CHAPTER 1

INTRODUCTION

1.1. Rabies

Rabies is one of the most feared zoonotic diseases with an almost invariably fatal encephalomyelitis, causes 70,000 human deaths each year (Dietzschold et al., 1996; Liu and Ertl 2012; www.rabiescontrol.net/news/news-archive/annual-number-of-deaths). Endemic rabies has been reported on every continent except Antarctica, but the vast majority of rabies deaths occur in Africa, Asia, Middle East and Latin America where, vaccination programmes and effective human post exposure prophylaxis (PEP) are either unavailable or not effectively applied (Kilic et al., 2006; Bourhy et al., 2010). In India, rabies is endemic and widespread in all the states except Andaman, Nicobar and Lakshadweep islands (WHO-APCRI, 2004). Although the prevalence of rabies in India is reported to be the highest in the world, it remains a totally neglected disease. Rabies is not a notifiable disease in India. There is no national programme for rabies control and eradication.

The rabies virus (RV) does not enter intact skin but can cross intact mucous surface. Animals and human beings usually acquire infection following a bite by a rabid animal (Constantine, 1962; Hemachudha et al., 2002). There are also other rare routes of transmission through inhalation of aerosols; ingestion of infected
animal meat and milk and organ transplantation (Gode and Bhide, 1988; Krebs et al., 1995; Dutta 1998; Hellenbrand et al., 2005; Smith and McDonald, 2006; www.cfsph.iastate.edu/Factsheets/pdfs/rabies.pdf). The incubation period of rabies is 1–3 months but may range from less than 7 days to more than 6 years (Smith et al., 1991; Hemachudha and Phuapradit, 1997). However, fulminant disease can become symptomatic within 5–6 days. The first signs of illness are nonspecific; fever, anxiety and malaise. Often there is tingling or pruritus at the site of the animal bite. This is followed by frank neurological signs, ranging from hyperactivity to paralysis. The disease is usually divided into encephalitic and paralytic forms. In the encephalitic form, signs of irritation of the central nervous system (CNS) predominate which includes hydrophobia and aerophobia. Within 2-12 days, the patient succumbs to the disease with initial signs of coma followed by cardio-vascular failure. Apart from human fatality, rabies adds to the economic burden in terms of losses incurred due to animal mortality and additional expenditure on account of rabies prophylactics. Although the disease is vaccine preventable, rabies continues to pose a major threat both to human and animal health in endemic countries like India.

1.2. Rabies virus

Rabies is caused by a bullet-shaped virus belonging to the genus *Lyssavirus* of family Rhabdoviridae. Rabies virions have a well defined inner, helical nucleocapsid and an outer, protein-studded
envelope. The RV possesses a 12 Kb long single-stranded, non-segmented RNA genome of negative polarity that encodes five structural proteins; the nucleoprotein (N), the phosphoprotein (P also termed NS), the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA polymerase (L) (Tordo et al., 1986). Rabies virus glycoprotein (RVG) which carries the B- and T-cell antigenic sites is the major target for virus neutralizing antibody responses. The RVG is anchored as a trimer (3×65 KDa) in the membrane context. G should be membrane bound to give better protection (Cox et al., 1980; Perrin et al., 1985) and both the ectodomain and transmembrane domain (TMD) of the RVG is required for generating high rabies virus neutralizing antibody (RVNA) titers (Rath et al., 2005). Moreover, preservation of the three dimensional conformation of G, is responsible for the consistent induction of virus neutralizing antibodies and protection (Wiktor et al., 1973; Desmezieres et al., 2003).

