A DNA VACCINE THAT ENCODES RABIES VIRUS GLYCOPROTEIN LACKING TRANSMEMBRANE DOMAIN ENHANCES ANTIBODY RESPONSE BUT NOT PROTECTION

P.K. GUPTA[1], S. SHARMA[1], S.S. WALUNJ[1], A.A. PATIL[1], A. RAI[1*], M. SAINI[2]

[1]Division of Veterinary Biotechnology, [2]Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar-243 122, UP, India

Received July 19, 2005; accepted April 7, 2006

Summary. – Rabies virus (RV) glycoprotein (gp) consists of three domains: cytoplasmic, transmembrane and ectodomain. It occurs in a complete, membrane-bound form within the infected cell, but it is released from them in a deleted, secreted form lacking the transmembrane domain. This study was performed to test the importance of the transmembrane domain for the capability of the RV gp gene, introduced into mice via a recombinant plasmid (DNA vaccine), to induce immune response and protection against challenge. Although the antibody response to the secreted form of gp was higher than that to complete gp, the protective efficacy of the respective DNA vaccine against challenge was not better than that of the DNA vaccine inducing complete gp. This indicates that the transmembrane domain of RV gp is important for generating protection against rabies and should be present in RV DNA vaccines.

Key words: Rabies virus; DNA vaccine; glycoprotein; antibody response; protection; mice; transmembrane domain

Introduction

RV causes a disease of central nervous system in humans as well as in domestic and wild animals. RV (the species *Rabies virus*, the genus *Lyssavirus*, the family *Rhabdoviridae*) contains a single-stranded negative-sense RNA genome of about 12 kb, which encodes 5 proteins, namely, the nucleoprotein, phosphoprotein, matrix protein, RNA-dependent RNA polymerase, and gp. RV gp, the only protein exposed on the surface of the viral particle, is the mediator of both the binding to cellular receptors and the entry into host cells (Coll, 1995). This type I transmembrane protein consists of three domains: cytoplasmic, transmembrane and ectodomain exposed as trimers at the virus surface (Gaudin *et al.,* 1992). The ectodomain is involved in the induction of both the virus neutralizing antibody induction and the protection after pre- and post-exposure vaccination (Cox *et al.,* 1977; Perrin *et al.,* 1985). RV gp also induces cytotoxic T lymphocytes (TLC) (Macfarlan *et al.,* 1986) and T helper cells (Celis *et al.,* 1988). Besides virus neutralizing antibodies also CTL and innate mechanisms are important for immune defence against rabies (Dietzschold and Ertl, 1991; Hooper *et al.,* 1998). Therefore, much attention has been focused on RV gp in the development of subunit vaccine against rabies.

Inoculation of mice with plasmids containing the gene encoding RV gp efficiently induced humoral and cellular immune responses resulting in protection against intracerebral challenge (Xiang *et al.,* 1994). Rabies DNA vaccines were effective in the pre-exposure and post-exposure protection of mice, dogs and non-human primates (Xiang *et al.,* 1994; Ray *et al.,* 1997; Lodmell *et al.,* 1998; Perrin *et al.,* 2000; Lodmell and Ewalt, 2001). Post-exposure vaccination against rabies requires high level and quick onset of neutralizing antibodies following the vaccination (Perry *et al.,* 1991; Xiang *et al.,* 1995). However, the immune response to a DNA vaccine is usually slow (Sedegah *et al.,*

*E-mail: raia1@rediffmail.com; fax: +91581-2301584.

Abbreviations: CTL = cytotoxic T-lymphocyte; gp = glycoprotein; RV = Rabies virus

1994; Ray *et al.,* 1997; Lodmell *et al.,* 1998). Therefore, several attempts have been made to increase this response. In particular, complexing plasmid DNA with positively charged vesicles, entrapping plasmid DNA in liposomes (Jones *et al.,* 1997; Chen *et al.,* 1998; Gregoriadis, 1998), improved gene expression, insertion of immunostimulatory sequences into the plasmid DNA backbone, co-administration of immunomodulators with DNA vaccine (Stingl, 1990; Xiang and Ertl, 1995; Iwasaki *et al.,* 1997), and boosting with inactivated virus or recombinant vaccinia virus after priming with DNA vaccine (Lodmell and Ewalt, 2000). Most of these attempts were successful.

