EVALUATION OF PROPHYLACTIC EFFICACY OF HUMAN ANTI-RABIES MONOCLONAL ANTIBODIES IN A MOUSE MODEL

by
CHAKRAVARTHY CHENNAREDDY

(**Under the direction of Zhen Fu)**

ABSTRACT

Rabies post exposure prophylaxis (PEP) includes administration of both vaccine and anti-rabies immunoglobulin. Rabies immunoglobulins are expensive and available in limited quantities. There is an inherent variability in quality and specificity between batches of immunoglobulin preparations and possibility of contamination with known or unknown pathogens. To provide a cost-effective and safe replacement for currently used human rabies immunoglobulin (HRIG) human anti-rabies monoclonal antibodies (huMAbs) were developed. The present study evaluated the overall prophylactic efficacy of SO57 and SOJB MAbs individually, in a mouse model when used in combination with rabies vaccine and compared with conventional HRIG.

When the protective effect of antibodies was examined in mice challenged with CVS-N2C virus, a dose-dependent survivorship response was observed in animals treated with antibodies and no animal survived in the control group. At the highest dose of antibody given 80% survivorship was recorded for SO57 and SOJB antibodies where as only 50% survived with HRIG. Serum half-lives were found to be approximately 16 days for SO57 (IgG1); 11 days for SOJB (IgG3) and 8 days for HRIG (polyclonal).

Furthermore, we investigated the potential interference of antibodies on vaccine mediated-immunity. When compared to the control group which was given only vaccine shots, the groups of animals given both antibody and vaccine developed lower virus neutralizing antibody (VNA) titers. The VNA titers appear to be a function of dose of antibody given and serum half-life of each antibody. The interference, as correlated with VNA titers developed in mice, is comparable to HRIG for both SO57 and SOJB MAbs.

When we investigated more on the induction of interference by antibodies on vaccine –mediated immunity with combinations of (i) antibody and different concentrations of vaccine (ii) administration of antibody and vaccine at different time intervals followed by intracerebral (i.c.) challenge all the groups of mice recorded higher survival rates. But the survival rates for SO57 and SOJB were comparable to that of HRIG.

Unlike in the previous studies of mouse monoclonal antibodies, we observed that passively administered human monoclonal antibodies and HRIG protects mice against intra cerebral challenge. The protective effect at the point of i.c. challenge may be a synergistic effect of passive and active immunities and account for higher survival among groups.
Together this data suggests that human anti-rabies monoclonal antibodies (huMAbs) viz: SO57 and SOJB have potential to replace the currently used human rabies immunoglobulin (HRIG) for rabies post exposure prophylaxis (PEP) regimen.

Key words**:** Rabies, Post Exposure Prophylaxis (PEP), Human anti-rabies monoclonal antibodies, Therapy.

EVALUATION OF PROPHYLACTIC EFFICACY OF HUMAN ANTI-RABIES MONOCLONAL ANTIBODIES IN A MOUSE MODEL

by

CHAKRAVARTHY CHENNAREDDY

B.V.Sc & A.H., Acharya N.G. Ranga Agricultural University, India, 1997

M.V.Sc., Tamilnadu Veterinary and Animal Sciences University, India, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2005

EVALUATION OF PROPHYLACTIC EFFICACY OF HUMAN ANTI-RABIES MONOCLONAL ANTIBODIES IN A MOUSE MODEL

By

CHAKRAVARTHY CHENNAREDDY

Major Professor: Zhen Fang Fu

Committee: Corrie Brown
Amelia Woolums

Electronic Version Approved:

Mureen Grasso
Dean of the Graduate School
The University of Georgia
December 2005

DEDICATION

To my parents and family members for all their unconditional support and making this work possible.

ACKNOWLEDGEMENTS

I sincerely thank my major professor, Dr. Zhen Fu for giving me an opportunity to work under him. His able guidance and inspiration through out my study is appreciated.

I also want to thank the members of my committee, Dr. Corrie Brown and Dr. Amelia Woolums for their invaluable suggestions and encouragement during my research.

I would also thank my lab colleagues Vikas Dhingra, Ellen (Xiaqing) Li, Pinghua liu, Luciana sarmento, Zhiwei Wang, Tesfai Tseggai, Haufusa toriumi, Zhao Ling, Kuang (Katie) Yi and Cathy Mallon for providing professional and healthy atmosphere in the lab and required technical help during my research work.

I extend my most sincere appreciation to the faculty, staff and students of the Department of Pathology. Special thanks to Amanda Crawford, Bonita Wagers, Mia Jackson, for their supportive help, which will always be remembered. I thank the faculty and staff of the Department of Animal Resources, University of Georgia, for their co-operation in animal experiments.

I am grateful to my parents, my brother Murali Mohan Reddy and to my friends for their moral support during the completion of this work.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Rabies is one of the oldest diseases known to mankind. Rabies virus infects all warm blooded animals. In the infected animals, it affects the central nervous system, resulting in fatal outcome in almost all the cases. Only few cases of rabies survivals have been reported so far. In spite of centuries of experience in animal rabies control and efficacious human post exposure prophylaxis (PEP), human rabies deaths continue to occur (**1**.Meslin *et.al.*1994). It is estimated that world wide over 70,000 people die of rabies annually (**2.**WHO 2001). More than 10 million people annually receive rabies post exposure prophylaxis (**3**.Ko *et.al.*2003). According to World Health Organization (WHO) guidelines, category 3 exposures which are considered with either single or multiple transdermal bites or contamination of mucous membranes with saliva of rabid animal, requires post exposure prophylaxis (PEP) (**4**.CDC 1999). Rabies PEP consists of immediate local treatment of the wound –washing and disinfection, followed by local infiltration and systemic administration with anti-rabies immunoglobulin (RIG) and injecting cell culture rabies vaccines, at a body site other than where immunoglobulin was given, in previously unvaccinated individuals. Such treatment has proven to be highly effective (**5**.WHO 1992). In cases of severe rabies exposure vaccination alone, i.e. without RIG, cannot be relied upon to prevent the disease (**6**.Wilde *et.al.*1989; **7**.Alvarez *et.al.*1994; **8**.Thraenhart *et.al.*1994; **9**.Wilde *et.al.*1996; **10**.Hemachudha *et.al.*1999). The passive administration of virus-neutralizing antibodies is believed to neutralize the virus present at the bite site as well as systemically and prevent spreading of virus to CNS (**11**.WHO 1997).

Currently, two types of anti-rabies immunoglobulin (RIG) are employed in human post exposure prophylaxis, equine rabies- immunoglobulin (ERIG) and human rabies- immunoglobulin (HRIG), which are prepared from pooled serum of rabies virus immunized horses and humans respectively. ERIG has been used for 45 years and even toady it is an essential component of rabies post exposure prophylaxis in many developing countries. ERIG preparations from various manufacturers have been shown to induce serum sickness in some recipients. In 1965, it was reported approximately 16% of persons treated with ERIG developed serum sickness; among persons over 15 years of age, the incidence was 46% (**12**.Karliner and Belaval 1965). Recently purified and heat- treated, safe ERIG have become available and the serum sickness incidence among recipients now reported to be <1-6.2% (**13**.Wilde *et.al.*1989).

Human rabies immunoglobulin (HRIG) have been developed and used for rabies post exposure prophylaxis mainly to avoid the risks associated with the administration of ERIG. In USA and Canada it has been used since 1975 and no cases of serum sickness have been reported so far (**14**.Fournier and Sikes 1996). Although HRIG is well tolerated it is prohibitively expensive and available only in limited quantities. There is an inherent variability in quality and specificity of antibodies in different batches of HRIG preparations. Like any other reagent prepared from human serum, with HRIG, there is a possibility of infection with known or unknown pathogens. In addition, the worldwide supply of HRIG and other polyclonal human and animal products is chronically restricted. So the worldwide demand for HRIG is increasing (**15**.Champion et. al., 2000).

Advances in hybridoma technology paved the way for production of monoclonal antibodies with predetermined target specificity. With the advances in production technology, monoclonal antibodies (MAb) now provide an efficacious and economical alternative to polyclonal RIG. Mouse monoclonal antibodies with appropriate specificities have been obtained and used in the study of neutralization and clearance of rabies virus from infected animals (**16**.Lafon *et.al.,* 1983; **17**.Schumacher et.al.1989 and **18**.Dietzschold, 1994) but their unknown half-life, compartmentalization and high immunogenicity in humans prevent these antibodies from being an ideal replacements for HRIG.

A panel of human anti-rabies monoclonal antibodies were produced and characterized by 19.Ukei *et.al.*1990, **20**.Dietzschold *et.al.*1990; **21**.Dorfman *et.al.*1994; **15**.Champion *et.al.*2000 and **22**.Hanlon *et.al.*2001. In these studies peripheral blood lymphocytes were collected from human volunteers immunized with commercial rabies vaccine and transformed with Epstein - Barr virus (EBV). Stable-antibody secreting hybridoma cells were produced by fusing EBV-transformed B-lymphocytes with SHM-D33 mouse myeloma cells. Several human MAbs resulting from stable mouse-human heterohybrid cell lines were evaluated. Monoclonal antibodies considered for anti-rabies therapy should fulfill following criteria (i) They should be of the IgG isotype because the IgG isotype has approximately five-fold longer half-life in vivo than IgM and superior biological properties in eliminating the virus (**23**.Waldman et.al. 1970) (ii) Should neutralize all rabies virus strains and other Lyssaviruses recommended by WHO (**24**.Prosniak *et.al.* 2003). (iii) Any neutralization- resistant virus variants (escape mutants) with slightly different epitope may go un-neutralized by MAbs. To overcome this problem and to achieve the protective activities comparable to HRIG, use of a cocktail of MAbs which differ in their epitope- recognition specificities, was suggested (**25**.WHO 2002). From the above studies

three hybridomas-JA, JB and J57- secreting rabies virus neutralizing human MAbs have been established. It is recognized that high production potential is not achieved either with murine hybridomas or with mouse-human heterohybrid cell line (**22**.Hanlon *et.al.* 2001). Cost-effective production is achieved by cloning heavy chain (H) and light chain (L) of MAb immunglobulins into rabies-virus based vectors and expressing them in mammalian cell lines. These recombinant –expressed MAbs are designated as SOJA, SOJB and SO57 (**26**.Morimoto *et.al*. 2001 and **24**.Prosniak *et.al*.2003). The recombinant MAbs, when used as a cocktail consisting of SO57, SOJB and SOJA in 1:1:1 protein ratio, showed protection levels in a mouse model comparable to that of conventional HRIG (**24**.Prosniak *et.al*.2003).

The present study aimed at evaluation of two MAbs Viz: SO57 and SOJB for their prophylactic efficacy in a mouse model when used in combination with rabies vaccine, as compared with conventional HRIG.

Specific objectives are to:

1. Compare protective activity of MAbs and HRIG.
2. Determine the biological half-life of MAbs and HRIG.
3. Characterize interference of MAbs on vaccine-mediated immunity.
4. Determine if interference of Antibodies on vaccine-mediated immunity depends on serum concentration and biological half-life of the administered antibodies.
5. Determine the correlation between antibody-dependent interference and reduction of protective immunity.

References:

1. Meslin FX, Fishbein DB, Matter HC. Rationale and prospects for rabies elimination in developing countries. Curr Top Microbiol Immunol.1994; 187: 1-26.

2. World Health Organization. Rabies. Fact Sheet. 2001; 99: 1-3.

3. Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A. 2003; 100(13): 8013-8018.

4. Center for Disease Control. Human rabies prevention-United States: Recommendations of the Advisory Committee on Immunization Practices (ACIP).MMWR Recom Report.1999; 48: 1-21.

5. World Health Organization. Expert Committee on rabies. Eighth report.WHO Tech Rep Ser 824. 1992; 1-84.

6. Wilde H, Choomkasien P, Hemachudha T, Supich C, Chutivongse S. Failure of rabies postexposure treatment in Thailand. Vaccine.1989; 7(1): 49–52.

7. Alvarez L, Fajardo R.Lopez E. Pedroza R. Hemachudha T. Kamolvarin N, et al. Partial recovery from rabies in a nine-year old boy. Pediatr Infect Dis J.1994; 13(12):1154–1155.

8. Thraenhart O, Marcus I, Kreuzfelder E. Current and future immunoprophylaxis against human rabies: reduction of treatment failures and errors. In: Rupprecht CE, Dietzschold B, Koprowski H, editors, Lyssaviruses. New York, Springer-Verlag. Curr Top Microbiol Immunol.1994; 187: 173–194.

9. Wilde H, Sirikawin S, Sabcharoen A, Kingnate D, Tantawichien T, Harischandra PA, Chaiyabutr N, de Silva DG, Fernando L, Liyanage JB, Sitprija V. Failure of post exposure treatment of rabies in children. Clin Infect Dis.1996; 22(2): 228–232.

10. Hemachudha T, Mitrabhakdi E, Wilde H, Vejabhuti A, Siripataravanit S, Kingnate D. Additional reports of failure to respond to treatment after rabies exposure in Thailand. Clin Infect Dis.1999**;** 28(1):143–144.

11. World Health Organization. WHO recommendation on rabies post-exposure treatment and the correct technique of intradermal immunization against rabies. 1996; WHO/EMC/ZOO.96.6 WHO, Division of Emerging and other Communicable Disease Surveillance and Control, Geneva.

12. Karliner JS, Belaval GS. Incidence of reactions following administration of antirabies serum: A study of 526 cases. JAMA.1965; 193: 359-362.

13. Wilde H, Chomchey P, Punyaratabandhu P, Phanupak P, Chutivongse S. Purified equine rabies immune globulin: a safe and affordable alternative to human rabies immune globulin. Bull World Health Organ.1989; 67(6): 731-736.

14. Fournier P, Sikes RK. Production of human rabies immunoglobulin. In: Laboratory techniques in rabies, F-X. Meslin, M.M.Kaplan and H.Koprowski (eds),4th ed., WHO.1996; 411-416.

15. Champion JM, Kean RB, Rupprecht CE, Notkins AL, Koprowski H, Dietzschold B, Hooper DC. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. J Immunol Methods.2000; 235(1-2): 81-90.

16. Lafon M, Wiktor TJ, Macfarlan RI. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. J Gen Virol.1983; 64 (4): 843-851.

17. Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H. Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest. 1989; 84(3): 971-975.

18. Dietzschold B. Monoclonal antibodies in rabies therapy. Clin. Immunother.1994; 1: 245.

19. Ueki Y, Goldfarb IS, Harindranath N, Gore M, Koprowski H, Notkins AL, Casali P. Clonal analysis of a human antibody response. Quantitation of precursors of antibody-producing cells and generation and characterization of monoclonal IgM, IgG, and IgA to rabies virus. J Exp Med.1990; 171(1): 19-34.

20. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H. Biological characterization of human monoclonal antibodies to rabies virus. J Virol.1990; 64(6): 3087-3090.

21. Dorfman N, Dietzschold B, Kajiyama W, Fu ZF, Koprowski H, Notkins AL. Development of human monoclonal antibodies to rabies. Hybridoma.1994;13(5): 397-402.

22. Hanlon CA, DeMattos CA, DeMattos CC, Niezgoda M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. Vaccine.2001; 19(28-29): 3834-3842.

23. Waldman TA, Strober W, Blaese RM, In: Immunoglobulins, Merler E. ed., National Academy of Science 1970; Washington, DC.

24. Prosniak M, Faber M, Hanlon CA, Rupprecht CE, Hooper DC, Dietzschold B. Development of a cocktail of recombinant-expressed human rabies virus-neutralizing monoclonal antibodies for postexposure prophylaxis of rabies. J Infect Dis.2003; 188(1): 53-56.

25. World Health Organization. WHO consultation on a monoclonal antibody cocktail for rabies posts exposure treatment. 23-24 May 2002.

26. Morimoto K, Schnell MJ, Pulmanausahakul R, McGettigan JP, Foley HD, Faber M, Hooper DC, Dietzschold B. High level expression of a human rabies virus-neutralizing monoclonal antibody by a rhabdovirus-based vector. J Immunol Methods.2001; 252(1-2): 199-206.

