 14 days p.i. with RV i.c., they were protected in 81.25% or 87.5%, respectively. A positive cell mediated immune response was observed by lymphocyte proliferation assay and macrophage migration inhibition test. These results indicate that the pTargeT.rabgp plasmid can be used as a rabies DNA vaccine.

Key words: Rabies virus; DNA vaccine; glycoprotein gene; protection; neutralization test; antibody titer

Introduction

Rabies is considered one of the oldest diseases of mankind worldwide which is a 100% fatal but, fortunately, easily preventable zoonosis. RV (the *Lyssavirus* genus, the *Rhabdoviridae* family) has a negative-sense RNA genome of about 12 kb, encoding five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoproptein (G) and large protein (L) (Ito *et al*., 2001). The N, P and L proteins form ribonucleoprotein together with the viral genomic and antigenomic RNA. The N protein is responsible for encapsidation of the genome, while the L protein in cooperation with the P protein function as an RNA-dependent RNA polymerase (Ito *et al*., 2001). The G and M proteins are located in viral envelope. The G protein, the only protein on the surface of the mature virion plays a critical role in viral pathogenicity (Sokol *et al*., 1971; Dietzschold *et al*., 1978). It is the viral attachment protein that is, therefore, important in determining the tissue tropism of the virus (Iwasaki *et al*., 1973; Perrin *et al*., 1982; Wunner *et al*., 1984). The G protein has several important functions; it is a major antigenic stimulus of the host immune system following RV infection or vaccination. The G protein forming trimers on the virion surface and determining the tropism of the virus by binding to receptors on neurons is the only viral protein that induces virus neutralizing antibodies (Cox *et al*., 1977; Wiktor *et al*., 1973). Furthermore, the G protein stimulates T helper (Th) as well as cytolytic T (Tc) cells (Ertl *et al*., 1990; Wiktor *et al*., 1988). DNA vaccines have many advantages over conventional vaccines because of the ease in their construction, their ability to induce a full spectrum of long lasting humoral and cellular immune responses, and their high temperature stability, which would represent a particular advantage for use in tropical areas where refrigeration is difficult. Moreover, the low cost for their mass production makes DNA vaccines ideally suited for developing countries.

The purpose of this study was to examine the immunogenicity of the pTargeT.rabgp plasmid for mice and dogs.

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Abbreviations: gp = glycoprotein; i.c. = intracerebral(ly); i.m. = intramuscular(ly); MMIT = macrophage migration inhibition test; p.i. = post immunization; RV = Rabies virus; T_c = cytolytic T cells; T_h = helper T cells

Materials and Methods

Amplification and cloning of RV G gene. The 1650 bp G gene of RV ERA strain was amplified by RT-PCR using viral RNA as template. The primers 5'-AGGAAAGATGGTTCCTCAGGCTA-3' (forward) and 5'-ATCTTCAGGACTTGGATCGT-3' (reverse) were used. The amplified G gene was cloned into the pTargeT vector (Promega) according to Rai and Yadav (2001). The recombinant plasmid was designated pTargeT.rabgp.

Plasmid DNA isolation. Escherichia coli DH_5 strain transformed with the pTargeT.rabgp plasmid was grown in LB broth with 100 µg/ml ampicillin at 37°C overnight. The bacterial culture, 1.5 ml or 50 ml, was processed using either the QIAprep Spin Miniprep Kit or HiSpeed Plasmid Midi Kit, respectively (both from QIAgen). The isolated plasmid DNA was electrophoresed on 0.7% agarose gel. The concentration and purity of plasmid DNA preparations were assayed spectrophotometrically.

Immunogenicity and protection studies in mice. Four-week-old Swiss albino mice were immunized i.m. into left quadriceps two times at seven-day-interval with 50 µg of tested plasmid DNA in 0.5 ml per animal. Serum RV antibodies were assayed 14 days p.i. by ELISA. Challenge of mice was done using a method described by Wibur and Aubert (1996) with modifications. The mice were challenged i.c. with 15 LD_{50} of RV in 0.03 ml per animal 14 days p.v. They were observed for 14 days to calculate the protection.

Immunogenicity studies in dogs. Four-week-old pups were immunized i.m. into the thigh two times at a 14-day-interval with 100 µg of tested plasmid DNA in 0.5 ml per animal. Twenty-one days p.i., blood was collected for assaying RV antibodies by ELISA and cell-mediated immunity response by MTT and MMIT assays. For the latter assays, heparin (SRL) was added to the blood (20 IU/ ml blood).

