Evaluation of the protective efficacy of a rabies DNA vaccine in mice using an intracerebral challenge model

Subhabrata Biswas, M. S. Ashok, G. S. Reddy[†], V. A. Srinivasan[†] and
P. N. Rangarajan*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India
[†]Indian Immunologicals, Hyderabad 500 019, India

An eukaryotic expression plasmid expressing the rabies virus surface glycoprotein (G protein) under the control of cytomegalovirus immediate early promoter and intron was constructed. This plasmid designated as pCMVRab also contains the gene conferring resistance to kanamycin (kanR) to bacteria harbouring this plasmid rather than that conferring resistance to ampicillin (ampR) and thus is devoid of the CpG immunostimulatory sequences (ISS) present in the latter. The ability of pCMVRab to induce a protective immune response was examined in outbred swiss mice using an intracerebral (i.c.) rabies virus challenge model. Following intramuscular (i.m.) inoculation, anti-G protein antibodies as well as virus neutralizing antibodies (VNA) are detected in the sera of immunized mice up to four months after immunization. Rabies virus-specific T helper cell proliferation could be demonstrated up to six months after immunization. Induction of these immune responses in pCMVRab-immunized mice results in significant protection against a subsequent lethal i.c. rabies virus challenge. Thus, we demonstrate for the first time that a rabies G protein expression plasmid devoid of the ISS present in the ampR gene confers significant protection against i.c. rabies virus challenge when inoculated into the skeletal muscle of outbred swiss mice.

VACCINES which can be produced and purified by inexpensive procedures and also stored at room temperature, are ideally suited for eradication of infectious diseases in developing countries. In this context the use of plasmid DNA as a vaccine assumes great significance, since it can be both produced at a very low cost and stored at room temperature. Inoculation of animals with purified plasmid DNA encoding antigenic proteins results in the transfection of host cells followed by the expression of the vector-encoded foreign proteins, leading to the stimulation of a specific immune response including T helper cells, cytolytic T cells and antibodies[1-8]. This methodology known as DNA vaccination, genetic immunization or nucleic acid immunization is a viable alternative to attenuated virus- or recombinant protein-based vaccines[9-12].

Rabies continues to be a serious problem in both developed and developing countries due to the reservoir of rabies virus in wild life and domestic animals. The disease causes a fatal encephalomyelitis in all warm-blooded animals, and there is no treatment once its symptoms have appeared. Protection against lethal infection can be achieved by pre- or, frequently, post-exposure vaccination. There are quite a few vaccines currently in use for humans, domestic and wild animals. However, limited access to high quality cell culture-based anti-rabies vaccines coupled with their high cost and lack of a cold chain are responsible for the deaths of more than 50,000 people and millions of animals in developing countries. In India, approximately 30,000 people die of rabies annually and 500,000 undergo rabies prophylaxis[13]. Thus in India, the demand for anti-rabies vaccine which is mostly imported, far exceeds the local production; this situation urgently calls for a vaccine that has a simple formulation, is cost effective and does not require a cold chain. In this study, we report our endeavour towards the development of such a vaccine for rabies.

Earlier studies[14-17] have demonstrated that inoculation of plasmid DNA encoding the rabies virus G protein by intramuscular (i.m.) or intradermal (i.d.) route protects mice and monkeys against peripheral virus challenge. Recently, inoculation of the rabies G protein expression plasmid into regenerating muscle was shown to protect mice against intracerebral (i.c.) rabies virus challenge[18]. The rabies G protein expression plasmid used in all these studies consists of the gene conferring resistance to ampicillin (ampR) to bacteria harbouring them. It is known that the ampR gene contains CpG immunostimulatory sequences (ISS)[19,20] and as a result, the plasmid vector, even in the absence of a gene encoding any antigen induces protective immune responses in mice. For example, a plasmid vector carrying no G protein gene elicited a non-specific immune response and conferred 60% protection in mice against i.c. rabies virus (CVS strain) challenge[18]. In case of plasmid DNA intended for use in clinical trials in humans and animals, the ampR gene is replaced by the gene conferring resistance to kanamycin (kanR)[21]. So far, the protective efficacy of a rabies G

protein expression plasmid lacking the *ampR* gene has not been reported. We therefore constructed such a plasmid vector and demonstrated for the first time that a rabies G protein expression plasmid in which the the *ampR* gene is replaced by *kanR* gene, still confers significant protection against i.c. rabies virus challenge in outbred swiss mice.