CHAPTER 2

LITERATURE REVIEW

2.1 Rabies Disease & History:

Rabies is one of the oldest diseases known to mankind. The word rabies derived from Latin *rabere* meaning "to rage or to rave" and some believe that it had originated from Sanskrit word *rabhas*, for "violence" **(1**.Jackson and Wunner 2002). In the legal documents of Mesopotamia as early as 2300 B.C., there is a mention about dangers and concerns associated with mad dog bites (**2**.Dietzschold *et.al.*, 1996). Greeks were familiar with rabies and the word "rabid dog" can be found in their books *The Iliad* 700 B.C. Aristotle (4^{th} century B.C) also recognized rabies as a disease of animals, but erroneously exempted humans from contracting the disease from the bite of a mad dog. Celsus, the Roman doctor, in first century A.D, perhaps the first time described the clinical picture of rabies infection in humans:" the patient is tortured at the same time by thirst and by invincible repulsion towards water" and mentioned a prophylaxis that could be done once hydrophobia become manifest- Cauterization and keeping the wound open to allow the "seeds of disease" to run out freely (**2**.Dietzschold *et.al.*, 1996). A century later, Galen reached the conclusion that only dogs were natural hosts to rabies and that a mere drop of saliva from a rabid dog on human skin could cause hydrophobia in humans (**1**.Jackson and Wunner 2002). In 1546, Girolamo Fracastoro, Italian physician in his treatise on contagious diseases clearly mentioned that rabies could not be transmitted to humans by "simple contact or by foams, or at a distance but only when the skin is torn by the bite of a dog that blood is drawn, as though contagion takes place in the blood itself through contact with teeth and foam from the mouth of the rabid

animal". He also mentioned that the incubation period of rabies as about 20 days or 30 days in most cases and sometimes extends up to a year. In North America, rabies was first reported by Fray Jose Gill Ramirez, in Mexico, in 1709.

Though, for centuries, people considered saliva of rabid dog as source of rabies infection, it was George Gottfried Zinke of Germany, in 1804 who was able to transmit rabies from it. Zinke successfully produced rabies in healthy dogs, cats, rabbits and fowl using a small brush to transfer saliva into incision. This began the era of animal experiments **(1**.Jackson and Wunner 2002).Willum Youatt (1776-1847) in London , took a special interest in the disease and made few suggestions like species quarantine in controlling the disease; need for the further experiments in developing vaccine for rabies. Youatte found that the rabbit develops the predominantly paralytic form of rabies and so could serve as less dangerous and more convenient animal model for experiments than the dog with furious form of the disease **(1**.Jackson and Wunner 2002). Victor Galtier, in 1879 at Lyon was credited with the transmission of rabies from rabbit to rabbit. He also used tissue obtained from rabbits to immunize sheep and goats (**2**.Dietzschold *et.al.*, 1996). The following year i.e, in 1880 Louis Pastuer started his experiments on rabies. By the time Pasteur became aware of Galtier's contribution, he himself has already established the value and prophylactic inoculation with attenuated material through the works on fowl cholera and Anthrax. Pasteur first adapted the street (wild-type) rabies virus to rabbits thus he was able to change the properties of virus. Then he used the desiccated spinal cords from rabies-infected rabbits for vaccination. July6th, 1885 was a milestone in the history of rabies: a 9-year old boy, Joseph Meister, who was bitten in 14 sites by a rabid dog, received first postexposure treatment with the Pasteur vaccine and survived. Pasteur's achievement is a milestone not just in the history of rabies but in virus research in general and vaccine

development in particular. Later on Pasteur's vaccine with all its modifications became the accepted treatment of rabies through out the world (**2**.Dietzschold *et.al.*, 1996).

2.2 Rabies Virus:

2.2.1 Rabies Virion structure:

Rabies virus is the prototype species of the genus *Lyssavirus* in the family *Rhabdoviridae*. The genome consists of a non-segmented, negative-stranded RNA so this family is placed under the order *mononegavirales* (**3.**Mayo and Pringle 1998). The Lyssavirus can be grouped into seven genotypes: Rabies virus, Lagos bat, Mokola virus, Duvenhage virus, European bat virus type 1 and 2, and Australian bat Lyssavirus (**4**.Bourhy *et.al.*, 1993). Rabies virus is highly neurotropic in the infected host causing fatal encephalomyelitis. Other six are rabies- related lyssaviruses reflecting the genetic diversity that they share with rabies virus (**1**.Jackson and Wunner 2002).

Under the electron microscope, the standard infectious rabies virions are bullet-shaped particles with average length of 180nm (130-250nm) and diameter 75nm (60-110nm) (**5**.Davis *et.al.*, 1963 and **6**. Hummeler *et.al.*, 1967).The virion consists of helical nucleocapsid and five structural proteins that include a nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase or large protein (L). The virions are composed of an external envelop derived from the cell membrane and an internal ribonucleoprotein (RNP) core. The single viral glycoprotein (G) spans the membrane once and forms an array of trimeric spikes that extends from the viral envelope. The helical RNP core consists of approximately 12,000 nucleotide long genomic RNA in a tight complex with nucleocapsid (N) protein. The viral RNA- dependent RNA polymerase, composed of the catalytic larger protein (L) and non-catalytic phosphoprotein (P), is associated with the RNP

core. The matrix protein is inside the viral envelope between the envelop and the RNP core (**2**.Dietzschold *et.al.*, 1996).

2.2.2 Virus Replication and Life cycle:

The sequence of events in rabies virus infectious cycle can be divided into three phases (**1**.Jackson and Wunner 2002). The early phase includes virus attachment to receptors on susceptible host cells, entry via endocytosis, fusion with endosomal membranes, uncoating of the viral particles and liberation of helical RNP to the cytosol. The middle phase includes transcription and replication of viral genome, and the late phase includes virus assembly and egress from the infected cell.

Adsorption: Rabies virus infection is initiated by attachment of the virus to a receptor on host cell surface. The receptor for rabies virus has been controversial and recent evidence indicates that several different receptors can be used. The first of such receptor candidates is nicotinic acetylcholine receptor (AchR). The AchR and rabies virus can be found at the same neuromuscular junction in situ (**7**.Burrage *et.al.*,1982 **8**.Lentz *et.al.*,1982). Further support for the hypothesis that the AchR may be biologically important in rabies virus infection *in vivo* comes from studies with an anti-idiotypic and virus overlay assay (**9**.Gaudin *et.al.*, 1992; **10**.Hanham *et.al.*,1993). Infection of cell lines that lack AchR, however, indicated that other molecules are also used by rabies virus for entry (**11**.Reagan and Wunner 1985). Recently, the neural cell adhesion molecule (NCAM) (**12**.Thoulouze *et.al.*,1998) and the low-affinity neurotrophin receptor p75NTR (**13**. Tuffereau *et.al.*,1998) have been proposed as rabies virus receptors.

Entry and uncoating: After binding to its cellular receptor, rabies virus is internalized by receptor-mediated endocytosis or clathrin-coated pits (**14**.Marsh and Helenius 1989; **15.**Matlin

et.al.,1982). After internalization, the viral glycoprotein mediates fusion of the viral envelope with the endosomal membrane at the low pH environment (**15.**Matlin *et.al.*,1982; **16.**Whitt *et.al.*, 1991). The threshold pH for fusion activity for rabies virus is about pH 6.3 and involves a series of specific and discrete conformational changes in G (**17**.Gaudin *et.al.*,1995; **18**.Gaudin 1997). Prior to virus binding to the cellular receptor, glycoprotein is in its initial native state. After virus attachment and internalization, glycoprotein is activated and exposes its hydrophobic domain to interact with hydrophobic endosomal membrane. Once entered into low pH endosomal compartment, the glycoprotein is activated and exposes its fusion domain to interact with the participating membrane (**17**.Gaudin *et.al.*,1995).

Genomic RNA transcription: Transcription of the viral genomic RNA occurs in the cytoplasm of the infected cell once the RNP core is released from endosome. The transcription process is carried out on the RNP complex by the virion-associated RNA polymerase complex (L-P) and is independent of host-cell functions (**19**.Banerjee 1987). The virion-associated RNA polymerase complex initiates transcription at the 3′ end of the genomic RNA, where the polymerase first synthesizes a small 55 nucleotide RNA called the leader. The leader RNA is neither capped, nor polyadenylated in contrast to the mRNA transcript (**20**.Colonno and Banerjee 1978; **21**.Leppert *et.al.*1979). Each of the five mRNAs encoding the viral proteins is then synthesized in the order it appears from the 3′ end of the genome (**22**.Arnheiter 1985; **19**.Banerjee 1987). At each inter junction, however, the polymerase pauses before continuing the down-stream mRNA transcription process, and an estimated 20-30% of the polymerase complex that reach the gene junction dissociates from the nucleocapsid (**23**.Emerson 1982). As a result, fewer polymerase molecules remain associated with the genome RNA-N template after each gene junction to resume the transcription process (**23**.Emerson 1982). Thus the number of mRNAs synthesized

from the remaining genes down stream in the genome gradually decrease in proportion to the number of polymerases that fall off. This phenomenon of self-regulating viral gene expression is a form of "localized" attenuation (**24**.Iverson and Rose 1981).

Each of the rabies virus N, M, G and L genes encodes only one protein (450 amino acids for N, 202 amino acids for M, 524 amino acids for G and 2142 amino acids for L) from a single open reading frame (ORF) of genome except the P gene. In addition to the full-length P (297 amino acids), three internal initiation sites on the P ORF are used to synthesize three smaller proteins, truncation of the first 19 amino acids (results 278 amino acids), the first 52 amino acids (results 242 amino acids), or the first 58 amino acids (249 amino acids). These P truncated forms have been found in purified virions, in virus-infected cells and in cells transfected with a plasmid encoding the complete P sequence. It is thought that a leaky scanning mechanism is responsible for translation of the P gene at the internal in-frame start codons and the functions of these P forms are unknown (**25**.Chenik *et.al*.1995).

Genome Replication: As soon as nascent soluble N protein is produced in the cytoplasm, it encapsidates the leader RNA (**26**. Galinski 1991; **27**. Horikami *et.al*. 1992; **28**. Yang *et.al*. 1998). Encapsidation of the leader RNA prevents termination of leader transcription at the leader-N gene junction. As a result, transcription is switched to replication to produce a full length antigenomic RNA (**29**. Banerjee and Barik 1992; **30**. Blumberg *et.al*. 1983; **28**. Yang *et.al*. 1998; **31**. Yang *et.al*. 1999). This antitermination model is widely accepted to explain negative strand RNA virus replication because immediately after virus entry, transcription would be favored over replication, at later times when the concentration of N increases replication would be favored (**2**.Dietzschold *et.al.* 1996). However, recent evidence from vesicular

stomatitis virus shows that transcriptase and replicase are two different complexes. The two-polymerase model arose from studying P mutants that were defective in transcription but able to support efficient replication of VSV DI RNAs (**32**. Das *et.al.* 1997; **33**.Pattnaik *et.al.* 1997). The P mutants either had substitutions in the conserved C-terminal basic residues and could bind N protein but did not interact efficiently with L (**32**. Das *et.al.* 1997), or were defective in domain I phosphorylation (**33**.Pattnaik *et.al.* 1997). Because phosphorylation is required for P oligomerization, transcription activation and interaction with L the transcriptase might be composed of an L-P3 complex, whereas the replicase might be composed of an L-(N-P) complex in which P is not phosphorylated. Regulation of P phosphorylation and ongoing viral protein synthesis would therefore affect the formation of the two different complexes at different times after infection (**34**.Rose and Whitt 2000).

Encapsidation of the genomic and antigenomic RNA by N occurs at the same time during replication. The 3′ terminal cis-acting signal in the genome acts as nucleation signal for the initial encapsidation (**35**.Barr *et.al*.2002). Once the initial encapsidation occurs, encapsidation of the genomic and antigenomic RNA is believed to advance rapidly, independent of the viral RNA sequence (**29**.Banerjee and Barik 1992). This fits well with the antitermination model for the switch from transcription to replication. The RNA encapsidation signal must reside in the 5′-Tr of the genome and antigenome(leader) because the leader RNA and progeny genomes are the only virus-specific products that are encapsidated (**22**.Arnheiter 1985; **30**.Blumberg *et.al*. 1983; **21**.Leppert *et.al.*1979). For rabies virus, leader RNA encapsidation may also be regulated by the phosphorylation status of N protein. Unlike the VSV N, which is not phosphorylated, rabies N is

phosphorylated on serine residue 389 and the unphosphorylated form of N protein binds more tightly to leader RNA in vitro (**31**.Yang *et.al*. 1999).

During infection, the two full-length genomic and anti genomic RNAs are produced in disproportionate amounts. The ratio of genome to antigenome is 49:1 (**36**.Finke and Conzelmann 1997). The bias for the excessive production of genomic RNA over antigenomic RNA in the rabies virus infected cells is attributed to the activity of their cis-acting sequences (**36**.Finke and Conzelmann 1997). In rabies virus and vesicular stomatitis virus, the 3′ end of the genomic and anti genomic RNA are important for polymerase binding, because the 3′-terminal 20 nucleotides of the antigenomic RNA serves as a specific enhancer for replication (**37**.Li and Pattnaik 1997).

Assembly and Budding: The process of virus assembly can be divide into three distinct phases: (1) encapsidation of newly synthesized RNA by N, (2) simultaneous condensation of the RNP core by matrix protein and (3) particle envelopment and release (**38**.Gaudin *et.al*. 1993). The process of virus assembly begins as soon as the viral progeny RNA is encapsidated. When N binds to genomic RNA phosphate-sugar backbone, the RNA becomes fully protected from degradation by cellular ribonuclease (**39**.Iseni *et.al*. 2000; **40**.Kouznetzoff *et.al*. 1998). After encapsidation, the RNP complex associates with M, the M plays critical role in virus assembly and budding. M protein binds to RNP and condenses the RNP core from the outside, a step that is sufficient to initiate virus budding (**41**.Lyles and McKenzie. 1998; **42**.Mebatsion *et.al*. 1996). Condensation of RNP core inhibits the transcription activity of RNA polymerase. The M then localizes the RNP core at the cellular membrane, where the nascent G protein is concentrated and M is able to interact with G (**43**.Mebatsion *et.al*. 1999; **44.**Simons and Garoff. 1980). In mature

rabies virion, M lies between the lipid membrane and RNP core, this is different to the VSV model which suggested the presence of M inside the RNP core. It was shown that M still binds to the RNP coil when VSV envelope was stripped off the virion (**45**.Barge *et.al*. 1993). In rabies virus, covering and condensation by M protein is thought to play an important role in virion morphogenesis. If M is missing from rabies particles, the budded particle will contain uncondensed RNP core (**43**.Mebatsion *et.al*. 1999). M deficient rabies virus causes increased cell-to-cell fusion and enhanced cell death, however wild type rabies virus causes relatively minor cytopathic effect to infected cells. In addition to the role of condensation of RNP core, M can also down regulate RNA polymerase activity (**44**.Clinton *et.al*. 1978; **45**.De *et.al*. 1982; **46**.Ito *et.al*. 1996; **47**.Flood *et.al*. 2000;). This is perhaps to preserve the potential of remaining active RNA polymerase complex in the RNP core to resume its activity when progeny virus infects a second cell (**48**.Emerson and Schubert 1987).

In the final stages of virus assembly, the mature virions acquire their lipid bilayer envelope during budding through the host cell membrane. Virus budding is observed both in extraneural tissue cells in vivo and in tissue culture system in vitro (**49**.Iwasaki *et.al*. 1973; **50**.Murphy *et.al*. 1973; **51**.Tsiang *et.al*. 1983).Occasionally virion maturation occurs intracellularly by budding through the cytoplasmic ER or Golgi apparatus (**52**.Matsumoto 1974; **53**.Gosztonyi 1994). If budding occurs at a site in the cell membrane where the nascent rabies virus trans-membrane glycoprotein is also targeted, then infectious virions will be produced. The mechanism by which M mediates the budding of virus appears to be associated with proline-rich (PPPY, PPxY or PY) domain located at residues 35-38 with in highly conserved 14-amino acid sequence near the N terminus of the rabies virus M (**21**.Leppert *et.al.*1979). A corresponding proline-rich motif is

found in the M of VSV (**34**.Rose and Whitt 2000), as well as M of Ebola and Marburg viruses (**32**.Das *et.al.* 1997). The PY motif is very similar to the late budding domain identified in viral proteins such as the Gag protein p2b of Rous sarcoma virus (**33**.Pattnaik *et.al.* 1997) and the p6Gag protein in human immuno deficiency virus (**22**.Arnheiter 1985), both of which are associated with virus budding. The unique function of the PY motif is that it interacts with a WW domain, a 38-40 amino acid long domain with two highly conserved tryptophans spaced 20-22 amino acids apart, found in a wide range of cellular proteins. Some of the WW domain-containing proteins are involved in cytoskeletal formation, where as others are involved in signal transduction and gene regulation (**30**.Blumberg *et.al*. 1983). It is therefore likely that the rabies virus M involves cellular proteins in the release of rabies virions from the cell (**37**.Li and Pattnaik 1997). Although exocytosis of virus is enhanced greatly by the interaction of the RNP-M complex with the envelop G, increased virion production as a result of direct interaction of the cytoplasmic domain of the G and viral RNP-M core suggests that a concerted action of both core and spike proteins is necessary for efficient recovery of virions. Interaction of G with M is essential for the stabilization of the G trimers on the virion surface and for efficient budding of rabies virus (**54**.Lyles *et.al*.1992; **42**.Mebatsion *et.al*. 1996; **43**.Mebatsion *et.al*. 1999).