In general, following intramuscular administration of a DNA vaccine an intense secretion of viral antigen is required for humoral immune response (Drew *et al.,* 2000). Several studies have demonstrated that DNA vaccines which expressed viral antigen in secreted form induced 5-10 times higher antibody titers compared with those which expressed viral antigen in membrane-bound form (Boyle *et al.,* 1997; Kuhrober *et al.,* 1997; Inchauspe *et al.,* 1997; Vinner *et al.,* 1999; Drew *et al.,* 2000).

In this study, we have attempted to increase the antibody response of mice to RV gp following immunization with a recombinant plasmid encoding the gp in secreted form. The protective efficacy of such a DNA vaccine was compared with that encoding the gp in membrane-bound (complete) form.

Materials and Methods

*Virus and cells***.** RV CVS strain was propagated in mouse brain. For transfection, MDBK cells were used. They were grown in Gibco Minimum Essential Medium (MEM) supplemented with 10% of heat-inactivated FBS, 50 µg/ml gentamicin at 37°C in 5% CO_2.

RT-PCR. Total RNA was isolated from RV-infected mouse brains using Trizol LS reagent (LifeTechnologies) according to the manufacturer's instructions. RNA was finally dissolved in 20 µl of DEPC-treated RNase-free distilled water per sample. cDNA was synthesized by reverse transcription from 8–10 µg of total RNA in a reaction mixture (20 µl) consisting of 50 pmoles of the primer 5'-AAGGAAAGATGGTTCCTCAG-3', 200 µmol/l dNTPs, 20 U of RNasin, and 200 U of MMLV reverse transcriptase in the 1x reaction buffer (Promega). After incubation at 42°C for 1 hr the cDNA was heated at 94°C for 5 mins and stored at -20°C until subjected to PCR. The PCR mixture (50 µl) contained 1 µl of cDNA, 50 pmoles of forward and reverse primers, 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$, and 3U of Expand™ high fidelity DNA polymerase (Roche) in the 1x reaction buffer. The primers for the amplicon of complete gp gene (1585 nts) were 5'-AAGGAAAGATGGTTCCTCAG-3' (forward) and 5'-CCTCACAGTCTGGTCTCACC-3'(reverse), and those for the amplicon of the gp gene lacking the transmembrane domain were 5'-CCCAAGCTTAAGTT CCCCATT TACACG-3' (forward, the *Hind*III site underlined) and 5'-CCTCACAGTCTGGTCTCACC-3' (reverse).The PCR consisted of 35 cycles of 94°C/1 min (denaturation), 60°C/1 min (annealing), and 72°C/2 mins (extension).

DNA vaccines consisted of recombinant plasmids pRab-G and pRab-Gsec, constructed using the pTargeT (Promega) expression vector carrying the Human cytomegalovirus (HCMV) immediate early enhancer/promoter. pRab-G contained complete RV gp gene, while in pRab-Gsec the tansmembrane domain was deleted and natural signal sequence was replaced with that for CD33.

To prepare pRab-G, the RV gp gene was amplified by PCR and inserted into pTargeT using T4 DNA ligase (LifeTechnologies) following the manufacturer's instructions. The presence of the insert in the construct was checked by the digestion with *Eco*RI. To prepare pRab-Gsec, the RV gp gene lacking the transmembrane domain was amplified by PCR. The PCR product was double digested with *Hind*III and *Pst*I, purified by gel electrophoresis and cloned into the *Hind*III and *Pst*I sites in the pUC19 vector. Further, this insert was cut out from the recombinant using *Hind*III and *Xba*I and cloned into the corresponding sites in the pCMV-CD33 vector containing a CD33 secretion signal. This positioned the CD33 signal peptide in frame with the N-terminal end of gp and stop codon within the *Xba*I site. The final construct pRab-Gsec was isolated and the presence of the insert was checked by the digestion with *Hind*III and *Xba*I. The sequences of the inserts in the constructs were determined using an ABI PRISM 377 Version 3.0 DNA sequencer.