Materials and methods

Rabies virus

The rabies virus of the challenge virus standard (CVS) strain propagated in Vero cells was concentrated by iso-pycnic centrifugation in a 15–50% sucrose density gradient. The virus particles forming a single band at a buoyant density of 1.17 g/cm^3 (equivalent to 40% sucrose) were inactivated with β-propiolactone and used for T helper (Th) cell proliferation assays. The same strain propagated in mouse brain was used for i.c. challenge of mice. Inactivated rabies virus vaccine (Raksharab, Indian Immunologicals, Hyderabad, India) was used for immunization of mice. All experiments involving live rabies virus were carried out at the Indian Immunologicals, Hyderabad following national biosafety guidelines.

Construction of rabies DNA vaccine plasmid and transfection of Vero cells

The plasmid DNA expressing the rabies virus G protein (pCMVRab) was constructed by isolating of cDNA encoding rabies virus G protein as a Bgl II fragment from the ptg155 plasmid[22] and its subsequent cloning into the BamHI site of the pVR1012 (ref. 23) downstream of cytomegalovirus immediate early promoter and intron sequences. Large-scale plasmid isolation and purification was carried out essentially as described earlier[24]. The purified plasmid was dissolved in saline and stored at − 80°C until further use in aliquots at a concentration of 1 mg/ml. Expression of mRNA encoding rabies G protein was examined by transfection of Vero cells with pVR1012 or pCMVRab using Lipofectamine (Life Technologies, USA), followed by RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) employing oligonucleotide primers (5′ CGCGGATCCGTTCCTCAGGC-TCTC 3′ and 5′ ACGCGTCGACTCACAGTCTGGTCTC 3′) specific for rabies G protein. The PCR products were analysed on a 1% agarose gel and visualized under UV light after ethidium bromide staining.

Detection of virus neutralizing antibodies

The presence of neutralizing anti-G protein antibodies in the mice sera was detected using the Platelia rabies kit (Diagnostics, Pasteur) following instructions given in the kit. This kit is based on the use of microplates coated with rabies virus glycoprotein extracted from inactivated and purified virus membranes. Different dilutions of a control serum titrated in IU/ml as well as the test sera are added to individual wells followed by washing and the addition of peroxidase conjugated protein A. After eliminating the unbound conjugate, the substrate is added and a spectrophotometric reading is taken at 492 nm. Comparison of absorbance values of the test sera with those of the control serum provides the titre of the test sera in units equivalent to the international units (EU) defined by seroneutralization. The ability of these anti-G protein antibodies to neutralize rabies virus infection was determined by Rapid Fluorescent Focus Inhibition Test (RFFIT)[25.] Briefly, different dilutions of the test and reference (WHO) sera were mixed with a fixed quantity of Street Alabama Dufrin (SAD) strain of rabies virus. The virus as well as the serum–virus mixture are then seeded along with BHK cells in the Labtrek counting chamber slides and incubated for 24 h. The cell sheet is then fixed with acetone and stained with fluorescein isothiocyanate conjugated rabies nucleocapsid antibodies (Diagnostics Pasteur, France) and observed under flourescent microscope for rabies inclusion bodies. Based on the presence of un-neutralized virus across the virus dilutions, the titre is expressed as the reciprocal of the dilution which neutralizes 50% of the virus. These titres were expressed as IU/ml by comparing the test serum titres with those obtained with the WHO reference serum.