2.2.3 Rabies Virus Pathogenesis:

Rabies is most commonly transmitted to both humans and animals through bites of rabid animals. After traumatic inoculation of virions from infectious saliva, that might occur with an animal bite, virus may persist and replicate at the bite site for hours to weeks (**55**.Charlton and Casey 1979; **56.**Charlton *et.al*. 1987) or follow a relatively rapid centripetal course directly to

central nervous system (CNS) with replication and dissemination prior to the development of a significant immune response (**57**.Charlton and Casey 1996). Several studies suggest that rabies virus can infect muscles cells and replicate before invasion of nervous system (**58**.Charlton *et.al*. 1997), while others demonstrate that virus can enter the nervous system without prior local replication (**59.**Baer 1975; 60.Shankar *et.al*. 1991). Thus during the incubation period, virus may reside in the periphery, it may remain sequestered within neurons, or perhaps it can persist in macrophages (**61**.Ray *et.al*.1995). Rabies virus exhibits exclusive neurotropism in natural infection. Virus may be taken up by unmyelinated sensory nerve endings of neuromuscular and neurotendinal spindles and motor end plates (**62**.Harrison and Murphy 1978; **8**.Lentz *et.al.*1982; **63**.Watson *et.al* 1981). Experimental evidences suggest that virus specific receptors in addition with growth factors, neural cell adhesion molecules, various phospholipids and glycolipids are probably operating in determining rabies viral neurotropism (**11**.Reagan and Wunner 1985; **12**.Thoulouze *et.al.*1998; **13**.Tuffereau *et.al.*1998).

After peripheral nerve entry, virus moves centripetally within axons to the CNS at an estimated rate of 3mm/hr (**50**.Murphy *et.al.* 1973a; **64**.Tsiang 1979). The virus spread in the nervous system may be facilitated by movement across cell-to-cell junctions, (**62**.Harrison and Murphy 1978) or through synaptic transfer (**65**.Gosztonyi *et.al.*1993). Recent evidence has shown that phosphoprotein of rabies virus interacts with dynein light chain 8 (LC8), a component of microtubules, and facilitates the axonal transport of rabies virus along microtubules through neuronal cells (**66**.Raux *et.al.*2000).

Once virus reaches the brain, it spreads centrifugally to a variety of organs. The spread into the salivary gland represents the transmission phase of the infection i.e., from animal to animal and from animal to human. Much of the virus is produced in mucogenic acinar cells and is delivered

into the saliva by normal secretary flow (**67**.Murphy 1985). Virus can also be found in sensory nerve end-organs in oral and nasal cavities, taste buds, adrenal glands, pancreas, kidney, heart muscle, brown fat, hair follicle, retina and cornea (**68**.Murphy *et.al.* 1973b).

Exact molecular mechanisms involved in rabies virus pathogenesis remain largely unresolved, but several studies indicate that G protein of virus plays an essential role in pathogenesis. The use of antigenic variants representing operationally defined antigenic sites on the G protein of CVS and ERA strains showed that the pathogenecity of the virus correlates with the presence of a determinant located within antigenic site III (**69**.Coulon *et.al.*1982; **70**.Coulon *et.al.*1983; **71**.Dietzschold *et.al.* 1983a). Sequence analysis showed that the change in pathogenecity corresponds to a single amino acid substitution on G protein, at position 333, from original arginine to glutamine or isoleucine in CVS and ERA variant strains respectively (**72**.Dietzschold *et.al.* 1983b). Syncytium-forming ability of the virulent type G protein but not the avirulent variant G protein is responsible for the more efficient spread of virulent virus in the brain (**73**.Morimoto *et.al.* 1992). Other investigators showed that nonpathogenic virus derivatives use different receptors or routes of entry from pathogenic viruses (**74**.Coulon *et.al.*1998; **75**. Kucera *et.al.* 1985), as well as differentially effect the expression of MHC class II mRNA in the CNS (**76**.Irwin *et.al.*1999).

2.2.4 Rabies Virus Neutralization:

The glycoprotein of rabies virus represents the only antigen that induces virus-neutralizing antibodies (VNA) (**77**.Cox *et.al.*1977).The mechanisms of viral neutralization by antibodies, have been proposed for many viruses. One simple mechanism is to block virus attachment to host cells by complete masking of all of the G protein spikes of the rabies virion which is named

as steric hindrance theory. It was estimated there are about 445 G protein spikes per rabies virion and 60% coverage of those virion spikes i.e., 200-250 antibody molecules per virion would be required to achieve steric hindrance (**78**.Falmand *et.al.*1993; **79**.Burotn *et.al.*2001; **80**.Irie and Kawai 2002). Irie and Kawai (**80**.) in 2002 explained another possible mechanism of viral neutralization by antibodies which would induce conformational changes of the G protein spikes and finally causing abolishment of the receptor-binding ability of the virion. They have shown that, antibodies that work in this way, achieve neutralization with lesser number of antibodies per virion. The VNAs can exert their protective effect by neutralization of extracellular virus, by complement-mediated lysis of virus –infected cells and by antibody-dependent cytotoxicity (**81**.Davies and Metzger 1983). VNA can mediate viral clearance from the CNS without other effectors (**82**.Dietzschold *et.al.* 1992).

2.3. Vaccines and Therapies for Rabies:

2.3.1 Human Rabies vaccines:

Rabies vaccine has long history. In 1885, Louis Pasteur, serially passaged the rabies virus collected from a rabid cow, in rabbit brain (for more than 1500 times) and the preparation made from desiccated spinal cords was used successfully as a vaccine. In Pasteur's vaccine inactivation of the virus was achieved partially by desiccation (**83**. Bunn1991).

Later on Fermi and Semple introduced phenol to chemically inactivate rabies virus infected nerve-tissue. Sir David Semple in 1911 while working at Central Research Institute in Kasauli, India, developed a rabies vaccine, which continued to be very popular in India, Pakistan and other developing countries in Asia and Africa. He used to inject a fixed strain of rabies virus

originating from Pasteur strain, intracerebrally into young sheep. After 6-7 days when sheep begin to demonstrate clinical signs of rabies sheep were euthanized and brains were removed and homogenized in a solution containing phenol or beta-propiolactone (BPL). This solution is filtered and used as vaccine. Vaccine made this way contains nerve tissue up to 5%. About 2-5 ml of vaccine is administered sub-cutaneously and 7-15 doses are recommended for post-exposure treatment. The volume of the vaccine dose is too much and painful to receive. Major disadvantage of this vaccine is it can cause severe neurologic adverse reactions in vaccines (reported from 1:142 to 1: 7000) because of the presence of myelinated tissue in the vaccine (**84**.Nicholson 1996). In the wake of recent reports, sheep brain vaccines may pose the potential risk for transmission of spongiform encephalopathy (**85**.Di Martino 1993; **86**.Arya 1994).

Fuenzalida and Palacios suggested the production of rabies vaccine in suckling mouse brain (SMB), because of lack of myelination, to reduce the complications of Semple's vaccine (**87**.Fuenzalida and Palacios 1955). They produced this vaccine in Chile, using a fixed rabies strain isolated in Chile. One-day-old mice are injected intracerebrally and brain tissue is harvested 4 days later and inactivated with ultraviolet light or BPL. The vaccine is supplied in 1-2 ml vials. Although adverse reactions associated with SMB vaccine are lower than Semple's vaccine (1 in 8000) the potency of the vaccine is less. It resulted in higher case mortality rate in affected patients who received the vaccine-22% over 4.8% resulted with Semple's vaccine (**88**.Nogueria 1988).

Introduction of cell-culture rabies vaccine has drastically reduced the allergic reactions that were associated with nerve tissue vaccine, increased the potency and reduced the number of doses

required for post-exposure treatment from 7-15 to 5 doses. With the cell culture vaccine very few vaccinees succumbed to rabies (**89**.Plotkin *et.al.*1999). The first highly successful cell culture rabies vaccine was produced in 1960s on human diploid cells at the Wistar Institute, Philadelphia (**90**.Wiktor *et.al.*1969). Human diploid cell culture vaccine (HDCV) was produced on human fibroblast cells using Pitmann-Moore strain (which is a Pasteur isolate), purified, concentrated by ultra centrifugation and inactivated with BPL. The safety and efficacy of HDCV vaccine was widely recognized and has been in use for both pre and post exposure treatments. However low virus yield and high production cost made this vaccine unaffordable in developing countries, where the majority of human rabies deaths occur (**89**.Plotkin *et.al.*1999). One report indicated that up to 10% of individuals previously vaccinated with HDCV developed severe immune-complex reactions after receiving boosters (**91**.Dreesen *et.al.*1986).

Barth and colleagues developed a rabies vaccine on purified chick embryo cells (PCECV) using Flury LEP strain, concentrated by zonal centrifugation and inactivated by BPL (**92**.Barth *et.al.*1984). Chicken fibroblasts produce high yields of virus and fewer risks compare to HDCV (**93**.Bijok 1984).

The use of heteroploid cell lines reduces the cost of production of viral vaccine. But cell lines can be potentially oncogenic. However, better purification processes now available eliminated this risk or reduced it to acceptable levels. Vero cell line (African green monkey kidney origin) was approved for the production of rabies vaccine. The heteroploid Vero cell line was introduced in 1982 for the production of inactivated rabies vaccine; it retained all the advantages of the Human Diploid Cell system, while offering the possibility of the large-scale industrial production of PVRV (**94**.Roumiantzeff 1988). This vaccine is widely used in Europe and developing countries, but it is not licensed for use in North America.

All modern high-quality cell-culture vaccines available for humans meet or exceed the published WHO standards (**95**.WHO 1981; **96**.WHO 1994). One could say that there is little need to develop a more efficacious human rabies vaccine. However, improvements certainly can be made to the present human rabies vaccines. For example, less expensive rabies vaccines are desperately needed in developing countries to replace the nerve tissue vaccines. It is unrealistic to imagine that the expensive vaccines and vaccination protocols currently used in industrialized countries will be able to be implemented widely in developing countries. Therefore, new generation of rabies vaccines must be developed that are inexpensive enough to vaccinate millions of people at risk in canine rabies-endemic countries.

2.3.2. Rabies vaccines for domestic animals:

Initially Pasteur's nervous tissue vaccine inactivated by desiccation was used to vaccinate dogs. In 1927, the First International Rabies Conference recommended that "fixed" virus for canine rabies vaccines be inactivated or attenuated so that they cause no disease in dogs with subcutaneous or intramuscular route. Later on all nervous tissue vaccines were inactivated using phenol (**83**.Bunn 1991). Nervous tissue vaccines sometimes resulted in postvaccinal nervous system reactions and death of few vaccinated animals (**83**.Bunn 1991). To improve the situation embryonated eggs were used to passage rabies virus. Flury strain, a human rabies virus isolate, was passaged for 40-50 times and used as Flury low-egg-passage (LEP) vaccine (**97**.Koprowski and Cox. 1948). This vaccine occasionally caused rabies in young pups, cats and cattle. When the passage level in eggs was increased to 205 times, designated as Flury high-egg-passage (HEP), the vaccine found to be safe for cats, cattle and pups of 3 months age (**98**.Koprowski and Black.1954). Killed cell-culture rabies vaccines, for veterinary use, are very much like vaccines

made for human use. But live rabies vaccines are available for only veterinary use. They are made from modified live virus (MLV) of Flury and Kelev strains (**93**.Bijok.1984; **99**.Arai *et.al.* 1991); SAD strain (**100**.Fenje.1960); and the ERA strain (**101**.Abelsheth.1964). Some of the rabies vaccines made from MLV strains have been adopted for oral immunization of carnivores, including domestic dogs and cats in Asia, Africa and some parts of Europe (**102**.Blancou and Meslin.1996). No MLV rabies vaccine is licensed for use in United States.

The epidemiological luxury provided by dog rabies control allowed the extension of this concept to wild life during 1960s. Using the oral rabies vaccines rabies was eliminated in red foxes in Europe (**103**.Wandeler 2000) and in North America (**104**.MacInnes *et.al.* 2001). Though present cell culture rabies vaccines are safe and effective, parenteral administration of these vaccines does not allow them to readily use in wild life vaccination. More over, in the wild life many animal reservoirs exist for rabies virus and each of these animal reservoirs carries an antigenically distinct virus variant. The currently available modified-live rabies virus vaccines have either safety problems or do not induce sufficient protective immunity in particular wild life species. Hence there is a need for the development of new live rabies virus vaccines that are very safe and highly effective in particular wild life species (**105**.Dietzschold and Schnell 2002**)**.

2.3.3. New Generation of Rabies Vaccines:

For the past two decades researchers are working on a new generation of rabies vaccines for both human and animal use.

In the1970s in Europe, oral rabies vaccination was practiced in an attempt to vaccinate free-ranging wild life species. The baits used for the purpose contained the live-virus of SAD-B19

strain. Unfortunately the residual virus in the baits was shown to be pathogenic to some rodents (**106**.Artois *et.al.* 1992). To combat the problem, low-virulent variants of SAD strain were selected using appropriate monoclonal antibodies. The variants SAG-1 and SAG-2 are slightly pathogenic in suckling mice, but avirulent in adult mice by all routes (**70**.Coulon *et.al.*1983; **72**.Dietzschold *et.al.* 1983b). It was found SAG-1 has a mutated serine instead of arginine on glycoprotein at position 333, where as in SAG-2 a two-nucleotide mutation at the same position codes for glutamine (**107**.Le Blois *et.al.* 1990; **108**.Lafay *et.al.* 1994). Both SAG-1 and SAG-2 are tested safe with no side effects and used extensively in oral vaccination in Europe (**109**.Artois *et.al.* 1997; **110**.Fekadu *et.al.* 1996).

Live recombinant rabies vaccines were developed by cloning the glycoprotein gene into viral vectors. Glycoprotein gene of ERA strain was inserted into thymidine kinase region of vaccinia virus (**111**.Kieny *et.al.* 1984; **112**.Wiktor *et.al.* 1984). This vaccinia-rabies glycoprotein (VRG) virus vaccine was licensed in 1995 by USDA APHIS for oral immunization of raccoons and is now available commercially. Similarly live canarypox virus that expresses rabies glycoprotein has been licensed as a combination-type vaccine for use in cats (**113**.Compendium 2001). Adenovirus- vectored recombinant rabies vaccines are developed using human adenovirus type 5 and type2. When the glycoprotein gene was cloned into the E3 locus of type 5 genome, the vaccine proved successful for oral immunization in several animal species (**114**.Prevec *et.al.*1990; **115**.Charlton *et.al.* 1992). These vaccines induced an immune response in very young animals and neonates even before maternal immunity against rabies subsides (**116**.Wang *et.al.*1997). Adeno-viral live recombinant rabies vaccine has potential for use in human vaccination (**117**.Xiang *et.al.*1996).

Recently using plant viruses, such as tobacco mosaic virus (TMV) and tomato bushy stunt virus, as vectors for expression of foreign antigens in plants, have provided prototypes of plant-derived, genetically manufactured vaccines (**118**.McGarvey *et.al.* 1995;**119**.Yusibov *et.al.*1997). Plant based production systems are inexpensive, safe and most of the time allow easy delivery of vaccine.

DNA-based rabies vaccines were developed by inserting rabies glycoprotein of ERA strain into plasmid vector. When used for immunization it protected animals against rabies challenge (**120**.Xiang *et.al.*1994). DNA-based vaccines are a simple yet versatile approach, as they can be administered by more than one route (**121**.Donnelly *et.al.*1994). Most significantly they provide an efficient way to induce CD8+ T-cell response apart from eliciting CD4+ and VNA (**122**.Germain 1994).

All these new generation vaccines would be appropriated for pre exposure vaccination because of robustness of live immunization. In infected animals, as the activation of CD8+ T-cells induces a pathological reaction that is associated with clinical paralysis, it discourages the use of these vaccines for post exposure regimens (**123**.Lodmell and Ewalt 2001).