1.3. Rabies Vaccines

Since Louis Pasteur’s first attempt to produce rabies vaccines, a number of approaches have been evolved from the usage of nervous tissue vaccines to the highly purified vaccines produced in defined cell lines. Despite the low cost of production of nerve tissue vaccines, the poor immunogenicity and safety related issues increased the risk of vaccine failure (Perrin et al., 1999). The current rabies vaccines contain inactivated rabies virus and these vaccines are produced by
growing the virus in large scale using primary or continuous cell lines/ cell strains. Administration of either pre- or post-exposure vaccination to achieve rabies prevention using modern tissue culture-based vaccine has drastically reduced the human rabies deaths worldwide. Though, the cell culture based vaccines are highly potent and entirely safe, the cost of cell culture vaccines are prohibitive. Also, the process of production of cell culture vaccines is difficult than the brain tissue vaccines (Horack, 1939; Sureau, 1992). Currently, the killed rabies vaccines alone are approved for use in humans because of the safety related issues associated with live vaccines. However, eradication of rabies from its wildlife reservoirs could only be feasible using live attenuated viruses in the form of baits (Wilhelm and Schneider, 1990; Rupprecht et al., 2004). Some European countries are free from rabies (especially in terrestrial animals) due to bait vaccination programmes (Potzsch et al., 2002; Matouch et al., 2007). Live attenuated RV vaccine Street alabama Gif-2 (SAG-2) has been licensed, particularly for oral immunization of wild animals. However, SAG-2 induces very low virus neutralizing antibody (VNA) responses in wild animals (Hanlon et al., 2002). Therefore, numerous attempts have been made to develop alternatives to traditional live RV vaccines (Schnell et al., 2005). Among these are recombinant viral vectors like canine adenovirus and poxviruses that express the RVG (Tordo et al., 2008; Weyer et al., 2009). Recombinant vaccinia virus expressing RVG (VRG) have also been licensed, particularly for oral immunization for wild animals (Paoletti, 1996). Oral bait vaccines containing the VRG
have been used effectively to eliminate rabies in many parts of the European and North American countries (Blancou et al., 1989; Hanlon et al., 1998; Potzsch et al., 2002). However, intensive skin inflammation and systemic vaccinia infection has been reported when human exposure to VRG occurred (Rupprecht et al., 2001; CDC, 2009). Other genetic vaccine based on the RVG (DNA rabies vaccines) for the control of rabies is not shown encouraging immunogenicity against PEP (Wunner and Briggs, 2010). The gene-deficient mutant RV are also developed and evaluated for their potential utility as live attenuated rabies vaccines (Faber et al., 2002; Shoji et al., 2004; Cenna et al., 2009; Gomme et al., 2010). However, absence of neurotoxicity for these mutants RV is not yet established convincingly. Also, the live rabies vaccines bear the intrinsic risk of mutating or reverting to a more virulent phenotype. Hence subunit vaccines based on RVG, which is the main protective antigen and the target of neutralizing antibodies provide substantial protection against RV challenge (Wiktor et al., 1973; Cox et al., 1977; Wiktor et al., 1984; Xiang et al., 1994; Ertl, 2009). However, handling and manufacturing of live RV to produce RVG poses biosafety concerns (Winkler et al., 1973). Also, creating and maintaining the bio-security procedures involve huge expenditure. Hence, the recombinant DNA technology could be an alternative to produce sub-unit rabies vaccines which can offer the advantages of obtaining scalable protein production without the necessity of handling live RV.
1.4. Recombinant rabies virus glycoprotein

The RVG has been expressed in various expression systems. Rabies G expressed in *E.coli* was not immunogenic, insoluble and failed to confer protection against rabies (Yelverton *et al.*, 1983; Lathe *et al.*, 1984). Whereas G expressed in yeast was able to protect against an intramuscular challenge but not against an intracerebral virus infection (Klepfer *et al.*, 1993). Moreover G molecules were not processed normally in yeast cells, resulting in abnormal folding and multimer formation (Sakamoto *et al.*, 1999). On the other hand, the mammalian cell expression systems can produce the correctly folded or post translationally modified proteins in its biologically active form, but the expression is at lower levels.

The Baculovirus expression vector is a thoroughly investigated eukaryotic expression system and is helper independent. Baculo viruses infect lepidopteron (butterflies and moths) insects and insect cells. Although, they are highly virulent for some insects, they are pathogenic neither for vertebrates nor for plants. Approximately 500 recombinant genes have been expressed in the Baculo virus expression vector system (BEVS). Many insect cells do not recognize the recombinant mammalian proteins, so that recombinant proteins expressed in BEVS can be obtained without host cell protein contamination. There are no preexisting antibodies for baculo viruses in mammals, which is a major problem associated with the recombinant virus vectors such as *Vaccinia*. Many human proteins
have been expressed using the BEVS for basic research and development of new bio-pharmaceuticals (Lucknow and Summers, 1988 et al., 1988; Hu, 2005). When mammalian proteins are expressed in insect cells, the protein folding and processing are more authentic compared to other prokaryotic expression systems albeit the differences in glycosylation. Different RV structural proteins have also been expressed in the BEVS. The nucleoprotein of RV (ERA strain) expressed using this system was antigenically and immunologically comparable to the native RV ribonucleoprotein. The G of the RV strains such as, Nishighara strain (genotype I), CVS strain, PV strain and Mokola virus expressed by this BEVS showed similar three dimensional structure and biological features as that of the native viral protein (Prehaud et al., 1989; Tuchiya et al., 1992; Tordo et al., 1993b). Large quantities of RVG were synthesized in insect cells infected with recombinant baculovirus, makes this BEVS as a potential economical source for the production of recombinant RVG (rRVG) (Tordo et al., 1993b).