T helper cell proliferation assay

Splenocytes and lymph node (LN) lymphocytes were isolated from mice immunized with pCMVRab and these cells were diluted to a final concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine and 5×10^{-5} M β-mercaptoethanol. A 100 µl aliquot containing 2×10^5 cells was added to each well of a 96-well flat-bottom microtitre plate (Nunc, Denmark). β-priopiolactone-inactivated rabies virus (CVS strain) suspended in 40% sucrose was used as the source of antigen. Cells were stimulated with either 40% sucrose alone or 40% sucrose containing inactivated rabies virus (1.5 µg total protein). All assays were carried out in triplicates. Three days after the addition of antigen, cells were pulsed with 1 µCi of ^{3}H thymidine (Du Pont NEN, USA) per well for 16 h. Cells were harvested, lysed and ^{3}H thymidine incorporation was measured in a liquid scintillation counter (Beckman, USA). The stimulation indices (SI) were calculated by the formula: SI = counts per minute (cpm) induced by rabies virus antigen/cpm induced by 40% sucrose.

Immunization of mice and i.c. rabies virus challenge

Four-week-old outbred swiss mice were inoculated twice at one month interval intramuscularly (i.m.) with 100 µg of pCMVRab or intraperitoneally (i.p.) with 0.5 ml of

inactivated rabies virus vaccine. Unimmunized mice were used as negative controls. Animals were challenged i.c. with 0.03 ml of $50LD_{50}$ rabies virus of the CVS strain. Mice were observed for 14 days post-challenge for development of symptoms of rabies and death. Mice dying within 48 h were considered as non-specific deaths and were eliminated from the study.

Results and discussion

The eukaryotic expression plasmid encoding the rabies G protein was generated by cloning the cDNA encoding rabies G protein into pVR1012 (ref. 23). The resulting plasmid was designated as pCMVRab. pVR1012 or pCMVRab was transfected into Vero cells and 24 h later RNA was isolated and subjected to RT-PCR analysis using oligonucleotide primers specific for rabies mRNA. A 1680 bp PCR product corresponding to mRNA encoding rabies G protein could be amplified from RNA isolated from pCMVRab-transfected cells (Figure 1; lane 2) but not pVR1012-transfected cells (Figure 1; lane 1). To examine the ability of pCMVRab to induce a protective