2.3.4 Rabies Post exposure prophylaxis (PEP):

A person can not be consider exposed to rabies simply being in the same area or room or touching the fur of a rabid animal since rabies virus can not enter the body through intact skin. An exposure to rabies occurs when virus enters the body through an open cut or wound or mucous membrane. A potential non bite exposure to rabies can happen with contamination of abrasions or existing wounds through virus-infected saliva or neural tissue (**124**.Constantine 1962). The World Health Organization (WHO) has categorized exposures according to their

severity (**125**.WHO.1992). According to WHO guidelines, category III exposures which are considered with either single or multiple transdermal bites or contamination of mucous membranes with saliva of rabid animal, requires post exposure prophylaxis (PEP) (**126**.CDC.1999). Every potential exposure to rabies should be evaluated on case-by-case basis considering rabies epidemiology of the area, species involved, type of contact between rabid animal and victim, severity of exposure and anatomic location (**127**.Moore *et.al.*2000).

Post exposure prophylaxis recommends immediate and thorough cleaning of bite site wound with water and soap and, if possible, irrigation with a virucidal agent such as povidone-iodine solution (**128**.Hatchett 1991). Washing of the wound helps to reduce rabies infection by eliminating or inactivating viral particles that might have inoculated into tissue otherwise. In addition, tetanus prophylaxis and antibacterial treatment also should be initiated for all animal bites that cause tissue damage (**129**.Fleisher 1999). Suturing and closing of the wound should be avoided unless is absolutely necessary. In order to implement immediate passive immunization in previously unvaccinated individuals, post exposure prophylaxis (PEP) should always include administration of anti-rabies immunoglobulin (RIG). RIG provides passive immunity until the immune system of vaccinated individual develops its own rabies virus neutralizing antibodies (VNA) by active immunity. In clinical trials, VNA was detectable from 7-10 days after immunization (**130**.Lang *et.al.*1998a). In the event RIG was not administered when PEP was initiated, it can be given up to 7 days after first vaccination, after which time it is presumed that VNA from active immunity is present (**131**.Khawplod *et.al.*1996). RIG should be infiltrated around the wound, if the volume of RIG is too small to infiltrate all open wounds, it can be diluted in PBS and used. Half of the total volume is infiltrated in and around wound site and

remaining half of the volume is injected intramuscularly (IM) at a site distant from the vaccine injection site. Then a series of five vaccines are given IM in the deltoid muscle on days 0, 3, 7, 14, 28. Currently there are two sources of RIG are employed in human post exposure prophylaxis, equine rabies- immunoglobulin (ERIG) and human rabies- immunoglobulin (HRIG), which are prepared from pooled serum of rabies virus immunized horses and humans respectively. The recommended dose is 20 IU/kg body weight for HRIG and 40 IU/kg body weight for ERIG. In clinical trials, the administration of RIG slightly decreased the production of VNA (**132**.Lang *et.al* 1998b). Therefore, no more than the recommended amount should be administered.

It could be detrimental to administer RIG to persons previously immunized with rabies vaccines because RIG can lower the anamnestic response to booster doses of rabies vaccine. If a previously immunized person is exposed to rabies virus, current recommendations state that the administration of two booster doses of vaccine is enough (**125**.WHO. 1992; **126**.CDC 1999). In the case of pregnant individuals exposed to a known rabid animal, they can be treated with regular PEP regimen without any contraindications. No abortions and congenital deformities have been reported so far (**133**.Varner *et.al.*1982; **134**.Sudarshan *et.al.*1999).

2.3.5 Anti-Rabies Immunoglobulin (RIG):

Passive immunization with anti-rabies immunoglobulins is an essential component of rabies post exposure prophylaxis (PEP). In cases of severe rabies exposure, life-saving benefits of adding RIG to PEP has been clearly established. In such cases vaccination alone without RIG can not be

relied upon to prevent the disease (**135**.Wilde *et.al.*1989; **136**.Alvarez *et. al.*1994; **137**.Thraenhart *et. al*.1994; **138**.Wilde *et.al.*1996; **139**.Hemachudha *et. al.*1999).

Equine rabies immunoglobulin (ERIG) has been used for over 45 years. It is widely used in developing countries because it is inexpensive compared to HRIG. Different ERIGs have been produced using various immunogenic preparations of rabies vaccines. A preparation of ERIG is produced at Queen Saovabha Memorial Institute (QMSI), Bankok Thailand, by immunizing 4-12 years age horses with purified Vero cell rabies (PVR) vaccine. The animals are given a series of vaccines (up to14 injections) subcutaneously into lateral aspect of neck. The immunization lasts 105 days and the first bleeding is made 14 days later (**140**.Luekrajang *et. al.*1996). Other rabies vaccines used for immunization of horses include horse nerve tissue vaccine and purified chick embryo cell vaccine (**141**.Goel *et. al.*2003). The animals are bled from the jugular vein and serum is collected. Protein fractionation and purification methods are adopted to bring down total serum protein levels to 5%. Potency of the immunoglobulin preparations is calculated by calibrating against international standard for rabies immunoglobulin (**142**.WHO.1985).

ERIG preparations from various manufacturers have been shown to induce serum sickness in some recipients ranging from 0.82% to 6.19% depending on the protein content of the ERIG (**135**.Wilde *et.al.*1989; **143**.Wilde and Chutivongse 1990). In 1965 it was reported approximately 16% of persons treated with ERIG developed serum sickness; among persons over 15 years of age, the incidence was 46% (**144**.Karliner and Belaval 1965). Recently available purified, heat-treated and even $F(ab')_2$ fractions of ERIG are safe and reduced the serum sickness incidence among recipients (**130**.Lang *et. al.* 1998a).

Equine origin immunoglobulins are heterologous for human therapy and associated anaphylactic reactions are always higher than a preparation made from human source (homologous). More over ERIG can be eliminated from the body more rapidly than HRIG in treated individuals. Human rabies immunoglobulin (HRIG) have been developed and used for rabies post exposure prophylaxis mainly to overcome the problems associated with ERIG. In USA and Canada it has been used since 1975 and no cases of serum sickness have been reported so far (**145**.Fournier and Sikes.1996).

The volunteer donors of plasma for production of HRIG were selected and vaccinated with HDCV as per pre-exposure or post exposure schedule. VNA titers of the donors are checked and should be at least 15 IU/ml, otherwise booster immunizations are recommended. Plasma or serum may be collected from donors. Then pooled plasma or serum is purified, fractionated and potency is calibrated. The potency of Imogam ® Rabies –HT, available in the market, is 150 IU/ml. Despite the fact that HRIG is well tolerated it is prohibitively expensive and available only in limited quantities. There is an inherent variability in quality and specificity of antibodies in different batches of HRIG preparations. Like any other reagent prepared from human serum, there is a possibility of infection with known or unknown pathogens with HRIG. In addition, the worldwide supply of HRIG and other polyclonal human and animal products is chronically restricted. So the worldwide demand for HRIG is increasing (**146**.Champion *et.al.*2000).

2.3.6 Treatment of a Rabies case:

Rabies is a fatal disease. Once rabies encephalitis develops, no therapy has proved effective. A working group of physicians consider potential treatment options in the management of human rabies. So far there is a record of five rabies survivals from acute illness. There may be a debate as to whether some of those patients actually had rabies (**147**.Jackson *et. al.*2003). Fatal clinical presentation of the disease tends to follow palliative therapy. In unusual circumstances, the attending physicians and relatives of the patients may wish to use an aggressive approach to therapy with an aim of curing the disease. No single therapeutic agent is likely to be effective, but a combination of specific therapies could be considered, including rabies vaccine, rabies immunoglobulin, monoclonal antibodies, ribavirin, interferon-α and ketamine. Therapy with corticosteroids should be avoided as their use may effectively close blood-brain barrier and reduce the entry of other therapeutic agents. Although this approach is mostly disappointing, in one case survival period was markedly prolonged to 133 days from the onset of symptoms (**148**.Emmons *et. al.*1973).

2.4 Monoclonal Antibodies against Rabies Virus.

In 1975 Kohler and Milstein (**149**.) discovered a method to produce monoclonal antibodies continuously, in vitro, from an antibody secreting B-cell by fusing it with an immortal mouse myeloma cell. The resulting somatic hybrid is now called hybridoma cell and produces monoclonal antibodies (MAb) against targeted, single epitope on the antigen. For this discovery Kohler and Milstein received the Noble Prize in 1984. This innovation paved the way to produce antibodies with predefined specificity, to speak literally against any antigen, in unlimited quantities. Thus hybridoma technology has virtually eliminated the need of using animal or

human donors for antibody or serum production. The monoclonal antibodies have enormous applications in research, diagnostics and cancer therapy.

Owing to the above mentioned problems associated with ERIG and HRIG, a possible alternative to rabies immunoglobulin (RIG) is human monoclonal antibodies (MAbs) with the ability to neutralize rabies virus. The advantages of MAb therapy are increased specific activity over hyper- immune serum, potential for large-scale production and decreased risk of transferring blood-borne pathogens during treatment.

In 1989,Schumacher *et.al.* (**150**) developed five mouse anti-rabies monoclonal antibodies that target different epitopes on G and N proteins. When cocktail of these mouse-MAbs was administered to mice and hamsters it protected them from lethal dose of rabies challenge.

Human anti-rabies monoclonal antibodies were developed initially by Ueki *et.al.*(**151**) and Lafon *et.al.*(**152**) in 1990. Peripheral blood lymphocytes were collected from donors previously immunized with rabies HDCV vaccine and transformed with Epstein-Barr virus (EBV) (**153**.Miller *et.al.*1972; **154**.Casali *et.al.* 1986). EBV-transformed B-cells were cultured and screened for specific antibody secretion and selected clones were fused with mouse- human heteromyeloma cell lines like F3B6 (**151**) or SPM4-0 (**152**) and selected by culturing in media containing Hypoxanthine, Aminopterin, and Thymidine (HAT). Using this approach, Ueki et.al reported the generation of 10 cell hybrids secreting IgM, IgG and IgA to the virus, one of these IgG MAbs (MAb57) efficiently neutralized a variety of rabies virus strains in vitro and in vivo (**155**.Dietschold *et.al.*1990). This MAb belongs to IgG1 subtype and targeted against the surface glycoprotein of rabies virus. The specific activity of MAb57 in vitro was found to be 38-8000 times better than HRIG. Using a similar approach Enssle et.al in 1991(**156**) developed TW-1

human monoclonal antibody which showed in vitro as well as in vivo rabies virus neutralization and protected rabies infected mice.

Recombinant Fab of MAb57 (rFab57) was prepared by cloning the heavy and light chains into bacterial vector and expressed in E coli (**157**.Cheung *et.al.*1992). Recombinant Fab57 had shown the same specificity as that of MAb57 for rabies virus but the dissociation constant was found to be 10 times lower than MAb57. It is known that the Fab domain of the antibody leaves the vasculature more rapidly (**158**.Mueller *et.al.*1990) and has better tissue accessibility (**159**.Mach *et.al.*1983). The ability of Fab domains to move out of the blood stream and into specific tissues, especially crossing the blood-brain barrier may act advantageously in the case of acute rabies virus infections. Thus an ideal therapeutic modality might consist not only of a cocktail of different intact antibodies but also a mixture of intact MAbs and monoclonal Fab domains (**157**.Cheung *et.al.*1992).

In 1994, Dorfman et.al (**160**) developed nine human monoclonal antibodies to rabies virus. In vitro assays revealed that one of the MAb (K4B9) is against G protein and neutralizes rabies virus. Western blot assay confirmed that K4B9 may be targeting discontinuous epitopes on G protein.

Champion et.al, in 2000 (**146**) produced a panel of human monoclonals that neutralized a variety of rabies strains in vitro. They followed similar approach but used SHM-D33 mouse-human heteromyeloma hybrid to fuse the EBV transformed B-cells. They found MAbs obtained from donors undergoing primary immunization were mostly IgM type and IgG type were obtained from previously immunized and boosted individuals. They established 29 clones that continuously secrete MAbs and 7 of them were IgG type. Evaluation studies on experimental

utility of these 7 MAbs in rabies PEP revealed that only two MAbs viz; JB.1 (IgG3) and JA-3.3A5 (IgG1) have broad range of rabies viral neutralization capacity (**161**.Hanlon *et.al.*2001). A specific limitation found with JA-3.3A5 was low level of antibody production in hybridoma supernatant. In hamsters, JB.1 had shown protection levels comparable to that of HRIG (**161**.Hanlon *et.al.*2001). When IgG heavy chain(H) and light chain(L) cDNA from JA-3.3A5 hybridoma cells was cloned into rhabdovirus- based vectors and expressed in BSR cells, much higher levels of functional MAb were obtained (**162**.Morimoto *et.al.*2001). Prosniak et.al, in 2003 (**163**) cloned all three hybridomas- JA-3.3A5, JB.1 and MAb57 in rhabdovirus vectors and expressed in either BSR or CHO cells and the secreted recombinant human monoclonal antibodies (rhuMAb) were designated as SOJA, SOJB and SO57 respectively. The yield of these rhuMAb was found to be at higher-level compared to that from hybridomas (≤ 40 μg/ml/48h). A cocktail of rhuMAb, consisting of SOJA:SOJB:SO57 in protein ratio 1:1:1, neutralized several fixed and street rabies viruses and protection levels obtained in mice, were comparable to that of commercial HRIG (**163**).

Monoclonal antibodies intended for human use would be better appreciated when produced in human cell lines, for immunogenic reasons, than in non-human BSR or CHO cells. Marissen et. al, in 2005, (**164**) cloned variable heavy and light chain coding regions of the SOJA, SOJB and SO57 antibodies into a single human immunoglobulin G1 (IgG1) expression vector, and expressed in human cell line PER.C6.The yielded antibodies are renamed as CRJA, CRJB and CR57. The PER.C6 cells are well characterized, produce higher-levels of recombinant IgG and do not add glycons, which are immunogenic in humans (**165**.Jones *et.al.* 2003).The CRJB antibody now belongs to IgG1 subtype and supposed to have longer half-life than IgG3 subtype to which it is originally belong to. The neutralization activities of CR57 and CRJB were found to

be higher than previously reported for the antibodies produced using a rhabdoviral vector. In contrast, CRJA had very low potency that it was decided to exclude from further experiments. More over it was found that CR57 and CRJB antibodies have overlapping epitope recognition for rabies virus, so CRJB is not suitable in combination with CR57 for PEP (**164**). To find an antibody that is complimentary with CR57 and fulfills criteria for rabies PEP, Bakker et. al. 2005 (**166**) used phase display selection using RV-immune antibody libraries. From the panel of 21 IgG1 antibodies raised, they selected MAb CR4098 as the best candidate that can complement CR57. It was shown that CR4098 recognized an epitope in antigenic site III of glycoprotein where as CR57 targets a linear epitope in antigenic site I. The in vitro exposure of rabies viruses to the combination of CR57 and CR4098 yielded no escape mutants and this obviates the need for inclusion another monoclonal antibody in the cocktail (**166**.Bakker *et.al.*2005).

Efforts to develop plant-derived antirabies monoclonal antibodies in transgenic tobacco, soybean and alfaalfa are underway as plant based production systems provide a safe and economically feasible alternative to animal systems (**167**.Modelska *et.al.*1998; **168**.Ko *et.al.* 2003; **169**.Tekoah *et.al.*2004; **170**.Ko *et.al.* 2004). But the glycosylation patterns and other innate natures of plant-derived monoclonal antibodies should be resolved before they are considered for parenteral administration for human therapy.

2.5 Monoclonal Antibodies Approved for Human Therapy.

It has been thirty-years since the development of the technology for production of monoclonal antibodies. In the initial years monoclonal antibodies were used extensively in the fields of diagnostics and research. In recent years, with clever manipulation of monoclonals from mouse

MAbs to→ Chimeric MAbs →Humanized MAbs →Fully human MAbs, their applications are extended into human therapeutic purpose. Defined target specificity, high- specificity, low toxicity and large-scale production made antibody-based therapies a reality. So far 18 MAbs have been approved by FDA for therapeutic use in United States and generated $5-6 billion revenue in 2003 (**171**.Stacy 2005). Despite the fact that antibodies have proven to be good antimicrobial agents, only one MAb, palivizumab- against respiratory syncitial virus (RSV), has been licensed for an infectious agent.

The table below enlists MAbs approved for human therapy in US.