1.5. Membrane extraction of recombinant rabies virus glycoprotein

The immunogenicity of the BEVS-expressed RVG was evaluated by immunizing either the intact cells or crude lysate of insect cells expressing RVG (Prehaud et al., 1989; Fu et al., 1993). However, vaccine preparations containing undefined quantity and composition of recombinant proteins may not be acceptable from the regulatory point of view. Extraction of membrane expressed G from the host cells
without altering its conformation and antigenicity is a challenging task indeed. The rabies vaccine development technology suffered a serious retard due to the lack of efficient solubilization methods for the extraction of the membrane bound rRVG without altering its biological activity. Several attempts to synthesize recombinant viral membrane G for immunization purposes have failed because of the difficulties in properly isolating them from the cell membrane without affecting their biological and antigenic properties (Astray et al., 2008). Cell lysis using detergents is a milder and easier alternative to physical disruption of cell membranes. However, there is no standard protocol available for selecting an appropriate detergent suitable for membrane lysis. The choice of detergent for cell lysis depends on various factors such as cell type, buffer, pH, salt concentration, temperature and nature of proteins (Garavito and Ferguson, 2001; Lin and Guidotti, 2009). In general, nonionic and zwitterionic detergents are milder and less denaturing than ionic detergents and are usually preferred as solubilizing agent, wherever preserving the protein structure is critical. Therefore, the efficiency of two nonionic detergents (Nonidet P-40 and Triton X-100) and a zwitterionic detergent (CHAPS-3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) in combination with six different buffers was evaluated for their solubilization ability of membrane bound rRVG from *Spodoptera frugiperda* (Sf-9) cells.
1.6. Purification of rabies virus glycoprotein

Though there are many chromatography methodologies available, purification of RVG is a cumbersome, often unsuccessful and expensive task. Most of the antigenic sites of G are conformational dependent based on the folding of the protein (Lafon et al., 1984; Seif et al., 1985; Prehaud et al., 1988; Benmansour et al., 1991). Thus, purification of RVG from the membrane fraction of cells without disturbing the conformation-dependent epitopes and trimeric structure is necessary to get complete protection to viral challenge. Moreover the recombinant proteins intended for in-vivo use should be devoid of any affinity tag to meet the regulatory requirements. Hence, a suitable alternative method is required for the purification of membrane bound and affinity tag free RVG without altering its immunogenic conformation.

The immuno-affinity chromatography (IAC) involves the immobilization of biological or synthetic ligands to inert resin and the resulting biospecific adsorbents will have high affinity for a single compound. The IAC technique using monoclonal antibodies (mAbs) are suitable for the purification of proteins from a variety of biological substances (Santucci et al., 1990). The mAbs are immunoglobulin molecules which are homogenous in structure and has specific binding property, secreted by a population of identical hybridomas. Ever since the 1970s, a multitude of different mAbs have been made and being exploited in almost every aspect of biomedical research, vaccine development, diagnosis and therapy. In vaccine research and
development, they are important tools for antigen discovery, quantification and characterization and for release of vaccine lots. The mAbs are commonly used in affinity chromatography as a versatile and specific means of isolating target molecules from complex mixtures. Immobilization of mAbs onto a solid support has found widespread application in affinity chromatography thereby generating powerful tools in biosensors, diagnostics and therapeutics (Lu et al., 1996). Purified antibodies are bound to commercially available beads that have been activated using a chemical reagent. In principle, the antibody immobilized on a column support is used to selectively adsorb antigen from a mixture containing many other proteins. The other proteins for which the antibody has no affinity may be washed away and the purified antigens are later eluted from the immunoadsorbent. In order to dissociate the antigen from its specific antibody, the conditions for elution are necessarily extreme and thus carefully chosen to permit isolation of active protein. The advantages of employing mAbs in immune-affinity purification are the unlimited availability and specific binding ability of the mAbs to a large proportion of the available antigen. The conditions to release the antigen from mAb will normally be gentler than those required for polyclonal antibody antigen interactions.

A significant number of researchers have contributed towards generation of mAbs against RV which could provide a wide range of applications in diagnostics, in-vitro potency determination and therapeutics (Wiktor and Koprowski, 1978; Nagarajan et al., 2006b).
Nagarajan *et al.* (2006b) had developed a conformational specific, anti-RVG mAb, M5B4, for the quantification of G from the rabies in-process samples. Santucci *et al.* (1990) has reported that IAC using antipeptide antibodies as a highly specific and sensitive method for the purification of RVG.

**OBJECTIVES OF RESEARCH**

Keeping in view the advantages of recombinant RVG based vaccines and BEVS; the proposed study was undertaken in order to develop a recombinant subunit vaccine for rabies with the following objectives;

1) **Production of recombinant RVG (rRVG)**
   a) Cloning and expression of RVG gene in BEVS
   b) Characterization of recombinant RVG

2) **Optimization of conditions for the membrane extraction of rRVG expressed in Sf-9 cells**

3) **Developing an alternative method for the purification of tag free rRVG**
   a) Immuno-affinity purification of membrane extracted rRVG using a conformational specific anti-RVG mAb, M5B4

4) **Potential application of BEVS expressed rRVG as a subunit vaccine**
   a) Confirming the immunogenicity and protective efficacy of the membrane extracted rRVG in mice
   b) Immunogenicity and protective efficacy of the IAC purified rRVG in mice.