immune response, outbred swiss mice of 4 weeks age were inoculated i.m. with 100 μg of pCMVRab twice at two-week intervals. Mice immunized i.m. with pVR1012 served as negative controls. Blood samples were collected by retroorbital puncture at regular intervals up to four months and sera from each group of mice were pooled. The sera samples were analysed for the presence of anti-glycoprotein antibodies using the Platelia rabies kit in which the ELISA plates are coated with rabies glycoprotein extracted from inactivated and purified virus membranes. Using a control serum (titrated in IU/ml) provided in the kit, the titre of the sera from pCMVRab-immunized mice was calculated and expressed as a unit equivalent to the international unit defined by seroneutralization (EU/ml). The results presented in Figure 2 a indicate that the level of anti-glycoprotein antibodies in the sera of pCMVRab-immunized mice is higher than the minimum level of 0.5 EU/ml needed to resist experimental infection induced by the injection of wild rabies virus. Thus, i.m. inoculation of pCMVRab results in the synthesis of rabies virus G protein in the host leading to the induction of protective levels of anti-glycoprotein antibodies in the immunized animals. Since virus neutralizing antibodies (VNA) are pivotal for protection against rabies[25], the ability of anti-glycoprotein antibodies in the sera of pCMVRab-immunized mice to neutralize rabies virus infection was examined by rapid fluorescent focus inhibition test (RFFIT), a widely used assay for determining the potency of rabies vaccines. The results presented in Figure 2 b indicate that protective VNA titre can be detected in the sera of pCMVRab-immunized mice up to four months after i.m. inoculation of two doses of this plasmid. It is known that DNA vaccination induces both humoral and cell-mediated immune responses[1-8]. Rabies virus-specific Th cell proliferative responses were analysed in mice immunized twice with pCMVRab in an *in vitro* Th cell proliferation assay.
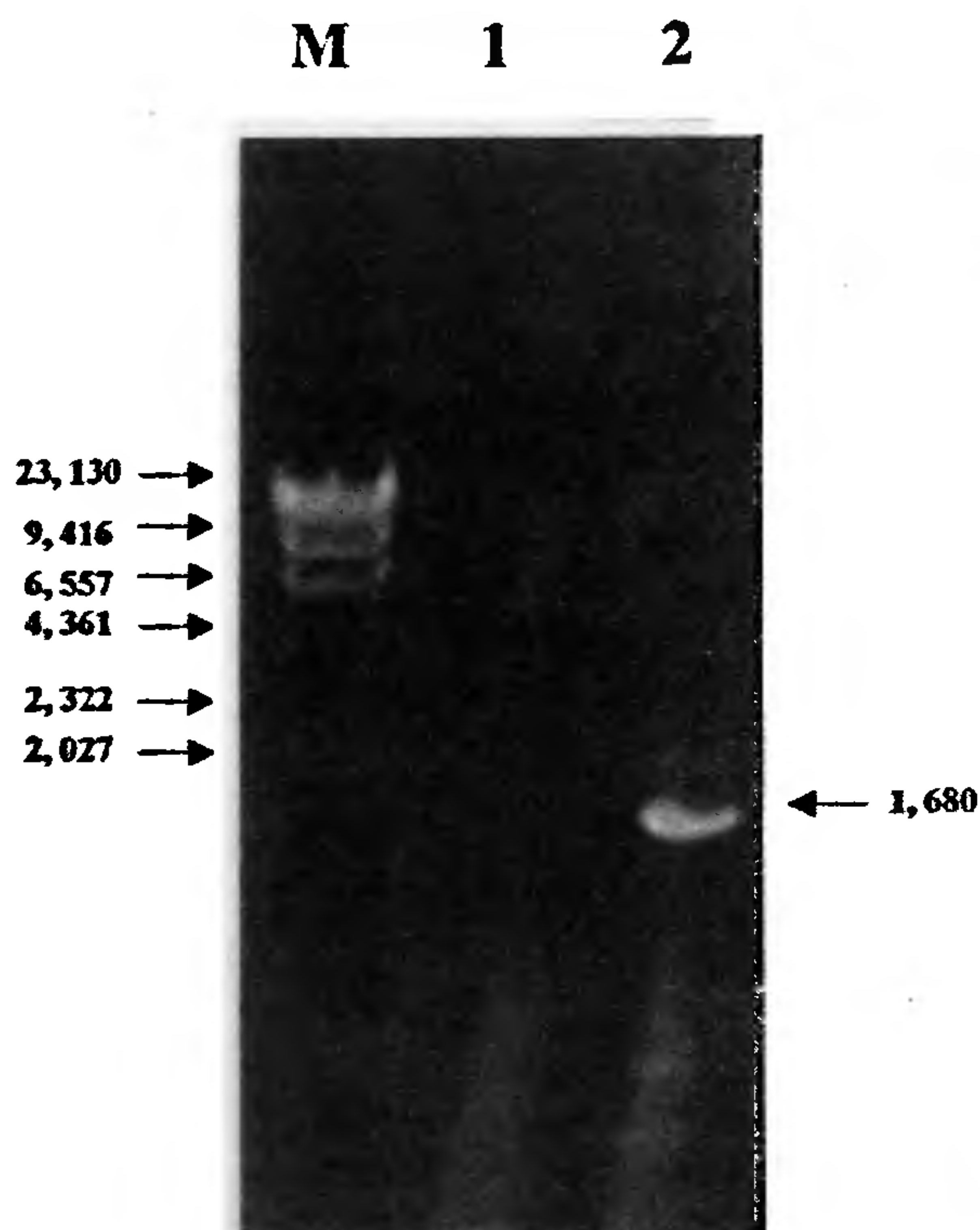