ELISA was used for assaying serum RV antibodies in mice (Talwar, 1983; Almeida *et al.,* 1979). Serial two-fold dilutions of sera from 1:16 to 1:256 were tested and A_{450} was read.

Virus-neutralization test (VNT) in mice was used. Aliquots (0.2 ml) of the serum samples collected from dogs under testing were diluted 1:10 with NSS and mixed with an equal volume of RV to make its concentration 10 LD_{50}/0.03 ml. The mixture was incubated at 37°C for 1 hr. Four-week-old mice were inoculated i.c. with 0.03 ml aliquots of the serum/virus mixtures. Control mice were injected with 10 LD_{50} of RV only. The mice were observed for 14 days to calculate the protection.

Cell-mediated immunity. The MTT colorimetric assay of cellular proliferation (Bounous *et al.,* 1992) with modifications was employed. The stimulation index (SI) was calculated using the following formula:

$$SI = A_{stimulated} / A_{unstimulated}$$

The macrophage migration inhibition test (MMIT) was employed to demonstrate the CMI response in dogs (Boyum, 1968; Clausen, 1973; Kalra *et al*., 1976). The test enabled to calculate the inhibition of migration in %.

Results and Discussion

The isolated recombinant plasmid pTargeT.rabgp, when analyzed by gel electrophoresis, showed clear band(s) of expected size of supercoiled DNA and a A_{260}/A_{280} value of 1.818. The mice and dogs vaccinated with the recombinant plasmid DNA did not show any adverse effects and high serum antibody titers were found by ELISA and VNT in mice (Tables 1–3). In immunized mice, an antibody ELISA titer of 256 was found, indicating a fair immune response. The challenge experiments revealed that the mice immunized with the recombinant plasmid with seppic oil as adjuvant were protected in 81.25% which was an apparently higher value than that for the empty vector (20%) and healthy control group (0%). This result showed that immune response to the DNA vaccine applied was evidently positive. The mice of control groups injected with the empty vector (pTargeT) or seppic oil adjuvant or unimmunized mice showed sudden paralytic symptoms, dullness and

Table 1. Immunogenicity of pTargeT.rabgp plasmid for mice

Plasmid/adjuvant/control	No. of mice	No. of mice protected	Protection (%)	Antibody titer (ELISA)
pTargeT.rabgp	14	11	78.57	128
pTargeT.rabgp with $Al(OH)_3$ adjuvant	8	6	75.00	≤16
pTargeT.rabgp with ISA50 adjuvant	16	13	81.25	256
pTargeT plasmid (empty)	10	2	20.00	≤16
ISA50 adjuvant	11	2	18.18	≤16
Healthy control	12	0	0	≤16

Mice were immunized i.m. with 50 µg of tested plasmid/adjuvant/control in 0.5 ml per animal.

Table 2. Immunogenicity of pTargeT.rabgp plasmid for dogs

Plasmid/adjuvant/control	No. of dogs	Antibody titer (ELISA)	VNT/MNT in mice
pTargeT.rabgp with ISA50 adjuvant	4	64	87.5%
PTargeT plasmid (empty)	1	≤16	37. 5%
Healthy control	1	≤16	0%

Dogs were immunized i.m. with 100 µg of tested plasmid/adjuvant/control in 0.5 ml per animal.

Table 3. Cell-mediated immunity in dogs vaccinated with pTarge. Trabgp plasmid

Plasmid/control	SI	Inhibition of migration (%)
pTargeT.rabgp with ConA	1.079	30
pTargeT.rabgp with RV	1.044	
Control: ConA	1.024	20
Control: RV	1.010	
pTargeT plasmid (empty)	Not done	20

SI = stimulation index. ConA = concavalin A.

unconsciousness resulting into death. Similar findings have been reported earlier by other workers (Biswas *et al*., 1999; Lodmell *et al*., 2000; Wunderli *et al*., 2003).

Induction of a long-lasting immunity to RV in mice by DNA vaccination has been reported by Xiang *et al*. (1995). Protection of mice against lethal RV infection by vaccination with nanogram quantities of plasmid DNA encoding RV gp has bee reported by Ray *et al*. (1997).

