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Alum adjuvanted rabies DNA vaccine confers 80% protection against lethal 50 LD_{50} rabies challenge virus standard strain

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ABSTRACT

Rabies is a serious concern world-wide. Despite availability of rabies vaccines for long; their efficacy, safety, availability and cost effectiveness has been a tremendous issue. This calls for improvement of rabies vaccination strategies. DNA vaccination has immense potential in this regard. The DNA vaccine pgp.LAMP-1 conferred 60% protection to BALB/c mice against 20 LD_{50} rabies challenge virus standard (CVS) strain challenge. Upon supplementation with Emulsigen-D, the vaccine formulation conferred complete protection against lethal challenge. To assess the feasibility of this vaccine formulation for human use, it was tested along with other FDA approved adjuvants, namely, Alum, Immuvac, Montanide ISA720 VG. Enhanced immune response correlated with high IgG antibody titer, Th2 biased response with a high level of rabies virus neutralizing antibodies (RVNAs) and IgG1/IgG2a ratio >1, observed upon alum supplementation of the rabies DNA vaccine. The total IgG antibody titer was 2 IU/ml and total RVNA titer was observed to be 4 IU/ml which is eight times higher than the minimum protective titer recommended by WHO. Furthermore, it conferred 80% protection against challenge with 50 LD_{50} of the rabies CVS strain, conducted in compliance with the potency test for rabies recommended by the National Institutes of Health (NIH), USA. Previously, we have established pre-clinical safety of this vaccine as per the guidelines of Schedule Y, FDA as well as The European Agency for evaluation of Medicinal Products. The vaccine showed no observable toxicity at the site of injection as well as at systemic level in Wistar rats when administered with 10X recommended dose. Therefore, supplementation of rabies DNA vaccine, pgp.LAMP-1 with alum would lead to development of a non-toxic, efficacious, stable and affordable vaccine that can be used to combat high numbers of fatal rabies infections tormenting developing countries.

1. Introduction

Rabies is a zoonotic disease, caused by rabies virus, a member of the Lyssavirus genus of Rhabdoviridae family. This virus uses mammals like domestic animals (dogs) and wild animals like raccoons, skunks, bats and foxes as its reservoir. The public health burden of rabies is huge; approximately 59,000 deaths occur every year (Hampson et al., 2015). WHO advises pre-exposure prophylaxis for high-risk area travelers, personnels occupationally involved with wild animal reservoirs, veterinarians or researchers working on rabies infected animals (WHO, Rabies, 2013). Post-exposure prophylaxis is used for people bitten by rabid animals. Currently, Human Diploid Cell Vaccine (HDCV), Purified Chick Embryo Cell (PCEC) vaccine, Purified Vero Cell Vaccine (PVCV) and Purified Duck Embryo Vaccine (PDEV) are advised by WHO for human usage. HDCV poses several local side effects like erythema in 85% patients, swelling in 61% patients and pruritus in 44% patients upon intradermal booster vaccination (Burridge et al., 1984). The high cost of HDCV is another factor demanding for alternative vaccination

Abbreviations: Ab, antibody; Ig, immunoglobulin; gp, glycoprotein; RVNA, rabies virus neutralization antibody; LAMP, 1 lysosomal-associated membrane protein-1.

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strategies (Dreesen et al., 1989). The adverse reactions of PVCV are similar but occur at lower frequency with symptoms of fever seen in only 6 of 100 vaccinees (Wang et al., 2000; WHO, 2010). DNA vaccine has emerged as one of the potent alternatives for rabies prevention. The beauty of DNA vaccine lies in the ease and cost-effectiveness of the procedure of generating DNA vaccine. It's capability to be stored at room temperature during transportation further enhances the scope of its usage in areas with limited cold storage accessibility. Plasmid DNA vaccinations have been used against a variety of infectious diseases including bacterial, viral and parasitic infections (Alves et al., 1998; Kaur et al., 2009; Wang et al., 1998) as they induce persistent cellular and humoral response (Liu, 2013). Novel adjuvants, targeting sequences, enhanced delivery methods, use of epigenetics and prime-boost DNA vaccine strategies have been further used to boost their immunogenicity (Li et al., 2012).

Previously, we have shown that DNA vaccine construct pgp.LAMP-1 (glycoprotein G gene with Lysosomal Associated Membrane Protein-1 targeting sequence) conferred partial protection (60%) against intracerebral challenge with 20 LD_{50} of rabies CVS strain in BALB/c mice (Kaur et al., 2009). To improve its protective efficacy, the vaccine was supplemented with Emulsigen-D which elicited enhanced total antibody as well as Rabies Virus Neutralizing antibody (RVNA) titer than the naked DNA. Further, the improved formulation imparted complete protection upon lethal challenge (Kaur et al., 2010). Our vaccine has been proven to be completely safe as per the guidelines of Schedule Y, FDA as well as The European Agency for evaluation of Medicinal Products (Garg et al., 2014). The vaccine alone as well as in combination with Emulsigen-D, was tested for local and systemic toxicity in Swiss Albino mice as well as Wistar rats and found to be safe even at 10X therapeutic dose (Garg et al., 2014).