Currently Approved MAbs for Human Therapy:

Brand name	Generic name	Type of MAb	Year Approved	Use
Orthoclone OKT_3	Muromonab-CD_3	Chimeric	1986	Prevention of organ rejection
ReoPro	Abciximab	Chimeric	1994	During cardiac catherization
Rituxan	Rituximab	Chimeric	1997	Treatment of non-Hodgkins lymphoma
Zenapax	Daclizumab	Humanized	1997	Kidney transplant rejections
Remicade	Infliximab	Chimeric	1998	Crohn's disease & rheumatoid arthritis
Herceptin	Trastuzumab	Humanized	1998	Treatment of breast cancer
Synagis	Palivizumab	Humanized	1998	Prophylaxis of RSV
Simulect	Basiliximab	Chimeric	1998	Kidney transplant rejections
Mylotarg	Gemtuzumab/ ozogamicin	Humanized	2000	Treatment of myelogenous leukemia
Campath	Alemtuzumab	Humanized	2001	Treatment of chronic lymphocytic leukemia
Humira	Adalimumab	Fully Human	2002	Treatment of rheumatoid arthritis
Zevalin	Ibritumomab-tituxetan- ^{90}Y*	Murine radio labeled	2002	Treatment non-Hodgkins lymphoma
Xolair	Omalizumab	Humanized	2003	Treatment of Asthma
Bexxar	Tositumomab/ tositumomab-^{131}I*	Murine radio labeled	2003	Treatment non-Hodgkins lymphoma
Raptiva	Efalizumab	Humanized	2003	Treatment of psoriasis
Avastin	Bevacuzumab	Humanized	2004	Treatment of colorectal cancer
Erbitux	Cetuximab	Chimeric	2004	Treatment of colorectal cancer
Tysabri	Natalizumab	Humanized	2004	Treatment of Multiple sclerosis

Source: The Scientist, 2005 February 14; Vol 19(3):17-19.

References:

1. Jackson AC, Wunner WH. (2002) In *Rabies*: Academic Press, An Elsevier Science Imprint .1-22.

2. Dietzschold B, Rupprecht C, Fu ZF, Kaprowski H. (1996) *Rhabdoviruses*: In Fields Virology 3rd edi. 1137-1159.

3. Mayo MA, Pringle CR. Virus taxonomy-1997. J.Gen.Virol. 1998; 79(4): 649-657.

4. Bourhy. H, Kissi B, Tordo N. Molecular diversity of the Lyssavirus genus. Virology.1993; 194(1): 70-81.

5. Davies MC, Englert ME, Sharples GR, Cabasso VJ. The electron microscopy of rabies virus in cultures of chicken embryo tissues. Virology.1963; 21: 103-114.

6.Hummeler K, Koprowski H, Viktor TJ. Structure and development of rabies virus in tissue culture J.Virol.1967; 1(1):152-170.

7.Burrage TG, Tignor GH, Hawrot E, Smith AL, Lentz TL. Co-Localization of rabies virus and regions of high density acetylcholine receptors. J.Cell Biol.1982; 95:620-630.

8. Lentz TL, Burrage TG, Smith AL, Crick J, Tignor GH. Is the acetylcholine receptor a rabies receptor? Science.1982; 215(4529): 182-184.

9.Gaudin Y, Ruigrok RWH, Tuffereau C, Knossow M, Flamand A. Rabies virus glycoprotein is a trimer. Virology.1992; 187(2): 627-631.

10.Hanham CA, Zhao F, Tignor GH. Evidence from the anti-idiotypic network that the acetylcholine receptor is a rabies virus receptor. J.Virol.1993; 67(1): 530-542.

11.Reagan KJ, Wunner WH. Rabies virus interacts with various cell lines is independent of the acetylcholine receptor. Arch.Virol.1985; 84(3-4): 277-282.

12. Thoulouze MI, Lafage M, Schachner M, Hartmann U, Cremer H and Lafon M. The neural cell adhesion molecule is a receptor for rabies virus. J.Virol.1998; 72(9): 7181-7190.

13. Tuffereau C, Benejean J, Blondel D, Kieffer B and Flamand A. Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO J. 1998; 17(24): 7250-7259.

14. Marsh M, Helenius A. Virus entry into animal cells. Adv Virus Res.1989; 36: 107-151.

15. Matlin KS, Reggio H, Helenius A, Simons K. Pathway of vesicular stomatitis virus entry leading to infection. J Mol Biol 1982; 156:609-631.

16. Whitt MA, Bounocore L, Prehaud C, Rose JK. Membrane fusion activity, oligomerization,and assembly of rabies virus glycoprotein. Virology 1991;185(2): 681-688.

17.Gaudin Y, Ruigrok RW, Brunner J. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. J Gen Viol 1995; 76(7): 1541-1556.

18. Gaudin Y. Folding of rabies virus glycoprotein: epitope acquisition and interaction with endoplasmic reticulum chaperones. J Virol 1997: 71(5): 3742-3750.

19. Banerjee AK. Transription and replication of rhabdoviruses. Microbiol. Rev 1987; 51(1): 66-87.

20. Colonno RJ, Banerjee AK. Complete nucleotide sequence of the leader RNA synthesized in vitro by vesicular stomatitis virus. Cell 1978; 15(1): 93-101.

21. Leppert M, Rittenhouse L, Perrault J, Summers DF, Kolakofsky D. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell 1979; 18(3): 735-747.

22. Arnheiter H, Davis NL, Wertz G, Schubert M, Lazzarini RA. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. Cell 1985; 41(1): 259-267.

23. Emerson SU. Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. Cell 1982; 31(3): 635-642.

24. Iverson LE, Rose JK. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell 1981; 23(2): 477-484.

25. Chenik M, Chebli K, Blondel D. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphoprotein mRNA is mediated by a ribosomal leaky scanning mechanism. J Virol.1995; 69(2): 707-712.

26. Galinski MS. Paramyxoviridae: transcription and replication. Adv Virus Res. 1991; 39: 129-162.

27. Horikami SM, Curran J, Kolakofsky D, Moyer SA. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J Virol. 1992;66(8): 4901-4908.

28. Yang J, Hooper DC, Wunner WH, Koprowski H, Dietzschold B, Fu ZF. The specificity of rabies virus RNA encapsidation by nucleoprotein. Virology. 1998; 242(1): 107-117.

29. Banerjee AK, Barik S. Gene expression of vesicular stomatitis virus genome RNA. Virology.1992; 188(2): 417-428.

30. Blumberg BM, Giorgi C, Kolakofsky D. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. Cell.1983; 32(2): 559-567.

31. Yang J, Koprowski H, Dietzschold B, Fu ZF. Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader RNA encapsidation. J Virol.1999; 73(2): 1661-1664.

32. Das T, Pattnaik AK, Takacs AM, Li T, Hwang LN, Banerjee AK. Basic amino acid residues at the carboxy-terminal eleven amino acid region of the phosphoprotein (P) are required for transcription but not for replication of vesicular stomatitis virus genome RNA. Virology.1997; 238(1): 103-114.

33. Pattnaik AK, Hwang L, Li T, Englund N, Mathur M, Das T, Banerjee AK. Phosphorylation within the amino-terminal acidic domain I of the phosphoprotein of vesicular stomatitis virus is required for transcription but not for replication. J Virol. 1997; 71(11): 8167-8175.

34.Rose J, Whitt MA. Rhabdoviridae: the viruses and their replication. *In* D.M. Knipe, P.M.Howley, D.E.Griffin, R.A.Lamb, M.A.Martin, B.Roizman and S E Straus(ed.), Fields Virology 4th edition, pp 1221-1244. Lippincott- Raven Publishers, Philadelphia,PA.

35. Barr JN, Whelan SP, Wertz GW. Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus. Biochim Biophys Acta. 2002;1577(2):337-353.

36. Finke S, Conzelmann KK. Ambisense gene expression from recombinant rabies virus: random packaging of positive- and negative-strand ribonucleoprotein complexes into rabies virions. J Virol. 1997; 71(10): 7281-7288.

37. Li T, Pattnaik AK. Replication signals in the genome of vesicular stomatitis virus and its defective interfering particles: identification of a sequence element that enhances DI RNA replication. Virology.1997; 232(2): 248-259.

38. Gaudin Y, Ruigrok RW, Knossow M, Flamand A. Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J Virol.1993; 67(3): 1365-1372.

39. Iseni F, Baudin F, Blondel D, Ruigrok RW. Structure of the RNA inside the vesicular stomatitis virus nucleocapsid. RNA.2000; 6(2): 270-281.

40. Kouznetzoff A, Buckle M, Tordo N. Identification of a region of the rabies virus N protein involved in direct binding to the viral RNA. J Gen Virol. 1998;79(5):1005-1013.

41. Lyles DS, McKenzie MO. Reversible and irreversible steps in assembly and disassembly of vesicular stomatitis virus: equilibria and kinetics of dissociation of nucleocapsid-M protein complexes assembled in vivo. Biochemistry.1998; 37(2): 439-450.

42. Mebatsion T, Konig M, Conzelmann KK. Budding of rabies virus particles in the absence of the spike glycoprotein. Cell.1996; 84(6): 941-951.

43. Mebatsion T, Weiland F, Conzelmann KK. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol.1999; 73(1): 242-250.

44. Simons K, Garoff H. The budding mechanisms of enveloped animal viruses. J Gen Virol.1980; 50(1): 1-21.

45. Barge A, Gaudin Y, Coulon P, Ruigrok RW. Vesicular stomatitis virus M protein may be inside the ribonucleocapsid coil. J Virol.1993; 67(12): 7246-7253.

44. Clinton GM, Little SP, Hagen FS, Huang AS. The matrix (M) protein of vesicular stomatitis virus regulates transcription. Cell. 1978;15(4):1455-1462.

45. De BP, Thornton GB, Luk D, Banerjee AK. Purified matrix protein of vesicular stomatitis virus blocks viral transcription in vitro. Proc Natl Acad Sci U S A. 1982;79(23):7137-7141.

46. Ito Y, Nishizono A, Mannen K, Hiramatsu K, Mifune K. Rabies virus M protein expressed in Escherichia coli and its regulatory role in virion-associated transcriptase activity. Arch Virol.1996; 141(3-4): 671-683.

47. Flood EA, McKenzie MO, Lyles DS. Role of M protein aggregation in defective assembly of temperature-sensitive M protein mutants of vesicular stomatitis virus. Virology. 2000; 278(2): 520-533.

48. Emerson SU, Schubert M. Location of the binding domains for the RNA polymerase L and the ribonucleocapsid template within different halves of the NS phosphoprotein of vesicular stomatitis virus. Proc Natl Acad Sci U S A. 1987; 84(16): 5655-5659.

49. Iwasaki Y, Wiktor TJ, Koprowski H. Early events of rabies virus replicaton in tissue cultures. An electron microscopic study. Lab Invest.1973; 28(2): 142-148.

50. Murphy FA, Bauer SP, Harrison AK, Winn WC Jr. Comparative pathogenesis of rabies and rabies-like viruses. Viral infection and transit from inoculation site to the central nervous system. Lab Invest.1973; 28(3): 361-376.

51. Tsiang H, Derer M, Taxi J. An in vivo and in vitro study of rabies virus infection of the rat superior cervical ganglia. Arch Virol.1983; 76(3): 231-243.

52. Matsumoto S, Schneider LG, Kawai A, Yonezawa T. Further studies on the replication of rabies and rabies-like viruses in organized cultures of mammalian neural tissues. J Virol. 1974; 14(4): 981-996.

53. Gosztonyi G. Reproduction of lyssaviruses: ultrastructural composition of lyssavirus and functional aspects of pathogenesis. Curr Top Microbiol Immunol.1994; 187: 43-68.

54. Lyles DS, McKenzie M, Parce JW. Subunit interactions of vesicular stomatitis virus envelope glycoprotein stabilized by binding to viral matrix protein. J Virol.1992; 66(1): 349-358.

55. Charlton KM, Casey GA. Experimental rabies in skunks: immunofluorescence light and electron microscopic studies. Lab Invest. 1979; 41(1): 36-44.

56. Charlton KM, Casey GA, Webster WA, Bundza A. Experimental rabies in skunks and foxes. Pathogenesis of the spongiform lesions. Lab Invest.1987; 57(6): 634-645.

57. Charlton KM, Casey GA, Wandeler AI, Nadin-Davis S. Early events in rabies virus infection of the central nervous system in skunks (Mephitis mephitis). Acta Neuropathol (Berl). 1996; 91(1): 89-98.

58. Charlton KM, Nadin-Davis S, Casey GA, Wandeler AI. The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure. Acta Neuropathol (Berl).1997; 94(1): 73-77.

59. Baer GM. Pathogenesis to the central nervous system. In: Baer GM, ed. The Natural History of Rabies. New York: Academic Press, 1975; 181-198.

60. Shankar V, Dietzschold B, Koprowski H. Direct entry of rabies virus into the central nervous system without prior local replication. J Virol.1991; 65(5): 2736-2738.

61.Ray NB, Ewalt LC, Lodmell DL. Rabies virus replication in primary murine bone marrow macrophages and in human and murine macrophage-like cell lines: implications for viral persistence. J Virol.1995; 69(2): 764-772.

62. Harrison AK, Murphy FA. Lyssavirus infection of muscle spindles and motor end plates in striated muscle of hamsters. Arch Virol.1978; 57(2): 167-175.

63. Watson HD, Tignor GH, Smith AL. Entry of rabies virus into the peripheral nerves of mice. J Gen Virol.1981; 56(2): 372-382.

64. Tsiang H. Evidence for an intraaxonal transport of fixed and street rabies virus. J Neuropathol Exp Neurol 1979; 38(3): 286-299.

65. Gosztonyi G, Dietzschold B, Kao M, Rupprecht CE, Ludwig H, Koprowski H. Rabies and borna disease. A comparative pathogenetic study of two neurovirulent agents. Lab Invest.1993; 68(3): 285-295.

66. Raux H, Flamand A, Blondel D. Interaction of the rabies virus P protein with the LC8 dynein light chain. J Virol. 2000; 74(21): 10212-10216.

67. Murphy FA. The pathogenesis of rabies virus infection. In:Plotkin SA, Koprowski H eds. World's Debt to Pasteur: New York: Alan Liss, 1985: 153-169.

68. Murphy FA, Harrison AK, Winn WC, Bauer SP. Comparative pathogenesis of rabies and rabies-like viruses: infection of the central nervous system and centrifugal spread of virus to peripheral tissues. Lab Invest.1973; 29(1): 1-16.

69. Coulon P, Rollin P, Aubert M, Flamand A. Molecular basis of rabies virus virulence. I. Selection of avirulent mutants of the CVS strain with anti-G monoclonal antibodies. J Gen Virol. 1982; 61 (l): 97-100.

70. Coulon P, Rollin PE, Flamand A. Molecular basis of rabies virus virulence. II. Identification of a site on the CVS glycoprotein associated with virulence. J Gen Virol. 1983; 64 Pt 3:693-696.

71. Dietzschold B, Wiktor TJ, Wunner WH, Varrichio A. Chemical and immunological analysis of the rabies soluble glycoprotein. Virology.1983; 24(2): 330-337.

72. Dietzschold B, Wunner WH, Wiktor TJ, Lopes AD, Lafon M, Smith CL, Koprowski H. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc Natl Acad Sci U S A.1983; 80(1): 70-74.

73. Morimoto K, Ni YJ, Kawai A. Syncytium formation is induced in the murine neuroblastoma cell cultures, which produce pathogenic type G proteins of the rabies virus. Virology.1992; 189(1): 203-216.

74. Coulon P, Ternaux JP, Flamand A, Tuffereau C. An avirulent mutant of rabies virus is unable to infect motoneurons in vivo and in vitro. J Virol. 1998; 72(1): 273-278.

75. Kucera P, Dolivo M, Coulon P, Flamand A. Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain. J Virol.1985; 55(1): 158-162.

76. Irwin DJ, Wunner WH, Ertl HC, Jackson AC. Basis of rabies virus neurovirulence in mice: expression of major histocompatibility complex class I and class II mRNAs. J Neurovirol.1999; 5(5): 485-494.

77. Cox JH, Dietzschold B, Schneider LG. Rabies virus glycoprotein. II. Biological and serological characterization. Infect Immun.1977; 16(3): 754-759.

78. Flamand A, Raux H, Gaudin Y, Ruigrok RW. Mechanisms of rabies virus neutralization. Virology.1993; 194(1): 302-313.

79. Burton DR, Saphire EO, Parren PW. A model for neutralization of viruses based on antibody coating of the virion surface. Curr Top Microbiol Immunol.2001; 260:109-143.

80. Irie T, Kawai A. Studies on the different conditions for rabies virus neutralization by monoclonal antibodies #1-46-12 and #7-1-9. J Gen Virol.2002; 83(12): 3045-3053.

81.Davies DR, Metzger H. Structural basis of antibody function. Annu Rev Immunol.1983; 1: 87-117.

82.Dietzschold B, Kao M, Zheng YM, Chen ZY, Maul G, Fu ZF, Rupprecht CE, Koprowski H. Delineation of putative mechanisms involved in antibody-mediated clearance of rabies virus from the central nervous system. Proc Natl Acad Sci USA 1992; 89(19): 7252-7256.