Large scale preparation of DNA vaccines. Recombinant constructs pRab-G and pRab-Gsec were prepared on large scale from respective transformed bacterial clones in standard manner. Plasmid DNA was isolated using the Plasmid Mega Kit (Qiagen) and assayed spectrophotometrically. For immunization, it was dissolved in 150 mmol/l NaCl. All DNA preparations were stored at -20°C until used.

In vitro expression of recombinant antigens. Expression of the two different forms of RV gp from pRab-G and pRab-Gsec was carried out in MDBK cells after transfection using Lipofectin reagent (Gibco BRL). In case of pRab-G, whole cell lysate of the transfected cells was prepared by resuspending the cell monolayer in 400 µl of Laemmli buffer. In case of pRab-Gsec, the serum containing medium from the transfected cell cultures was removed 24 hrs post transfection and the cells were incubated with 4 ml of serum-free medium for 24 hrs. The cell culture supernatant was clarifies at 10,000 rpm for 10 mins and the proteins were precipitated and resuspended in 100 µl of Laemmli buffer (Drew *et al*., 2000). The samples of both types were boiled for 5 mins and subjected to SDS-PAGE.

SDS-PAGE and Western blot analysis. SDS-PAGE (10% gel) was carried out in standard manner. The gels were stained with Coomassie Blue and, for Western blot analysis, blotted onto nitrocellulose membranes and blocked with 5% skim milk in PBS containing 0.05% Tween 20 for 1 hr. The blots were probed with a mouse polyclonal anti-RV serum for 1 hr, and the antibodies were detected using an anti-mouse antibody conjugated with alkaline phosphatase (Sigma) and visualized with NBT/BCIP (Ameresco).

Vaccination of mice. Groups of six 4–6 week-old BALB/c mice were vaccinated each with 100 µg of DNA vaccine per dose at one site in quadriceps muscle. The first dose was followed by the second after 21 days. A control group of mice received saline. The mice were bled on days 0 and 28 and the obtained sera were assayed for specific antibodies using ELISA.

ELISA. Microplates (Nunc) were coated overnight at 4°C with inactivated purified RV antigen (Rai, 1985) in 0.05 mol/l carbonate/bicarbonate buffer pH 9.6 (1 µg/100 µl/well) and blocked with 5% skim milk in PBS-T for 1 hr. Aliquots of serum samples diluted 1:50 or 1:200 in 2% skim milk in PBS-T were added (50 µl/well) and the plates were incubated for 1 hr. Then an anti-mouse antibody conjugated with HRPO (Sigma) was (50 µl) added. After 1 hr the color was developed using OPD, the reaction was stopped with 5 N H_2SO_4 and A_{492} was read. All samples were run in triplicate.

Challenge of vaccinated mice. Two weeks after the second dose of DNA vaccine, the vaccinated mice were challenged intracerebrally with 15 LD_{50} of fixed RV CVS strain. Six healthy non-vaccinated mice (control) were challenged too. All the challenged mice were observed for 14 days for development of rabies-specific paralytic symptoms or death.

Results

In vitro expression of the two forms of RV gp in MDBK cells

The ability of the DNA vaccines pRab-G and pRab-Gsec to direct the expression of the respective forms of RV gp was investigated by transfecting MDBK cells (Fig. 1). SDS-PAGE revealed an additional protein of 67 K (lane 1) in the cell lysate of pRab-G-transfected cells compared with the lysate of mock-transfected cells (lane 2). The M_r of this protein was similar to that predicted for complete RV gp. The expression of the secreted RV gp of 59 K was observed in the lysate of the cells transfected with pRab-Gsec (lane 3). The predicted M_r of the secreted RV gp (59 K) should be by 8 K less than that for complete RV gp (67 K). The 59 K protein was also found in the supernatant from the culture of cells transfected with pRab-Gsec (lane 5).