Figure 1. RT-PCR analysis of mRNA encoding rabies G protein in Vero cells transfected with pVR1012 (lane 1) or pCMVRab (lane 2). The numbers on the left indicate the size (in bp) of DNA fragments generated by *Hind*III digestion of λ DNA (lane M).
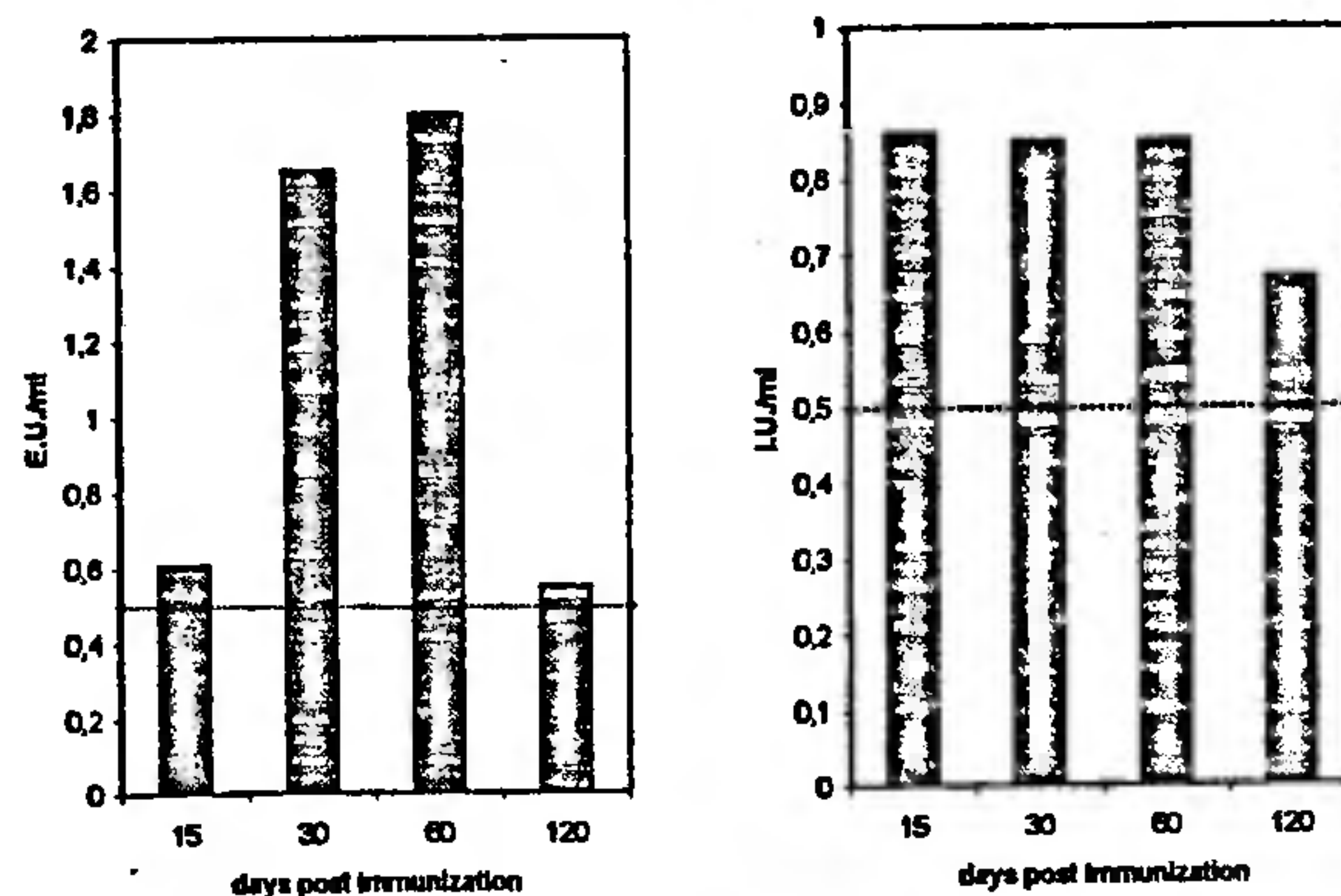