The dogs vaccinated with pTargeT.rabgp developed an antibody ELISA titer of 64 and were protected in 87.5% (Table 2). This finding showed that dogs can be protected by DNA vaccine against rabies in accordance with the findings of Perrin *et al*. (1999) and Shimazaki *et al*. (2003). All the vaccinated as well as control dogs remained healthy and did not show any untoward reaction. The control with the empty pTargeT plasmid showed no antibody titer and a 37.5% protection only, while healthy unimmunized dogs showed neither antibody nor protection.

The cell-mediated immunity response to the DNA vaccination applied, as determined by the MTT assay, is shown in Table 3. The SI value for the pTargeT.rabgp plasmid was apparently insignificantly higher than that for the healthy control. It showed that cell-mediated immunity does play a role although not to the extent of humoral immunity; anyway, they both together play an important role in protection against rabies, which is in accordance with the observations reported by Biswas *et al*. (1999). The MMIT assay using lymphocytes from immunized and control healthy dogs revealed that the inhibition of migration for pTargeT.rabgp with ISA 50 adjuvant was slightly higher (30%) than that for the healthy and empty plasmid controls (20%) (Table 3).

The present study shows that pTargeT.rabgp DNA can be used as DNA vaccine against RV administered along with ISA50 adjuvant to dogs. This conclusion is in agreement with the findings reported earlier by several workers (Butts *et al*., 1998; Biswas *et al*., 2001; Yamanouchi *et al*., 1998). It has also been demonstrated that DNA vaccines against RV have several advantages, namely they do not need cold chain storage but can be stored at room temperature, are economic to produce, safe in handling, highly effective (Fodor *et al*., 2000; Hassett *et al*., 2000; Gendon, 1999; Diogo *et al*., 2001) and thus most suitable for prevention and control of rabies in developing countries like India.

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References

Almeida JD, Atanasiu P, Bradley DW, Gardner PS, Maynard J, Schuurs AW, Voller A, Yolken RH (1979): *Manual for Rapid Laboratory Viral Diagnosis*. WHO, Geneva, pp. 22–25.

Biswas S, Askok MS, Reddy GS, Srinivasan VA, Rangarajan PN (1999): Evaluation of the protective efficacy of a rabies DNA vaccine in mice using an intracerebral challenge model. *Curr. Sci*. **76**, 1012–1016.

Biswas S, Reddy GS, Srinivasan VA, Rangarajan PN (2001): Preexposure efficacy of novel combination DNA and inactivated rabies virus vaccine. *Hum. Gene Ther.* **12**, 1917–1922.

Bounous OI, Campergnoh RP, Brown J (1992): Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. *Avian Dis.* **36**, 1022.

Boyum A (1968): Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**, 77.

Butts C, Zubkoff I, Robbins DS, Cao S, Sarzotti M (1998): DNA immunization of infants, potential and limitations. *Vaccine* **16**, 1441–1449.

Clausen JE (1973): Migration inhibitory effect of cell free supernatants from Tuberculin-stimulated cultures of human mononuclear leukocytes demonstrated by two step MIF agarose assays. *J. Immunol*. **110**, 546–551.

Cox JM, Dietzschold B, Schneider LG (1977): Rabies virus glycoprotein. II. Biological and serological characterization. *Infect. Immun.* **16**, 754–759.

Dietzschold B, Cox JH, Schneider LG, Wiktor TJ, Koprowski H (1978): Isolation and purification of a polymeric form of the glycoprotein of rabies virus. *J. Gen. Virol*. **40**, 131–139.

Diogo MM, Ribeiro SC, Queiroz JA, Monteiro GA, Tordo N, Perrin P, Prazeres DM (2001): Production, purification and analysis of an experimental DNA vaccine against rabies. *J. Gene Med*. **3**, 577–584.

Ertl HCJ, Dietzschold B, Gore M, Otvos L, Jr, Koprowski H (1990): Induction of rabies virus specific T cells by synthetic peptides that carry dominant T helper cell epitopes of the viral ribonucleoprotein. *J.Virol.* **63**, 2885–2892.

Fodor I, Kucsera L, Fodor N, Palfi V, Grabko VI (2000): Gene immunization of mice with plasmid DNA expressing rabies virus glycoprotein. *Acta Vet. Hung*. **48**, 229–236.