The reactivity of the expressed proteins was confirmed by Western blot analysis using a RV-specific serum (Fig. 1B).

Antibody response to DNA vaccines

The immunogenicity of the DNA vaccines pRab-G and pRab-Gsec expressing the two forms of Rv gp was followed in mice (Fig. 2). All mice vaccinated with both of these vaccines generated a detectable antibody response on day 28 post vaccination. pRab-Gsec generated a higher antibody response compared with pRab-G; in particular, the mean ELISA absorbance for pRab-Gsec was approximately 1.5 times higher.

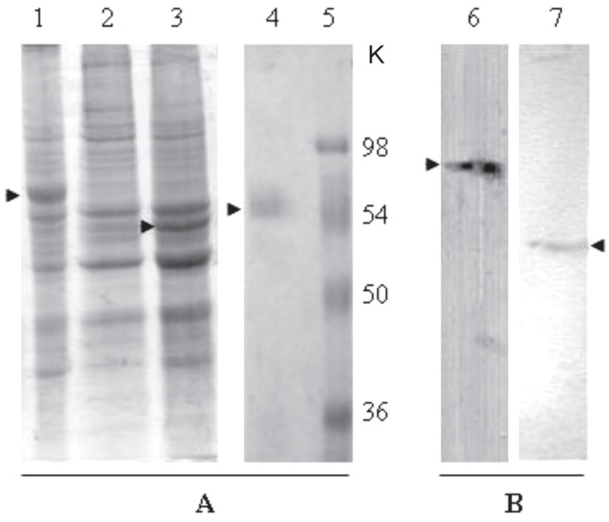

Fig. 1

SDS-PAGE (A) and Western blot analysis (B) of complete and secreted form of RV gp in transfected MDBK cells

pRab-G-transfected cells (lane 1); mock-transfected cells (lane 2); pRab-Gsec-transfected cells (lane 3); supernatant from pRab-Gsec-transfected cell culture (lane 4); DNA size marker (K) (lane 5); pRab-G-transfected cells (lane 6); pRab-Gsec-transfected cells (lane 7).

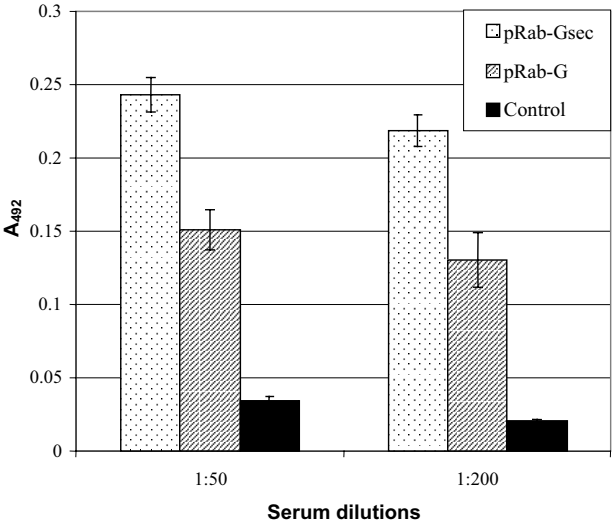

Fig. 2

Antibody response to pRab-Gsec and pRab-G in mice

Groups of six 4–6 week-old BALB/c mice were vaccinated with 100 µg/dose of plasmid DNA intramuscularly on days 0 and 21. Serum IgG antibodies from day 28 post vaccination were assayed by ELISA. A_{492} values represent means from six mice.