Figure 2. Detection of virus neutralizing antibodies (VNA) in the mice sera. The VNA titre was determined using (*a*), the Platelia rabies kit, or (*b*) RFFIT. The dotted line indicates the minimum VNA titre (0.5 IU) required for protection against rabies as recommended by WHO.
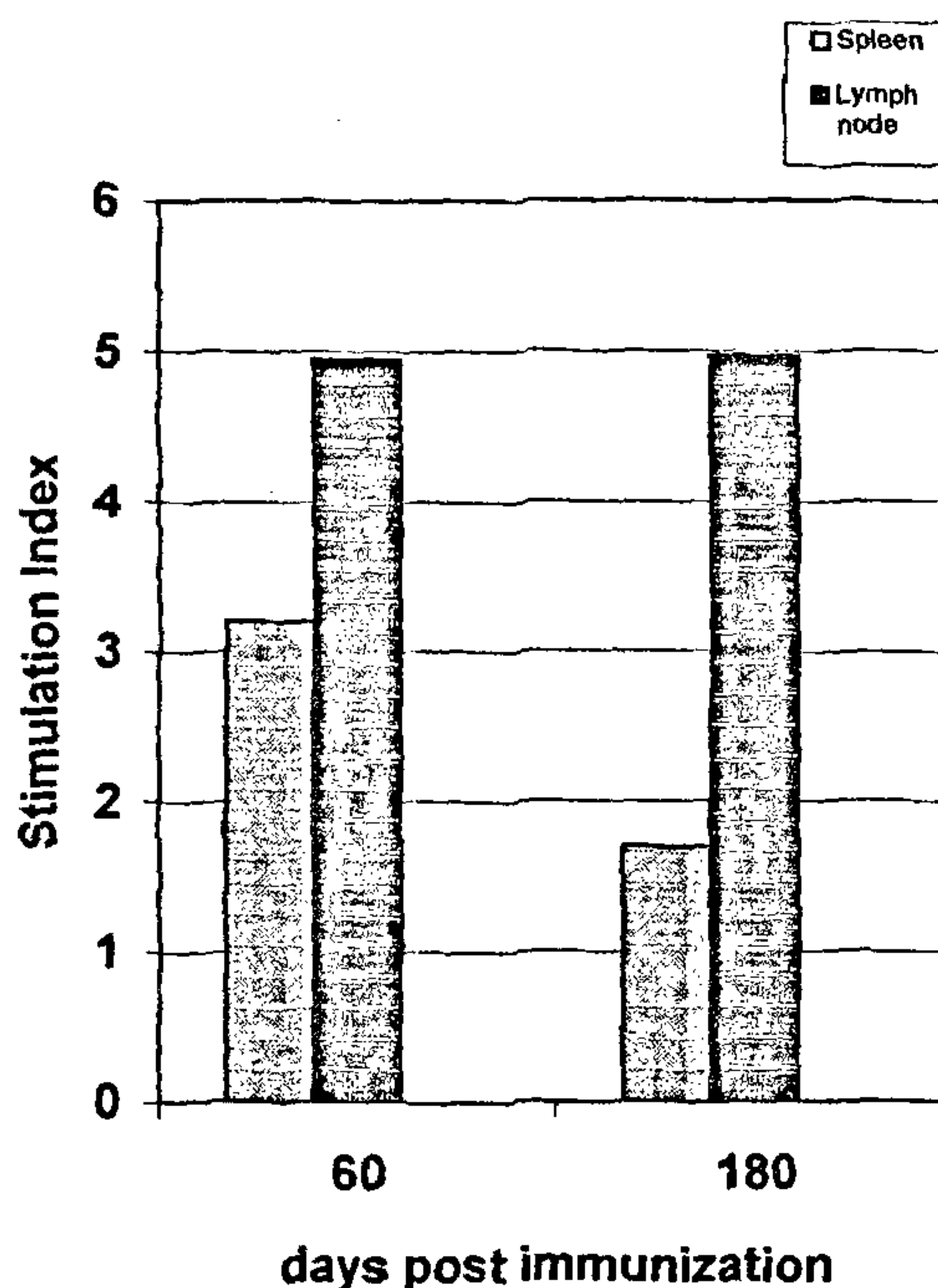
Figure 3. Analysis of rabies virus-specific Th cell proliferative responses in splenocytes and lymph node lymphocyes of mice immunized with pCMVRab.