Gendon IZ (1999): Progress in developing viral polynucleotide (DNA) vaccines. *Vopr. Virusol*. **44**, 148–154 (in Russian).

Hassett DE, Zhang J, Slifka M, Lindsay-Whitton J (2000): Immune responses following neonatal DNA vaccination are long-lived, abundant and qualitatively similar to those induced by conventional immunization. *J. Virol*. **74**, 2620–2627.

Ito N, Takayama M, Yamada K, Sugiyama M, Minamoto N (2001): Rescue of rabies virus from cloned cDNA and identification of the pathogenicity related gene: Glycoprotein gene is associated with virulence for adult mice. *J. Virol*. **75**, 9121–9128.

Iwasaki Y, Wiktor TZ , Koprowski H (1973): Early events of rabies virus replication in tissue culture: an electron microscopic study. *Lab. Invest.* **28**, 142–148.

Kalra SK, Dogra SC, Singh SB, Sharma VK (1976): Demonstration of cell mediated immunity in experimentally infected Guinea pigs by macrophage migration inhibition test. *Indian J. Microbiol*. **16**, 185–188.

Lodmell DL, Ray NB, Ulrich JT, Ewalt LC (2000): DNA vaccination of mice against rabies virus: effects of the route of vaccination and the adjuvant monophosphoryl lipid (MPL). *Vaccine* **18**, 1059–1066.

Perrin P, Jacob Y, Agiular SA, Loja RE, Jallet C, Desmejieres E, Aubert M, Cliquet F, Tordo N (1999): Immunization of dogs with a DNA vaccine induces protection against rabies virus. *Vaccine* **18**, 479–486.

Perrin P, Portnoi D, Sureau P (1982): Etude de I' adsorption et de la penetration du virus rabique: interactions avec les cellules BHK-21 et des membranes artificielles. *Annal. Inst. Pasteur Virologie* **133E**, 403–422.

Rai A,Yadav M (2001): Cloning of rabies virus glycoprotein gene in mammalian expression vector pTargeT. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **22**, 137–139.

Ray NB, Ewalt LC, Lodmell, DL (1997): Nanogram quantities of plasmid DNA encoding the rabies virus glycoprotein protect mice against lethal rabies virus infection. *Vaccine* **15**, 892–895.

Shimazaki Y, Inoue S, Takahashi C, Gamoh K, Etoh M, Kamiyama MH (2003): Immune response to Japanese rabies vaccine in domestic dog. *J. Vet. Med. B. Infect. Dis. Vet. Public. Health.* **50**, 95–98.

Sokol F, Stancek D, Koprowski H (1971): Structural protein of rabies virus. *J. Virol.* **7**, 241–249.

Talwar GP (1983): *A Handbook of Practical Immunology*. Vikas Publishing House, New Delhi, pp. 246–251.

Wibur LA, Aubert MFA (1996): The NIH test for potency. In Meslin FX, Kaplan MM, Koprowski H (Eds): *Laboratory Techniques in Rabies.* 4th ed., WHO Geneva, pp. 360–368.

Wiktor TJ, Cohen GH, Schlumberger D, Sokol F, Koprowski H (1973): Antigenic properties of rabies virus Components. *J. Immunol.* **110**, 269–276.

Wiktor TJ, Kieny MP, Lathe R (1988): New generation of rabies vaccine, vaccinia- rabies glycoprotein recombinant virus. In Kurstak E, Marusyk R G, Murphy FA, van Regenmortel, MHV (Eds): *Applied Virology Research.* VoI l, Plenum, New York, pp. 115–137.

Wunderli PS, Dreesen DW, Miller TJ, Baer GM (2003): Effect of heterogeneity of rabies virus strain and challenge route efficacy of inactivated rabies vaccine in mice. *Am. J. Vet. Res.* **64**, 499–505.

Wunner WH, Reagen KJ, Koprowski H (1984): Characterization of saturable binding sites for rabies virus. *J. Virol.* **50**, 691–697.

Xiang ZQ, Spitanik SL, Cheng J, Erikson J, Wojczyt B, Ertl HC (1995): Immune responses to nucleic acid vaccines to rabies virus. *Virology* **209**, 569–579.

Yamanouchi K, Barrett T, Kai C (1998): New approaches to the development of virus vaccines for veterinary use. *Rev. Sci. Tech.* **17**, 641–653.