In the present study, we have explored the effect of novel FDA approved adjuvants on pgp.LAMP-1 vaccination and compared their protective efficacy as per the NIH guidelines in BALB/c mice (Meslin et al., 1996). In addition to Emulsigen-D (MVP Lab. Inc); we investigated other potent and novel adjuvants, namely Alum (Sigma), Immuvac (Cadila Pharmaceuticals Ltd.), Montanide ISA720 VG (Seppic) in order to achieve a highly potent and efficacious vaccine formulation for human usage. Emulsigen-D is an oil-in-water emulsified adjuvant containing DDA immunostimulant which was previously used in our study, where optimized rabies DNA vaccine formulation imparted complete protection against challenge with 20 LD_{50} of CVS (Kaur et al., 2010). Emulsigen-D has been widely used for adjuvanting DNA vaccines such as in vaccine against *Toxoplasma gondii* as well as inactivated vaccines like Foot and mouth disease vaccine resulting in a heightened immune response (Hiszczyńska-Sawicka et al., 2010; Park et al., 2014). Despite its immense potential in enhancing immune response, Emulsigen-D is still not approved by FDA for human use. This prompted us to assess supplementation of RDV with FDA approved adjuvants so that the vaccine can be improvised for human usage. Amongst the different adjuvants, alum has been most extensively used in vaccines, including human vaccines for more than seven decades. Though, the exact molecular mechanism of its action is still not known, it has been postulated that adsorption to alum leads to increased antigen availability at the site of injection thereby elevating uptake by antigen-presenting cells (APCs) as well as dendritic cells (DCs) *in vitro* (Hem and Hogenesch, 2007; Morefield et al., 2005). The intraperitoneal administration of alum induced recruitment of monocytes which after internalization of vaccine antigen, migrate to the draining lymph nodes, and differentiate into inflammatory DCs (Kool et al., 2008). However, its inability to induce Th1 antibody isotypes or cellular immune responses, and poor impact on polysaccharide antigens limits its applicability in some scenarios (Asherson, 1995). Immuvac consisting of heat – killed *Mycobacterium w* (now referred to as *Mycobacterium indicus pranii*, MIP) has been approved for human use against leprosy, where it resulted increased immune responses to *M. leprae* antigens and enhanced bacterial clearance in patients (Sharma et al., 2005). Additionally, MIP has been shown to induce protection against tuberculosis (TB) in animal models and early sputum conversion in TB patients (Patel et al., 2002; Singh et al., 1991). Furthermore, it has shown potential for tuberculin conversion and increased CD4+ cell count in human immunodeficiency virus (HIV)-infected people (Nyasulu, 2010). Montanide ISA-720 VG, water-in-squalene emulsion, has been shown to elicit high antibody response in several animal species. It has been evaluated in human vaccine trials in combination with malaria (Lawrence et al., 1997) and HIV vaccines (Toledo et al., 2001). Montanide ISA 720 VG co-administered with *Plasmodium vivax* circumsporozoite protein based polypeptide was highly immunogenic in BALB/c mice and *Aotus* monkeys (Arévalo-Herrera et al., 2011). Moreover, it has established its safety profile in various studies for human vaccines (Ascarateil et al., 2015). Its mode of action is proposed to be the formation of a depot at the injection site from where antigen is released slowly (Miles et al., 2005). In the current study, the impact of these FDA approved adjuvants on rabies DNA vaccine pgp.LAMP-1 was assessed in BALB/c mice. Various immune parameters elicited along with protective efficacy imparted were compared in view of developing a highly efficacious, stable and cost effective rabies vaccine.

2. Materials and methods

2.1. Cells, virus, vaccine and mice

Baby hamster kidney (BHK)-21 cells were procured from National Centre for Cell Science (NCCS), Pune, India and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Life Technologies) containing 100 U/ml Penicillin (Amersham) and 100 μg/ml Streptomycin (Amersham), in a humidified 5% CO_2 incubator at 37 °C. Mice brain infected with Rabies Challenge Virus Standard Strain (CVS) was procured from Indian Veterinary Research Institute (IVRI), Izzatnagar, Bareilly. The rabies DNA vaccine encoding glycoprotein G gene of rabies virus (pgp.LAMP-1, GenBank Accession Number EU715588) used in this study was generated by our group (Kaur et al., 2010). Three to four weeks BALB/c mice weighing 20–22 g were obtained from National Centre for Laboratory Animal Sciences, NIN, Hyderabad, India and were maintained in animal holding room of BSL3 laboratory. All the animal experiments were done in compliance with Institutional Animal Ethics committee, Jawaharlal Nehru University and Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India; as per the Three Rs guidelines of Russell and Burch (http://3rs.ccac.ca/en/about/three-rs.html, Romanian Parliament, 2002).

2.2. Standardization of rabies virus: culture, titration, lethal dose

2.2.1. Rabies Challenge Virus Standard culture in BHK-21 cells

At 80% confluence, BHK-21 cells were infected with rabies virus at multiplicity of infection (MOI) range of 0.1–0.5. The infection was allowed to proceed for 60 min at 37 °C with gentle stirring after every 10–15 min. The cells were harvested after 2 days of infection and trypsinized to produce the CVS. The supernatant collected was used to infect fresh BHK-21 cells to get Passage 1 cells (P1). Likewise generated, Passage 5 (P5) BHK-21 cells were trypsinized and the cell supernatant was collected in complete DMEM. The supernatant was centrifuged at 800–1000*g* for 15 min at 4 °C, aliquoted