83. Bunn TO.1991. Canine and Feline Rabies vaccines past and present. In: The natural History of Rabies, GM Baer (ed) 2nd ed. 415-425. CRC Press, Boca Raton, FL.

84.Nicholson KG. Cell-culture vaccines for human use: General considerations. 1996. In laboratory techniques in rabies, F-X. Meslin, M.M.Kaplan and H.Koprowski(eds),4th ed., WHO, 271-279.

85.Di Martino A. Transmissible spongifrom encephalopathies and safety of naturally derived biologicals. Biologicals.1993; 21(1): 61-66.

86. Arya SC. Transmissible spongiform encephalopathies and sheep-brain derived rabies vaccines. Biologicals.1994; 22(1): 73-74.

87. Fuenzalida ER. Palacios. 1955**.** An improved method for preparation of rabies vaccine. Bol. Inst. Bacteriol.Chile.1955; 8: 3-10

88. Nogueria YL. Adverse effect verses quality control of the Fuenzalida-Palacios antirabies vaccine. Rev.Inst.Med.Trap.Sao Paulo.1988; 40: 295-299.

89. Plotkin SA, Rupprecht CR, Koprowski H. Rabies vaccine. In :Vaccines Plotkin SA, Orenstein WA (eds.) 3rd ed.1999; 743-766. W.B. Saunders, Philadelphia.

90.Wiktor TJ, Sokol F, Kuwert E, Koprowski H. Immunogenicity of concentrated and purified rabies vaccine of tissue culture origin. Proc Soc Exp Biol Med.1969; 131(3): 799-805.

91. Dreesen DW, Bernard KW, Parker RA, Deutsch AJ, Brown J. Immune complex-like disease in 23 persons following a booster dose of rabies human diploid cell vaccine. Vaccine.1986; 4(1): 45-49.

92. Barth R, Gruschkau H, Bijok U, Hilfenhaus J, Hinz J, Milcke L, Moser H, Jaeger O, Ronneberger H, Weinmann E. A new inactivated tissue culture rabies vaccine for use in man. Evaluation of PCEC-vaccine by laboratory tests. J Biol Stand.1984; 12(1): 29-46.

93. Bijok U, Vodopija I, Smerdel S, Thongcharoen P, Nicholson K, Dietrich M, Gonzalez de Cosio A. Purified chick embryo cell (PCEC) rabies vaccine for human use: clinical trials. Behring Inst Mitt.1984; (76): 155-164.

94. Roumiantzeff M. The present status of rabies vaccine development and clinical experience with rabies vaccine. Southeast Asian J Trop Med Public Health.1988; 19(4): 549-561.

95. World Health Organization. Expert Committee on biological Standardization. Thirty-First Report. Requirements for Rabies Vaccine for Human Use.1981; WHO technical Report Series No.658: WHO, Geneva.

96. World Health Organization. Expert Committee on biological Standardization. Forty-Third Report.1994; WHO technical Report Series No.840: WHO, Geneva.

97. Koprowski H, Cox HR. Studies on chick embryo adapted rabies virus: I. Culture characteristics and pathogenecity. J.Immunol.1948; 60: 533-536.

98. Koprowski H, Black J. Studies on chick-embryo-adapted rabies virus. VII. Immunological responses of animals to vaccination with high egg passage Flury strain. J Immunol.1954; 72(6): 503-510.

99. Arai YT, Ogata T, Oya A. Studies on Japanese-produced chick embryo cell culture rabies vaccines. Am J Trop Med Hyg.1991; 44(2): 131-134.

100. Fenje P. Propagation of rabies virus in cultures of hamster kidney cells. Can J Microbiol.1960; 6: 479-484.

101. Abelsheth MK. Propagation of rabies virus in pig kidney cell culture. Can Vet J. 1964; 5: 84-86.

102. Blancou J, Meslin FX. Modified live- virus rabies vaccine for oral immunization of carnovores. In laboratory techniques in rabies: Meslin FX, Kaplan KK, Koprowski H (edi) 4th ed. WHO.1996; 324-337.

103. Wandeler AI. Oral immunization against rabies: afterthoughts and foresight. Schweiz Arch Tierheilkd. 2000; 142(8): 455-462.

104. MacInnes CD, Smith SM, Tinline RR, Ayers NR, Bachmann P, Ball DG, Calder LA, Crosgrey SJ, Fielding C, Hauschildt P, Honig JM, Johnston DH, Lawson KF, Nunan CP, Pedde MA, Pond B, Stewart RB, Voigt DR. Elimination of rabies from red foxes in eastern Ontario. J Wildl Dis.2001; 37(1):119-132.

105. Dietzschold B, Schnell MJ. New approaches to the development of live attenuated rabies vaccines. Hybrid Hybridomics.2002; 21(2): 129-34.

106. Artois M, Guittre C, Thomas I, Leblois H, Brochier B, Barrat J. Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparison. Vaccine.1992; 10(8): 524-528.

107. Le Blois H, Tuffereau C, Blancou J, Artois M, Aubert A, Flamand A. Oral immunization of foxes with avirulent rabies virus mutants. Vet Microbiol. 1990; 23(1-4): 259-266.

108. Lafay F, Benejean J, Tuffereau C, Flamand A, Coulon P. Vaccination against rabies: construction and characterization of SAG2, a double avirulent derivative of SAD Bern. Vaccine.1994; 12(4): 317-320.

109. Artois M, Cliquet F, Barrat J, Schumacher CL. Effectiveness of SAG1 oral vaccine for the long-term protection of red foxes (Vulpes vulpes) against rabies. Vet Rec.1997; 140(3): 57-59.

110. Fekadu M, Nesby SL, Shaddock JH, Schumacher CL, Linhart SB, Sanderlin DW. Immunogenicity, efficacy and safety of an oral rabies vaccine (SAG-2) in dogs. Vaccine. 1996; 14(6): 465-468.

111.Kieny MP, Lathe R, Drillien R, Spehner D, Skory S, Schmitt D, Wiktor T, Koprowski H, Lecocq JP. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature.1984; 312(5990): 163-166.

112.Wiktor TJ, MacFarlan RI, Reagan KJ, Dietzschold B, Curtis PJ, Wunner WH, Kieny MP, Lathe R, Lecocq JP, Mackett M, Moss M, Koprowski H. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc Natl Acad Sci U S A. 1984; 81(22): 7194-7198.

113. Compendium of animal rabies vaccines, 2001. J Am Vet Med Assoc.2001; 218: 26-31.

114. Prevec L, Campbell JB, Christie BS, Belbeck L, Graham FL. A recombinant human adenovirus vaccine against rabies. J Infect Dis. 1990; 161(1): 27-30.

115. Charlton KM, Artois M, Prevec L, Campbell JB, Casey GA, Wandeler AI, Armstrong J. Oral rabies vaccination of skunks and foxes with a recombinant human adenovirus vaccine. Arch Virol.1992; 123(1-2): 169-179.

116. Wang Y, Xiang Z, Pasquini S, Ertl HC. The use of an E1-deleted, replication-defective adenovirus recombinant expressing the rabies virus glycoprotein for early vaccination of mice against rabies virus. J Virol. 1997; 71(5): 3677-3683.

117. Xiang ZQ, Yang Y, Wilson JM, Ertl HC. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. Virology.1996; 219(1): 220-227.

118. McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprowski H, Michaels FH. Expression of the rabies virus glycoprotein in transgenic tomatoes. Biotechnology (N Y).1995; 13(13): 1484-1487.

119. Yusibov V, Modelska A, Steplewski K, Agadjanyan M, Weiner D, Hooper DC, Koprowski H. Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. Proc Natl Acad Sci U S A. 1997; 94(11): 5784-5788.

120. Xiang ZQ, Spitalnik S, Tran M, Wunner WH, Cheng J, Ertl HC. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus.Virology.1994; 199(1): 132-140.

121. Donnelly JJ, Ulmer JB, Liu MA. Immunization with DNA. J Immunol Methods.1994; 176(2): 145-152.

122. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation.Cell.1994; 76(2): 287-299.

123. Lodmell DL, Ewalt LC. Post-exposure DNA vaccination protects mice against rabies virus. Vaccine.2001; 19(17-19): 2468-2473.

124. Constantine DG. Rabies transmission by nonbite route. Public Health Rep.1962; 77: 287-289.

125. World Health Organization. Expert Committee on rabies. Eighth report.WHO Tech Rep Ser 824.1992; 1-84.

126. Center for Disease Control. Human rabies prevention-United States: Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Report.1999; 48: 1–21.

127. Moore DA, Sischo WM, Hunter A, Miles T. Animal bite epidemiology and surveillance for rabies postexposure prophylaxis. J Am Vet Med Assoc.2000; 217(2): 190-194.

128. Hatchett RP. Rabies: the disease and the value of intensive care treatment. Intensive Care Nurs. 1991; 7(1): 53-60.

129. Fleisher GR. The management of bite wounds. N Engl J Med. 1999; 340(2): 138-140.

130. Lang J, Gravenstein S, Briggs D, Miller B, Froeschle J, Dukes C, Le Mener V, Lutsch C. Evaluation of the safety and immunogenicity of a new, heat-treated human rabies immune globulin using a sham, post-exposure prophylaxis of rabies. Biologicals.1998; 26(1): 7-15.

131. Khawplod P, Wilde H, Chomchey P, Benjavongkulchai M, Yenmuang W, Chaiyabutr N, Sitprija V. What is an acceptable delay in rabies immune globulin administration when vaccine alone had been given previously? Vaccine.1996; 14(5): 389-391.

132. Lang J, Simanjuntak GH, Soerjosembodo S, Koesharyono C. Suppressant effect of human or equine rabies immunoglobulins on the immunogenicity of post-exposure rabies vaccination under the 2-1-1 regimen: a field trial in Indonesia. MAS054 Clinical Investigator Group. Bull World Health Organ.1998; 76(5): 491-495.

133. Varner MW, McGuinness GA, Galask RP. Rabies vaccination in pregnancy. Am J Obstet Gynecol.1982; 143(6): 717-718.

134. Sudarshan MK, Madhusudana SN, Mahendra BJ. Post-exposure prophylaxis with purified vero cell rabies vaccine during pregnancy--safety and immunogenicity. J Commun Dis.1999; 31(4): 229-236.

135. Wilde H, Choomkasien P, Hemachudha T, Supich C, Chutivongse S. Failure of rabies postexposure treatment in Thailand. Vaccine.1989; 7(1): 49–52.

136. Alvarez L, Fajardo R.Lopez E. Pedroza R. Hemachudha T. Kamolvarin N, et al. Partial recovery from rabies in a nine-year old boy. Pediatr Infect Dis J.1994; 13(12):1154–1155.

137. Thraenhart O, Marcus I, Kreuzfelder E. Current and future immunoprophylaxis against human rabies: reduction of treatment failures and errors. In: Rupprecht CE, Dietzschold B, Koprowski H, editors, Lyssaviruses. New York, Springer-Verlag. Curr Top Microbiol Immunol.1994; 187:173–194.

138. Wilde H, Sirikawin S, Sabcharoen A, Kingnate D, Tantawichien T, Harischandra PA, Chaiyabutr N, de Silva DG, Fernando L, Liyanage JB, Sitprija V. Failure of post exposure treatment of rabies in children. Clin Infect Dis.1996; 22(2): 228–232.

139. Hemachudha T, Mitrabhakdi E, Wilde H, Vejabhuti A, Siripataravanit S, Kingnate D. Additional reports of failure to respond to treatment after rabies exposure in Thailand. Clin Infect Dis.1999**;** 28(1):143–144.

140. Luekrajang T, Wangsai J, Phauphak P. Production of antirabies serum of equine origin. In: Laboratory techniques in rabies, F-X. Meslin, M.M.Kaplan and H.Koprowski (eds),4th ed., WHO.1996; 401-410.

141.Goel SK, Sharma S, Singh US. Antibody response to purified chick embryo cell vaccine in equines for production of equine rabies immune globulin. Biologicals. 2003; 31(4): 233-236.

142. World Health Organization. Expert Committee on biological standardization. Thirty-Fifth Report. 1985; WHO Tech Rep Ser 725: WHO, Geneva.

143. Wilde H, Chutivongse S. Equine rabies immune globulin: a product with an undeserved poor reputation. Am J Trop Med Hyg.1990; 42(2): 175-178.

144. Karliner JS, Belaval GS. Incidence of reactions following administration of antirabies serum: A study of 526 cases. JAMA.1965; 193: 359-362.

145. Fournier P, Sikes RK. Production of human rabies immunoglobulin. In: Laboratory techniques in rabies, F-X. Meslin, M.M.Kaplan and H.Koprowski (eds),4th ed., WHO.1996; 411-416.

146. Champion JM, Kean RB, Rupprecht CE, Notkins AL, Koprowski H, Dietzschold B, Hooper DC. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute

for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. J Immunol Methods.2000; 235(1-2): 81-90.

147. Jackson AC, Warrell MJ, Rupprecht CE, Ertl HC, Dietzschold B, O'Reilly M, Leach RP, Fu ZF, Wunner WH, Bleck TP, Wilde H. Management of rabies in humans. Clin Infect Dis. 2003; 36(1):60-63.

148. Emmons RW, Leonard LL, DeGenaro F Jr, Protas ES, Bazeley PL, Giammona ST, Sturckow K. A case of human rabies with prolonged survival. Intervirology.1973; 1(1): 60-72.

149. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature.1975; 256(5517): 495-497.

150. Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H. Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest. 1989; 84(3): 971-975.

151. Ueki Y, Goldfarb IS, Harindranath N, Gore M, Koprowski H, Notkins AL, Casali P. Clonal analysis of a human antibody response. Quantitation of precursors of antibody-producing cells and generation and characterization of monoclonal IgM, IgG, and IgA to rabies virus. J Exp Med. 1990; 171(1): 19-34.

152. Lafon M, Edelman L, Bouvet JP, Lafage M, Montchatre E. Human monoclonal antibodies specific for the rabies virus glycoprotein and N protein. J Gen Virol.1990; 71(8): 1689-1696.

153.Miller G, Shope T, Lisco H, Stitt D, Lipman M. Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc Natl Acad Sci U S A.1972; 69(2):383-387.

154.Casali P, Inghirami G, Nakamura M, Davies TF, Notkins AL. Human monoclonals from antigen-specific selection of B lymphocytes and transformation by EBV. Science.1986; 234(4775): 476-479.

155. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H. Biological characterization of human monoclonal antibodies to rabies virus. J Virol. 1990; 64(6):3087-3090.

156. Enssle K, Kurrle R, Kohler R, Muller H, Kanzy EJ, Hilfenhaus J, Seiler FR. A rabies-specific human monoclonal antibody that protects mice against lethal rabies. Hybridoma. 1991; 10(5): 547-556.

157.Cheung SC, Dietzschold B, Koprowski H, Notkins AL, Rando RF. A recombinant human Fab expressed in Escherichia coli neutralizes rabies virus. J Virol.1992; 66(11): 6714-6720.

158.Mueller BM, Reisfeld RA, Gillies SD. Serum half-life and tumor localization of a chimeric antibody deleted of the CH2 domain and directed against the disialoganglioside GD2. Proc Natl Acad Sci U S A. 1990; 87(15): 5702-5705.

159.Mach JP, Chatal JF, Lumbroso JD, Buchegger F, Forni M, Ritschard J, Berche C, Douillard JY, Carrel S, Herlyn M, Steplewski Z, Koprowski H. Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. Cancer Res. 1983; 43(11): 5593-5600.

160. Dorfman N, Dietzschold B, Kajiyama W, Fu ZF, Koprowski H, Notkins AL. Development of human monoclonal antibodies to rabies. Hybridoma.1994; 13(5): 397-402.

161. Hanlon CA, DeMattos CA, DeMattos CC, Niezgoda M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. Vaccine. 2001; 19(28-29): 3834-3842.

162. Morimoto K, Schnell MJ, Pulmanausahakul R, McGettigan JP, Foley HD, Faber M, Hooper DC, Dietzschold B. High level expression of a human rabies virus-neutralizing monoclonal antibody by a rhabdovirus-based vector. J Immunol Methods.2001; 252(1-2): 199-206.

163. Prosniak M, Faber M, Hanlon CA, Rupprecht CE, Hooper DC, Dietzschold B. Development of a cocktail of recombinant-expressed human rabies virus-neutralizing monoclonal antibodies for postexposure prophylaxis of rabies. J Infect Dis.2003; 188(1): 53-56.

