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

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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].

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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 isopycnic 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′ CGCGGATCCGTTCCTCAGGCTCTC 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