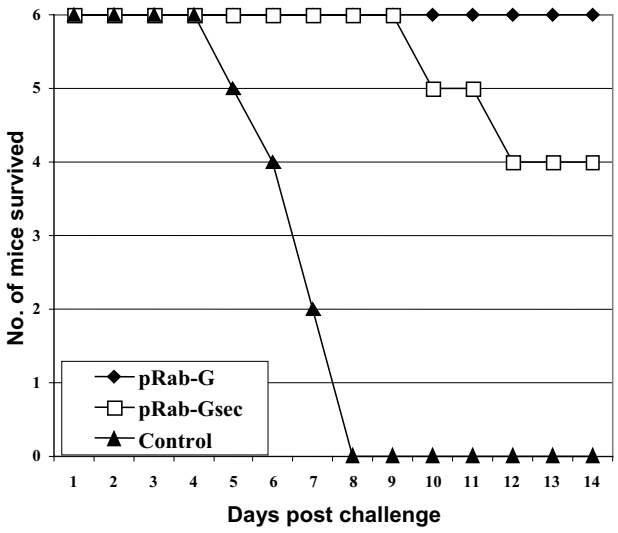

Fig. 3

Survival of immunized mice after challenge

The immunized mice (see Fig. 2) were challenged 2 weeks after the second dose with 15 LD_{50} of RV i.c. Survival was monitored daily for 14 days post challenge.

Protective response to DNA vaccines

The mice vaccinated with pRab-G encoding complete RV gp were protected against the challenge by 100%, while the mice vaccinated with pRab-Gsec encoding the deleted form of RV gp (secreted gp) showed only a 66.6% protection (Fig. 3). All the non-vaccinated mice died.

Discussion

In this study, the immunogenicity and protective efficacy of a complete and a truncated (secreted) form of RV gp was investigated. The pRab-Gsec plasmid constructed in this study did not encode the hydrophobic anchor (transmembrane) domain of full length RV gp. This type of truncation in the gp gene was made with the aim to construct the gp in the form secreted out from the cells. The natural signal sequence of the gene was replaced with the CD33 signal sequence to ensure maximum secretion of the expressed protein from the expressing cells as described earlier for other proteins (Day *et al*., 1999; Cik *et al*., 2000). The plasmid construct pRab-Gsec carrying this type of RV gp gene led to the expression of the 59 K protein. The latter protein could not be found in the pRab-G-transfected or mock-transfected cells, confirming thereby that the expressed protein was secreted out from the cell through the CD33 signal.

Both plasmid constructs induced antibody responses in immunized mice. The secreted form of gp generated a higher antibody response compared with complete gp. This difference was ascribed to the secretion of the expressed protein. Earlier studies on different forms of expressed antigens have demonstrated that the location of the expressed protein influences the nature of the induced response. When the expressed protein remained cell-associated, either in the cytoplasm or the membrane, Th1 cytokines and IgG2a antibodies were produced (Boyle *et al.,* 1997; Lewis *et al.,* 1999). In contrast, when the protein was secreted, Th2 cytokines and IgG1 antibodies predominated (Boyle *et al.,* 1997; Lewis *et al.,* 1999).

The increased antibody response in the mice vaccinated with pRab-Gsec leading to the secreted form of RV gp, observed in this study, might be due to the release of the antigen from transfected cells. In general, after DNA immunization, induction of respective antibody response occurs following the movement of free antigen to lymph nodes or the capture of antigen by professional APCs (Robinson and Torres, 1997). Furthermore, the dose of the antigen delivered to the cells of the immunity system is responsible for the magnitude of respective immune response (Barry and Johnston, 1997). In this study, the secretion of RV gp might have allowed its release from transfected cells into extracellular space where from it could either passively migrate into draining lymph nodes or be captured by infiltrating and migrating APCs. The other DNA vaccine, pRab-G was able to accomplish release of the respective antigen into extracellular space only upon lysis or death of transfected cells, and thereby it generated a low antibody response. Several studies have also reported higher antibody titers in response to DNA vaccines expressing a secreted form of proteins compared with DNA vaccines expressing membrane bound form of proteins (Boyle *et al*., 1997; Kuhrober *et al*., 1997; Drew *et al*., 2000; Morel *et al*., 2004).