164. Marissen WE, Kramer RA, Rice A, Weldon WC, Niezgoda M, Faber M, Slootstra JW, Meloen RH, Clijsters-van der Horst M, Visser TJ, Jongeneelen M, Thijsse S, Throsby M, de Kruif J, Rupprecht CE, Dietzschold B, Goudsmit J, Bakker AB. Novel rabies virus-neutralizing epitope recognized by human monoclonal antibody: fine mapping and escape mutant analysis. J Virol.2005; 79(8): 4672-4678.

165. Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van der Kraats S, van der Helm E, Smits S, Schouten J, Brouwer K, Lagerwerf F, van Berkel P, Opstelten DJ, Logtenberg T, Bout A. High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog.2003; 19(1): 163-168.

166. Bakker AB, Marissen WE, Kramer RA, Rice AB, Weldon WC, Niezgoda M, Hanlon CA, Thijsse S, Backus HH, de Kruif J, Dietzschold B, Rupprecht CE, Goudsmit J. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. J Virol.2005; 79(14): 9062-9068.

167. Modelska A, Dietzschold B, Sleysh N, Fu ZF, Steplewski K, Hooper DC, Koprowski H, Yusibov V. Immunization against rabies with plant-derived antigen. Proc Natl Acad Sci U S A.1998; 95(5): 2481-2485.

168. Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A.2003; 100(13): 8013-8018.

169. Tekoah Y, Ko K, Koprowski H, Harvey DJ, Wormald MR, Dwek RA, Rudd PM. Controlled glycosylation of therapeutic antibodies in plants. Arch Biochem Biophys. 2004; 426(2): 266-278.

170. Ko K, Wei X, Crooks PA, Koprowski H. Elimination of alkaloids from plant-derived human monoclonal antibody. J Immunol Methods.2004; 286(1-2): 79-85.

171. Stacy KM. Therapeutic MAbs: Saving Lives and Making Billions. The Scientist.2005; 19(3): 17-19.

CHAPTER 3

EVALUATION OF PROPHYLACTIC EFFICACY OF HUMAN ANTI-RABIES MONOCLONAL ANTIBODIES IN A MOUSE MODEL[1]

Chakravarthy Reddy[1], Chris Pak[2], Jeffrey Mattis[2], Bernhard Dietzschold[3]and Zhen F. Fu[1.] To be submitted to Vaccine.

Abstract

To provide a cost-effective and safe replacement for currently used human rabies immunoglobulin (HRIG), human anti-rabies monoclonal antibodies (huMAbs) were developed. In the present study, we evaluated the prophylactic efficacy, half-lives, and interference with vaccination of two huMAbs SO57 and SOJB MAbs individually, in a mouse model when compared with conventional HRIG. Potency test revealed that SO57 and SOJB protected 80% of mice against challenge infection when given at 20 IU whereas only 50% survived with HRIG at the same dose. Serum half-lives were found to be 16 days for SO57 (IgG1); 11 days for SOJB (IgG3), and 8 days for HRIG (polyclonal). Like HRIG, huMAbs interfered with the production of active immune responses after vaccination and the levels of interference is greater than HRIG. However the protection levels of huMAbs are comparable to HRIG. In contrast to previous observations we found that passively administered antibodies protected mice against intracerebral (i.c.) challenge. Thus our studies indicate that huMAbs have biological functions comparable to or better than HRIG and could provide an alternative to HRIG in post-exposure prophylaxis.

1. Introduction

In spite of centuries of experience in animal rabies control and efficacious human post exposure prophylaxis (PEP), human rabies deaths continue to occur (**1**.Meslin *et.al.*1994). It is estimated that world wide over 70,000 people die of rabies annually (**2.**WHO 2001). More than 10 million people receive rabies post exposure prophylaxis each year (**3**.Ko *et.al.*2003). According to World Health Organization (WHO) guidelines, single or multiple transdermal bites or contamination of mucous membranes with saliva of rabid or rabid-suspected animal, requires post exposure prophylaxis (PEP) (**4**.CDC 1999). Rabies PEP consists of immediate local treatment of wounds – washing and disinfection, followed by local infiltration and systemic administration with anti-rabies immunoglobulin (RIG) and injecting cell culture rabies vaccines, at a body site other than where immunoglobulin was given, in previously unvaccinated individuals. Such treatment has proven to be highly effective (**5**.WHO 1992). In cases of severe rabies exposure vaccination alone, i.e. without RIG, cannot be relied upon to prevent the disease (**6**.Wilde *et.al.*1989; **7**.Alvarez *et.al.*1994; **8**.Thraenhart *et.al.*1994; **9**.Wilde *et.al.*1996; **10**.Hemachudha *et.al.*1999). The passive administration of virus-neutralizing antibodies is believed to neutralize the virus present at the bite site as well as systemically and prevent spreading of virus to CNS (**11**.WHO 1997).

Currently, two types of anti-rabies immunoglobulin (RIG) are employed in human post exposure prophylaxis, equine rabies- immunoglobulin (ERIG) and human rabies- immunoglobulin (HRIG), which are prepared from pooled serum of immunized horses and humans respectively. The use of ERIG is often associated with adverse effects ranging from local reactions at the injection site to systemic allergic reactions (**12**.Karliner and Belaval 1965). To avoid the risks associated with the administration of ERIG, HRIG has been developed and used in North

America since 1975 and no cases of serum sickness reported so far (**13**.Fournier and Sikes 1996). Despite the fact that HRIG is well tolerated it is prohibitively expensive and available only in limited quantities. There is an inherent variability in quality and specificity of antibodies in different batches of HRIG preparations. Like any other reagent prepared from human serum, there is a possibility of infection with known or unknown pathogens with HRIG. In addition, the worldwide supply of HRIG and other polyclonal human and animal products is chronically restricted. So the worldwide demand for HRIG is increasing (**14**.Champion et. al., 2000). The need to replace these hyperimmune serum preparations is widely recognized (**15**.WHO 2002), and human monoclonal antibodies that neutralize rabies virus offer an efficacious and economical alternative.

In the past panels of human anti-rabies monoclonal antibodies were produced and characterized (**16**.Ukei *et.al.*1990, **17**.Dietzschold *et.al.*1990; **18**.Dorfman *et.al.*1994; **14**.Champion *et.al.*2000 and **19**.Hanlon *et.al.*2001). From the above studies three hybridomas-JA, JB and J57- that specifically secrete rabies virus neutralizing MAbs have been established. It is recognized that high production potential is not achieved either with murine hybridomas or with mouse-human heterohybrid cell line (**19**.Hanlon *et.al.* 2001). Cost-effective production is achieved by cloning heavy (H) and light (L) chains of the human MAb immunglobulins into rabies-virus based vectors and expressing them in mammalian cell lines. These recombinant –expressed MAbs are designated as SOJA, SOJB and SO57 (**20**.Morimoto *et.al*. 2001 and **21**.Prosniak *et.al.*2003). The recombinant MAb cocktail consisting of SO57, SOJB and SOJA has been shown to have the level of protection as comparable to conventional HRIG in mouse model (**21**.Prosniak *et.al.*2003).

In the present study, we evaluated the overall prophylactic efficacy and half-lives of SO57 and SOJB MAbs, individually, in mouse model when used in combination with rabies vaccine and compared with conventional HRIG.

2. Materials and methods

2.1 Cells, Viruses and Antibodies

Mouse neuroblastoma (NA) and BSR cells were grown at 37^0C in 5% CO_2 in Dulbecco's modified Eagle's medium (Cambrex) supplemented with 10% FBS. Rabies strains CVS-11 and CVS-N2C (**22**.Morimoto et al.1998) viruses were propagated individually, by infecting monolayer of NA or BSR cells respectively at a multiplicity of infection (MOI) 0.1 for 1h 30 min at 37^0C and 5% CO_2. The virus was then removed, fresh medium was added to the cells and the mixture was incubated for 72 h at 34^0C and 5% CO_2. The culture supernatants were collected and stored at -80^0C until further use. CVS-24 was passaged in suckling mouse brain as described by Yan et al. 2001(**23**).

Human anti-rabies monoclonal antibodies (huMAb) SO57 (A-03-15) and SOJB (A-04-06) were expressed by rhabdoviral vectors in BSR or CHO cells as described by Prosniak *et.al.*2003 (**21**). Human rabies immunoglobulin (HRIG), Imogam® Rabies- HT, was purchased from Aventis Pasteur SA.

2.2 Animals

Four to six- weeks old female Swiss Webster mice (Harlan Sprague-Dawley, Inc., Indianapolis) were housed in temperature- and light-controlled quarters in the Animal Facility, College of

Veterinary Medicine, University of Georgia. They had access to food and water ad libitum. After inoculation animals were examined twice daily for clinical signs of rabies and were euthanized by CO_2 intoxication when moribund. Survivors were observed for 5 weeks after rabies virus inoculation at which time they were similarly euthanized. Control group of mice received phosphate buffered saline (PBS). Blood was with drawn from saphenous veins of mice at different time points and serum was collected and preserved at -20^0C until further use.

2.3 Virus titration and determination of LD_{50}

To determine the virus titers, monolayer of BSR cells in 96-well plates were infected with serial 10-fold dilutions of virus suspension and incubated at 37^0C. At 24 h post-infection cells were fixed in ice-cold 80% acetone and stained with FITC-labeled rabies virus N protein-specific antibody (Fujirebio Inc, Malvern, PA). Foci were counted using fluorescence microscopy. All titrations were carried out in triplicate. Titers were expressed as focus forming units (FFU) / ml. To determine the fifty percent lethal dose (LD_{50}) for viruses, a group of 10 mice were infected with (intranasally or intracerebrally) a serial 10-fold dilution of virus suspension and animals were observed twice daily for 5 weeks and moribund animals were euthanized. LD_{50} values were calculated as per the method described by Reed and Muench (**24**).

2.4 Vaccine preparation

Rabies vaccine was prepared from gradient-purified SPBN virus. Inactivation of concentrated SPBN virus was carried out with β-propiolactone (BPL) treatment according to the method described by Nicholoson.1996 (**25**). The protein concentration of the vaccine was 1mg/ml.

2.5 ELISA

A sandwich ELISA was performed to determine the serum half-lives of monoclonal antibodies and HRIG in mice described by Ko et al. 2003 (**26**).A flat-bottom 96-well plates (Nunc MaxiSorp™) were coated with 1µg per well of inactivated SPBN strain of rabies virus diluted in carbonate-bicarbonate buffer (pH 9.6) overnight at 4^0C. The plates were then blocked with 5% powdered milk in PBS and washed with PBS containing 0.05% Tween $_{20}$ (PBS-Tween). Serial dilutions of serum samples were then added in the well of the plates. Serum samples were diluted to achieve a linear response of absorbance with machine. Following 1 h incubation at 37^0C, plates were washed with PBS-Tween to remove unbound primary antibody. Goat anti-human antibody conjugated with HRP (Jackson Immuno Research) was used as secondary antibody as per the manufacturers specifications. After 1 h incubation at 37^0C, plates were washed and bound antibody was detected with TMB substrate. Absorbance values were read at 540nm in a SPECTRAmax 340PC microplate spectrophotometer (Molecular Devices).

2.6 Virus Neutralization assay

Rabies virus neutralization assay was performed in 96-well plate using the rapid fluorescent focus inhibition test (RFFIT) as previously described (**27**.Smith et al.1996; **28**.Zalan et al.1979). Serum was heated at 56^0C for 30 min to inactivate complement proteins. A serial two-fold dilutions of positive, negative and test sera were made in 96-well plates and then working concentration of the virus was added to each well. The plate was incubated at 37^0C for 90 min. Following the incubation, 2.5X 10^4 BSR cells were added to each well and plates were incubated for 24 h. At the end of incubation the cells were fixed in ice-cold 80% acetone and stained with FITC-labeled rabies virus N protein-specific antibody (Fujirebio Inc, Malvern, PA). Foci were

observed using fluorescence microscope. The highest dilution of the serum at which 100% inhibition of foci occur was recorded. The reciprocal of this dilution was considered as the neutralization titer. In vitro specific virus-neutralizing titer values for the MAbs and HRIG were obtained by comparing with the titer of WHO reference serum run along and expressed in IU/ml.

3. Results

3.1 In vitro characterization of antibodies

The antibody preparations were characterized in vitro for their protein concentration by Bradford method and the specific virus-neutralizing activity was determined comparing with WHO reference serum. Monoclonal antibodies have very high specific virus-neutralizing activity per milligram of protein compared to HRIG. Results are summarized in *Table1*. The molecular weight and purity of antibody preparations were checked by SDS-PAGE. Two clear bands corresponding to heavy and light chains of immunoglobulin appear in the gel and their molecular weights are in accordance for IgG class of antibodies (**29**.Abbas and Litchman. 2003) (Figure 1*)*.

3.2 Protective effect of MAbs and HRIG

To compare the protective effect of SO57 and SOJB with that of HRIG, groups of ten Swiss Webster mice were infected intranasally (i.n.) with $10LD_{50}$ CVS-N2C virus, a highly neurovirulant mouse adapted challenge virus strain (22.Morimoto et al.1998). One hour later the groups were injected intraperitoneally (i.p.) with doses of 20, 10, 5 and 2.5 IU of each antibody preparations per mouse. Control group were mock inoculated with PBS. The mice were observed for 5 weeks for appearance of clinical signs of rabies and survival was recorded. As summarized

in Table 2 eighty percent of mice were protected against challenge when given 20 IU of SO57 or SOJB monoclonal antibodies where as only 50% survival was recorded for HRIG. Thus SO57 and SOJB protected more animals against infection than HRIG for all groups.

3.3 Serum half-life of MAbs and HRIG in mouse

To determine the serum half-lives of human monoclonal (SO57 & SOJB) and polyclonal HRIG in mouse model, groups of ten Swiss Webster mice were injected intramuscularly (i.m) with 50 IU per mouse of each antibody and control group were sham treated. Serum was collected from mice on 1, 3, 7, 14, 21and 28 days after post inoculation and ELISA was performed to measure the levels of antibody present in the serum. Sample absorbance values were plotted against the day of the serum collection and a linear regression was computed. Half of the absorbance maximum for each antibody was predicted from the regression equation and considered as half-life. As shown in *Figure 2*, antibody SO57 has the longest serum half-life followed by SOJB and HRIG in mouse. Computing with linear regression equation for each antibody, serum half-lives were found to be 16 days for SO57 (IgG1); 11 days for SOJB (IgG3), and 8 days for HRIG (polyclonal).

3.4 Interference of antibodies on vaccine- mediated immunity

To demonstrate the interference of human MAbs with active immunization when compared with HRIG, groups of ten Swiss Webster mice were given intraperitoneally (i.p.) 5, 1, 0.2, 0.04 IU of antibody per mouse. The groups of mice then received intramuscularly (i.m) four vaccinations with 5µg SPBN-BPL vaccine per mouse on 2, 12, 62 and 92 days after antibody treatment. Mice were bled 2 days after antibody treatment and 10 days after each vaccination. Serum was used

for measurement of virus neutralization antibody (VNA) titers by RFFIT. Titers were expressed in geometric mean values. The results are summarized in *Table 3*. In contrast to the control group which had already exhibited vaccine induced immune response after first vaccination with a geometric mean titer (GMT) of 1:453, all groups of mice pretreated with antibodies showed increase in VNA titers only after third vaccination. The VNA titers of all antibody treated groups were always lower than the control group for the corresponding time point. In the group that was given SO57 antibody a significant level of interference was observed even after the fourth vaccination. When mice were given 0.2 and 0.04 IU of each antibody, no antibody was detected at 2 days after antibody administration. Yet, such low concentrations of antibodies showed a significant level of interference on vaccine-mediated immunity. Therefore, Interference of vaccine-mediated immunity is not dependent on doses of antibodies administered.

3.5 Dependence of interference level on concentration of the vaccine administered.

To assess if the antibody interference on the development of protective immunity is dependent on the dose of vaccine, groups of ten Swiss Webster mice were injected intraperitoneally (i.p.) with 10 IU of SO57, SOJB and HRIG antibodies per mouse. Control group mock injected with PBS. Twenty four hours later, each group was vaccinated intramuscularly with 2, 0.4, 0.08, 0.016 µg doses of SPBN-BPL vaccine per mouse. Seven days after primary vaccination all groups were re-vaccinated with same dose as that of primary vaccination. Seven days after second vaccination all groups were challenged intracerebrally (i.c) with 25 MIC LD_{50} of CVS-24virus. The i.c route of challenge was chosen because, as shown in previous studies for mouse monoclonal antibodies (**30**.Schumacher et al. 1989; **31.** Schumacher et al.1992), only active immunized animals, but not passive immunized animals are protected against an i.c challenge.