Table 1. Protection of mice immunized with pCMVRab or inactivated rabies virus vaccine against i.c. rabies virus challenge

Vaccine	No. of mice immunized	No. of mice survived	% Protection
None	10	0	0
pCMVRab	14	9	64
Inactivated rabies virus vaccine	16	14	88

Mice were inoculated once with 0.5 ml of inactivated rabies virus vaccine (i.p.) or twice with 100 μg of pCMVRab (i.m.) at one month interval. Mice were challenged i.c. with $50LD_{50}$ CVS virus one month later.

Splenocytes and LN lymphocytes isolated from these mice were stimulated *in vitro* with inactivated rabies virus antigen. Rabies virus-specific Th cell proliferation was seen in the splenocytes as well as LN lymphocytes of mice sacrificed 2 months or 6 months after primary immunization (Figure 3).

The potency of rabies vaccines can be correlated with their ability to induce the production of VNA and the results presented above clearly indicate that rabies DNA vaccine induces VNA as well as Th cell responses in mice. The protective efficacy of rabies virus vaccines is assessed by the most widely used NIH potency test which was developed originally at the United States National Institutes of Health (NIH)[26]. This test involves i.c. chal-

lenge of the immunized mice with a standardized virus dose of the CVS strain of fixed rabies virus. The NIH test was adopted by the WHO expert committee on rabies and has become a part of many national and international requirements for testing the potency of several inactivated rabies vaccines. Although several laboratories including the United States Center for Disease Control (CDC) have developed alternate peripheral challenge models, these protocols have not yet been given official recognition[27]. Since i.c. challenge using the CVS strain is a widely accepted method for testing the potency of inactivated rabies vaccines, we investigated the ability of plasmid DNA immunization to protect mice from i.c. challenge. Mice were inoculated i.m. twice with pCMVRab and then challenged i.c. with $50LD_{50}$ CVS strain of rabies virus. Unimmunized mice and mice immunized with inactivated rabies virus vaccine served as negative and positive controls respectively. The results presented in Table 1 indicate that DNA vaccination confers significant protection against i.c. rabies virus challenge.

This study clearly demonstrates that i.m. inoculation of a plasmid DNA encoding rabies virus G protein induces humoral and cell-mediated immune responses in outbred swiss mice and confers significant protection against i.c. rabies virus challenge. pCMVRab used in this study consists of *kanR* gene instead of the *ampR* gene and thus is devoid of the ISS present in the *ampR* gene. However, the

protective efficacy of pCMVRab is lower than that recently reported by Bahloul et al.[18]. The plasmid constructs used by these authors consist of the ampR gene, cytomegalovirus promoter and intron as well as an SV40 enhancer and is therefore different from pCMVRab used in the present study. While it is tempting to speculate that the higher level of protection observed by these authors is due to presence of ampR gene in the G protein expression plasmid, one cannot rule out other possibilities such as differences in the promoter sequences, strain of mice, etc. Further, these authors have injected cardiotoxin prior to the injection of G protein expression plasmid and thus have used a regenerating muscle for plasmid DNA inoculation, while we have injected pCMVRab into a normal skeletal muscle. We are now examining the role of ISS of the ampR gene in protective immunity against rabies by introducing oligonucleotides containing ISS into pCMVRab.

Immunization of plasmid DNA encoding rabies G protein using a gene gun or by a combination of intradermal (i.d.) and i.m. inoculations protects rhesus monkeys against peripheral rabies virus challenge[17]. Surprisingly, inoculation via the i.d. route does not induce VNA in monkeys although i.d. inoculation induces a protective immune response in mice[17]. The potency of i.m. inoculation of plasmid DNA expressing rabies G protein has not been examined in primates. These studies indicate that despite a large number of reports on the efficacy of rabies DNA vaccine, several parameters such as vector design, route of immunization, challenge model, etc. have to be optimized for each mammalian species. Eradication of rabies in India will depend to a great extent on the immunization of animal reservoirs, primarily dogs, and therefore the efficacy of plasmid DNA immunization should be examined in a canine model. The knowledge gained from these studies will be of paramount importance in the design and development of a protocol for clinical trials of rabies DNA vaccine for both veterinary and human use.

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