In this study, the DNA vaccine leading to the secreted RV gp increased the antibody response but not protection compared to that producing complete membrane-bound RV gp. Similar results have been reported earlier with purified soluble form of RV gp (Dietzschold *et al.*, 1983).

When this manuscript was in press, Rath *et al.* (2005) reported that, using a baculovirus as vector (DNA vaccine), a RV gp lacking the transmembrane domain induced lower antibody titers compared with complete gp. However, their results are hardly comparable with ours, as (i) they used in ELISA an antigen expressed in *Escherichia coli*, hence an unglycosylated unnatural antigen, (ii) they employed a non-mammalian vector (baculovirus pAC vector) as DNA vaccine, and (iii) they did not perform a protective test. In contrast, we used a purified natural RV as ELISA antigen and a mammalian expression vector as DNA vaccine, and we added a challenge/protection test on the vaccinated mice.

In conclusion, this study indicated that a DNA vaccine encoding complete RV gp conferred to mice a 100% protection against challenge thereby indicating that the transmembrane domain of RV gp is important for inducing protective immunity.

Acknowledgements. The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar, India, for providing necessary facilities to carry out the work. The help extended by Scientist, Quality Control, National Project on Rinderpest Eradication, is highly acknowledged.

References

Barry MA, Johnston SA (1997): Biological features of genetic immunization. *Vaccine* **15**, 788–791.

Boyle JS, Koniaras C, Lew AM (1997): Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int. Immunol.***12**, 1897–1906.

Celis E, Ou D, Dietzschold B, Koprowski H (1988): Recognition of rabies and rabies-related virus by T cells derived from human vaccine recipients. *J. Virol.* **62**, 3128–3134.

Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JC, Greenberg HB, Herrmann JE (1998): Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J. Virol.* **72,** 5757–5761.

Cik M, Masure S, Lesage ASJ, Linden IV, Gompel PV, Pangalos MN, Gordon RD, Leysen JE (2000): Binding of GDNF and neurturin to human GDNF family receptor α 1 and 2. *J. Biol. Chem.* **275**, 27505–27512.

Coll JM (1995): The glycoprotein G of rhabdoviruses. *Arch. Virol.* **140**, 827–851.

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

Day JM, Murdoch AD, Hardingha TE (1999): The folded protein modules of the C-terminal G3 domain of aggrecan can each facilitate the translocation and secretion of the extended chondroitin sulfate attachment sequence. *J. Biol. Chem*. **274**, 38107–38111.

Dietzschold B, Ertl HC (1991): New developments in pre- and post-exposure treatment of rabies. *Crit. Rev. Immunol.* **10**, 427–439.

Dietzschold B, Wiktor TJ, Wunner WH, Varrichio A (1983): Chemical and immunological analysis of the rabies soluble glycoprotein. *Virology* **124**, 330–337.

Drew DR, Lightowlers M, Strugnell RA (2000): Humoral immune responses to DNA vaccines expressing secreted, membrane bound and non-secreted forms of the Taenia ovis 45W antigen. *Vaccine* **18**, 2522–2532.

Gaudin Y, Ruigrok R, Tuffereau C, Knossow M, Flamand A (1992): Rabies glycoprotein is a trimer. *Virology* **187**, 627–632.

Gregoriadis G (1998): Genetic vaccines: strategies for optimization. *Pharm. Res.* **15**, 814-870.

Hooper DC, Morimoto K, Bette M, Weihe E, Koprowski H, Dietzschold B (1998): Collaboration of antibody and inflammation in the clearance of rabies virus from the CNS. *J. Virol.* **72**, 3711–3719.

Inchauspe G, Vitvitski L, Major ME, Jung G, Spengler U, Maisonnas M, Trepo C (1997): Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA Cell Biol.* **16**, 185–195.

Iwasaki A, Stiernholm BJ, Chan AK, Berinstein NL, Barber BH (1997): Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J. Immunol.* **158**, 4591–4601.

Jones DH, Corris S, McDonald S, Clegg JC, Farrar GH (1997): Poly (DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* **15**, 814–817.

Kuhrober A, Wild J, Pudollek HP, Chisari FV, Reimann J (1997): DNA vaccination with plasmid encoding the intracellular (HBsAg) or secreted (HBcAg) from of the core protein of hepatitis B virus primes T cell responses to two overlapping Kb and Kd-restricted epitopes. *Int. Immunol.* **9**, 1203–1212.

Lewis PJ, van Drunen Littel-van den Hurk S, Babiuk LA (1999): Altering the cellular location of an antigen expressed by a DNA-based vaccine modulates the immune response. *J. Virol*. **12**, 14–23.

Lodmell DL, Ewalt LC (2000): Rabies vaccination: comparison of neutralizing antibody responses after priming and boosting with different combinations of DNA, inactivated virus, or recombinant vaccinia virus vaccines. *Vaccine* **18**, 2394–2398.

Lodmell DL, Ewalt LC (2001): Post-exposure DNA vaccination protects mice against rabies virus. *Vaccine* **19**, 2468–2473.

Lodmell DL, Ray NB, Ewalt LC (1998): Gene gun particle-mediated vaccination with plasmid DNA confers protective immunity against rabies infection. *Vaccine* **16**, 115–118.

Macfarlan RI, Dietzschold B, Koprowski H (1986): Stimulation of cytotoxic T-lymphocyte responses by rabies virus glycoprotein and identification of an immunodominant domain. *Mol. Immunol.* **23**, 733–741.

Morel PA, Falkner D, Plowey J, Larregina AT, Falo LD (2004): DNA immunisation: altering the cellular localisation of expressed protein and the immunisation route allows manipulation of the immune response. *Vaccine* **22**, 447–456.

Perrin P, Jacob Y, Aguilar-Setien A, Loza-Rubio E, Jallet C, Desmezieres E, Aubert M, Cliquet F, Tordo N (2000): Immunization of dogs with a DNA vaccine induces protection against rabies virus. *Vaccine* **18**, 47–56.

Perrin P, Thibodeau L, Sureau P (1985): Rabies immunosomes (subunit vaccine) structure and immunogenicity. Pre- and post exposure protection studies. *Vaccine* **3**, 325–332.

Perry LL, Lodmell DL (1991): Role of CD4+ and CD8+ T cells in murine resistance to street rabies virus. *J. Virol.* **65**, 342–344.

Rai A (1985): *Methods in Cell Culture and Virology.* Allied Publishers, New Delhi.

Rath A, Choudhury S, Batra D, Kapre SV, Rupprecht CE, Gupta SK (2005): DNA vaccine for rabies: relevance of the trans-membrane domain of the glycoprotein in generating an antibody response. *Virus Res.* **113**, 143–152.

Ray NB, Ewalt LC, Lodmell DL (1997): Nanogram quantities of plasmid DNA encoding the virus glycoprotein protect mice against lethal rabies virus infection. *Vaccine* **15**, 82–85.

Robinson HL, Torres CA (1997): DNA vaccines. *Semin. Immunol.* **9**, 271–283.

Sedegah M, Hedstrom R, Hobart P, Hoffman SL (1994): Protection against malaria by immunization with plasmid DNA encoding circumsporozoit protein. *Proc. Natl. Acad. Sci. USA* **91**, 9866–9870.

Stingl G (1990): Dendritic cells of skin. *Dermatol. Clin.* **8**, 673–679.

Vinner L, Nielsen HV, Bryder K, Corbet S, Nielsen C, Fomsgaard A (1999): Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp-160 envelope gene using mammalian codons. *Vaccine* **17**, 2166–2175.

Xiang Z, Ertl HC (1995): Manipulation of immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* **2**, 129–135.

Xiang ZQ, Knowles BB, McCarrick JW, Ertl HC (1995): Immune effector mechanisms required for protection to rabies virus. *Virology* **214**, 398–404.

Xiang ZQ, Spitalnik S, Tran M, Wunner WH, Heng J, Ertl HC (1994): Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* **199**, 132–140.