As shown in *Table 4,* 80% mice survived in the control group with the highest (2 µg) dose of vaccine and a dose-dependent survival response was observed for remaining doses of the vaccine administered. In the groups of animals that were administered both antibodies and vaccine, a surprisingly higher rate of survival was observed (80-100%), irrespective of the vaccine dose administered, indicating that passively administered antibody is at least partially protective.

3.6 Correlation between antibody dependent interference and reduction of protective immunity.

To demonstrate a correlation between antibody-dependent interference and reduction of protective immunity in response to vaccine, groups of ten Swiss Webster mice were injected intraperitoneally with 10 IU per mouse of SO57, SOJB or HRIG antibodies. Control group were mock injected with PBS. Groups of mice then received 2µg dose of SPBN-BPL vaccine per mouse, intramuscularly, at different time intervals from 0-21 days (Table 5). Mice were revaccinated with the same dose of vaccine 7 days after the first vaccination. Seven days after second vaccination, serum was collected and all groups were challenged intracerebrally (i.c) with 25 $MICLD_{50}$ of CVS-24 virus. As shown in *Table 5*, 90-100% survival was observed in all groups of mice including those were given both vaccine and antibody preparation.

3.7 Passive immunity protects against intra cerebral (i.c.) challenge.

Similar levels of protection was observed in mice pre-treated with antibody and subsequently immunized with different concentrations of vaccine (Table 4) or vaccinated at different time intervals (Table 5) indicating that passively administered antibodies may be offering protection to mice against intracerebral challenge. To investigate this phenomenon, groups of eight Swiss Webster mice were injected intraperitoneally with 10 IU per mouse of SO57, SOJB and HRIG

antibodies. Vaccine and control groups were not given any antibody preparation but mock injected with PBS. Vaccine group was given two doses of 2µg of SPBN-BPL vaccine per mouse, intramuscularly on 0 and 7 days. All other groups were mock vaccinated intramuscularly with PBS on same days. Seven days after the second vaccine all groups were challenged intracerebrally with 25 $MICLD_{50}$ of CVS-24 virus. The results were shown in *Table 6*. In control group which is not given either antibody or vaccine no mice survived and in vaccine group that was given only two doses of vaccine on 0 and 7 days all animals survived. Surprisingly the groups of animals that were given only one dose of antibody preparation survived the intracerebral challenge and the survival rates were similar among the groups of mice treated with antibodies SO57, SOJB or HRIG.

4. Discussion

In rabies post exposure prophylaxis (PEP) the need to find a replacement for currently used polyclonal immunoglobulins (HRIG/ERIG) is widely recognized (**15**.WHO 2002). Human monoclonal antibodies developed against rabies virus have shown the potential to replace polyclonal preparations (**16**.Ukei *et.al.*1990, **17**.Dietzschold *et.al.*1990; **18**.Dorfman *et.al.*1994; **14**.Champion *et.al.*2000 and **19**.Hanlon *et.al.*2001). A cocktail of human anti-rabies monoclonal antibodies (MAbs) consisting of SO57, SOJB and SOJA when used in mouse model has shown the protection levels comparable to that of conventional HRIG (**21**.Prosniak *et.al.*2003). But the overall prophylactic efficacy of these antibodies when used in combination with rabies vaccines need to be evaluated. Monoclonal antibodies when used as a cocktail the prophylactic properties of one antibody may over dominate the other antibody, because of the subtle inherent biological variations between MAbs, in such cases deductions from evaluation studies may not truly hold

for each antibody. In this paper we evaluated the prophylactic efficacy of MAbs individually by comparing them with HRIG and found that SO57 and SOJB had comparable efficacy as that of HRIG.

In vitro characterization of antibodies reveals that SO57 and SOJB have very high specific virus-neutralizing activity per milligram of protein compared to HRIG (Table1). So using monoclonal antibodies the desired therapeutic doses can be achieved with significantly lower amounts of protein compared to polyclonal preparations. SDS-PAGE results confirm that the antibody preparations used in the study are pure and free from any other proteins (figure1).

The protection levels offered by SO57 and SOJB in mice are higher or comparable to that of HRIG and a dose dependent survival response with antibodies was observed among group's mice. It is found that protection efficacy each MAb, not necessarily as a cocktail, is comparable to that of HRIG.

It was characterized that SO57 antibody belongs to IgG1 subclass where as SOJB to IgG3 subclass (**21**.Prosniak *et.al.*2003) and HRIG preparations consist of polyclonal immunoglobulins made from pooled serum. In humans the IgG1 subclass has the longest half-life with approximately 21 days whereas for the IgG3 subclass it is approximately 7 days (**32**). In our studies, the serum half-life of SO57 was also found to be longer than SOJB with approximately 16 days and 11 days respectively (Fig2). These results indicate that human antibodies may have different half-lives in the mouse because of the heterologous environment.

Impairment of immune response to vaccine by administered virus-specific antibodies has been described for respiratory syncytial virus (**33**.Murphy et al. 1989) and rabies virus (**31.** Schumacher et al.1992; **34.** Wiktor et al.1971; **35**.Wiktor et al.1977). The mechanisms by which passively administered antibodies induce a suppression of protective immune response to rabies vaccine are not fully understood. One plausible mechanism is the formation of antigen-antibody complexes (**31.** Schumacher et al.1992. **36.** Rowly et al.1973). The interference of antibodies over vaccine-mediated immunity is clearly evident from the experiment that the groups of mice given both antibody and vaccine developed VNA titers lower than the mice that received vaccine only (Table 3). It is not surprising to observe that SO57 antibody continues to interfere even after the fourth vaccination because of its longest serum half-life.

It was shown in previous studies for mouse monoclonal antibodies (**30**.Schumacher et al. 1989; **31.** Schumacher et al.1992), that only actively immunized, not passive immunized animals are protected against an i.c challenge. So i.c challenge is considered a way to differentiate between passive and active components of the immunity and the protection from i.c challenge is an indicator of vaccine-mediated (active) immunity. When both antibody and vaccine are administered, a higher level of interference was expected in those groups that were given antibody and higher concentration of vaccine dose and a much lower survival rate. But contrastingly all groups recorded higher survival rates (Table 4). Similarly higher level of interference was expected, in the groups of mice that were given antibody followed by two doses of vaccines in shortest time gap (i.e, on 0 and 7 days). Surprisingly all these groups recorded 90-100% survival (Table 5).

It is possible that the higher survival rates might be because of the protection offered by the passively administered human monoclonal antibodies against i.c challenge. When antibodies alone were administered and mice challenged through i.c route after 14 days surprisingly 6-7 out of 8 mice in the groups survived (Table 6). In contrasting to the results observed by Schumacher et al. in 1989 and 1992 (**30**, **31**) for mouse monoclonal antibodies, we observed the human anti-rabies monoclonal antibodies (SO57, SOJB) and HRIG protected mice against i.c challenge. It is possible that SO57, SOJB and HRIG can cross blood brain barrier (BBB) and confer protection against i.c. challenge.

Overall our study showed that huMAb SO57 and SOJB have comparable protective efficacy against challenge and longer half-lives than HRIG. Although huMAb showed stronger interference with active induction of VNA than HRIG they showed similar level of protection when used in combination with vaccine. Together our studies indicate that human anti-rabies monoclonal antibodies have potential to serve as an alternative to HRIG.

References:

1. Meslin FX, Fishbein DB, Matter HC. Rationale and prospects for rabies elimination in developing countries. Curr Top Microbiol Immunol.1994; 187: 1-26.

2. World Health Organization. Rabies. Fact Sheet. 2001; 99: 1-3.

3. Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A. 2003; 100(13): 8013-8018.

4. Center for Disease Control. Human Rabies Prevention-United States: Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recom Report.1999; 48: 1-21.

5. World Health Organization. Expert Committee on rabies. Eighth report.WHO Tech Rep Ser 824. 1992; 1-84.

6. Wilde H, Choomkasien P, Hemachudha T, Supich C, Chutivongse S. Failure of rabies postexposure treatment in Thailand. Vaccine.1989; 7(1): 49–52.

7. Alvarez L, Fajardo R.Lopez E. Pedroza R. Hemachudha T. Kamolvarin N, et al. Partial recovery from rabies in a nine-year old boy. Pediatr Infect Dis J.1994; 13(12):1154–1155.

8. Thraenhart O, Marcus I, Kreuzfelder E. Current and future immunoprophylaxis against human rabies: reduction of treatment failures and errors. In: Rupprecht CE, Dietzschold B, Koprowski H, editors, Lyssaviruses. New York, Springer-Verlag. Curr Top Microbiol Immunol.1994; 187: 173–194.

9. Wilde H, Sirikawin S, Sabcharoen A, Kingnate D, Tantawichien T, Harischandra PA, Chaiyabutr N, de Silva DG, Fernando L, Liyanage JB, Sitprija V. Failure of post exposure treatment of rabies in children. Clin Infect Dis.1996; 22(2): 228–232.

10. Hemachudha T, Mitrabhakdi E, Wilde H, Vejabhuti A, Siripataravanit S, Kingnate D. Additional reports of failure to respond to treatment after rabies exposure in Thailand. Clin Infect Dis.1999**;** 28(1):143–144.

11. World Health Organization. WHO recommendation on rabies post-exposure treatment and the correct technique of intradermal immunization against rabies. 1996; WHO/EMC/ZOO.96.6 WHO, Division of Emerging and other Communicable Disease Surveillance and Control, Geneva.

12. Karliner JS, Belaval GS. Incidence of reactions following administration of antirabies serum: A study of 526 cases. JAMA.1965; 193: 359-362.
13. Fournier P, Sikes RK. Production of human rabies immunoglobulin. In: Laboratory techniques in rabies, F-X. Meslin, M.M.Kaplan and H.Koprowski (eds),4th ed., WHO.1996; 411-416.

14.Champion JM, Kean RB, Rupprecht CE, Notkins AL, Koprowski H, Dietzschold B, Hooper DC. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. J Immunol Methods.2000; 235(1-2): 81-90.

15. World Health Organization. 2002. Monoclonal antibody cocktail for rabies post exposure treatment: report of a WHO consultation, 23-24 May 2002. World Health Organization document R2-370-15.WHO. Geneva, Switzerland.

16. Ueki Y, Goldfarb IS, Harindranath N, Gore M, Koprowski H, Notkins AL, Casali P. Clonal analysis of a human antibody response. Quantitation of precursors of antibody-producing cells and generation and characterization of monoclonal IgM, IgG, and IgA to rabies virus. J Exp Med.1990; 171(1): 19-34.

17. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H. Biological characterization of human monoclonal antibodies to rabies virus. J Virol.1990; 64(6): 3087-3090.

18. Dorfman N, Dietzschold B, Kajiyama W, Fu ZF, Koprowski H, Notkins AL. Development of human monoclonal antibodies to rabies. Hybridoma.1994;13(5): 397-402.

19. Hanlon CA, DeMattos CA, DeMattos CC, Niezgoda M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. Vaccine.2001; 19(28-29): 3834-3842.

20. Morimoto K, Schnell MJ, Pulmanausahakul R, McGettigan JP, Foley HD, Faber M, Hooper DC, Dietzschold B. High level expression of a human rabies virus-neutralizing monoclonal antibody by a rhabdovirus-based vector. J Immunol Methods.2001; 252(1-2): 199-206.

21. Prosniak M, Faber M, Hanlon CA, Rupprecht CE, Hooper DC, Dietzschold B. Development of a cocktail of recombinant-expressed human rabies virus-neutralizing monoclonal antibodies for postexposure prophylaxis of rabies. J Infect Dis.2003; 188(1): 53-56.

22. Morimoto K, Hooper DC, Carbaugh H, Fu ZF, Koprowski H, Dietzschold B. Rabies virus quasispecies: implications for pathogenesis. Proc Natl Acad Sci U S A.1998; 95(6): 3152-3156.

23. Yan X, Prosniak M, Curtis MT, Weiss ML, Faber M, Dietzschold B, Fu ZF. Silver-haired bat rabies virus variant does not induce apoptosis in the brain of experimentally infected mice. J Neurovirol.2001; 7(6): 518-527.

24. Reed LJ, Muench H. A simple method of estimation of fifty percent end points. The Am J Hygiene.1938; 27(3): 493-497.

25. Nicholson KG. Cell culture vaccines for human use: general considerations. In: Meslin FX, Kaplan MM, Koprowski H, editors, Laboratory Techniques in Rabies (4th ed.), World Health Organization, Geneva, Switzerland, 1996: 271-279.

26. Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A. 2003;100(13): 8013-8018.

27. Smith JS, Yager PA, Baer GM. A rapid tissue culture test for determining rabies-neutralizing antibody. In: Meslin FX, Kaplan MM, Koprowski H, editors, Laboratory Techniques in Rabies (4th ed.), World Health Organization, Geneva, Switzerland, 1996: 181–192.

28. Zalan E, Wilson C, Pukitis D. A microtest for the quantitation of rabies virus neutralizing antibodies. J Biol Stand.1979; 7(3): 213-220.

29. Chapter 3- Antibodies and Antigens In: Abbas AK, Litchman AH editors, Cellular and Molecular Immunology (5th ed.) Saunders, Philadelphia, PA, 2003: 43-64.

30. Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H.Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest.1989; 84(3): 971-975.

31. Schumacher CL, Ertl HC, Koprowski H, Dietzschold B. Inhibition of immune responses against rabies virus by monoclonal antibodies directed against rabies virus antigens.Vaccine.1992; 10(11): 754-760.

32. Chapter 6- Antibodies and their Receptors In: Riott I, Brostoff J and Male D editors, Immunology (5th ed.) Mosby International Ltd, London, UK, 1998: 71-82.

33. Murphy BR, Collins PL, Lawrence L, Zubak J, Chanock RM, Prince GA. Immunosuppression of the antibody response to respiratory syncytial virus (RSV) by pre-existing serum antibodies: partial prevention by topical infection of the respiratory tract with vaccinia virus-RSV recombinants. J Gen Virol.1989; 70(8): 2185-2190.

34. Wiktor TJ, Lerner RA, Koprowski H. Inhibitory effect of passive antibody on active immunity induced against rabies by vaccination. Bull World Health Organ. 1971; 45(6): 747-753.

35. Wiktor TJ, Doherty PC, Koprowski H. In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus. Proc Natl Acad Sci U S A. 1977; 74(1): 334-338.

36. Rowley DA, Fitch FW, Stuart FP, Kohler H, Cosenza H. Specific suppression of immune responses.Science.1973; 181(105): 1133-1141.

Table 1

In vitro Characterization of Antibodies

Antibody	Isotype	Conc.* mg/ml	Specific Virus- neutralizing activity†	
			IU/ml	IU/mg
SO57	IgG1	6.8	10,000	1470
SOJB	IgG3	7.4	6,000	811
HRIG	polyclonal	90	150	1.67

***** Protein concentrations of monoclonal and polyclonal preparations were calculated by Bradford method.

† Specific in vitro virus-neutralizing activity of antibodies was determined by RFFIT. The titer of the MAbs and HRIG was obtained by comparing with titer of WHO reference serum run along and expressed in IU/ml.

Table 2

Protective effect of MAbs and HRIG

Dose* IU/mouse	Survival			
	SO57	**SOJB**	**HRIG**	**Control†**
20	8/10	8/10	5/10	0/10
10	6/10	9/10	4/10	
5	3/10	3/10	2/10	
2.5	1/10	1/10	1/10	

* Groups of ten Swiss Webster mice were infected intranasally with $10LD_{50}$ CVS-N2C virus and 1 h later the groups were injected intra peritoneally with different doses of antibodies. The mice were observed for 5 weeks for appearance of clinical signs of rabies and survival was recorded.

† Control group was only infected, not given any of the antibody preparations.

Table 3

Interference of Antibodies on Vaccine- mediated Immunity

Treatment[a]	Dose IU/mouse	VNA titer[b]				
		Days after antibody treatment				
		2	**12**	**22**	**72**	**102**
Control	0	0	453	640	905	1416
SO57	5	67	226	226	380	554
	1	15	135	298	842	905
	0.2	0	57	113	460	538
	0.04	0	190	226	460	596
SOJB	5	40	149	269	842	1280
	1	10	160	269	596	905
	0.2	0	80	160	453	453
	0.04	0	190	269	842	905
HRIG	5	74	34	85	660	805
	1	14	10	180	460	640
	0.2	0	80	280	596	761
	0.04	0	110	160	905	1280

[a]Groups of ten Swiss Webster mice were treated intra peritoneally with different doses (5, 1, 0.2, 0.04 IU per mouse) of MAbs and HRIG and then vaccinated intra muscularly with 5µg SPBN-BPL vaccine per mouse, four times, on days-2, 12, 62 and 92 after antibody treatment.
[b]Mice were bled and serum was collected 2 days after antibody treatment and 10 days after each vaccination. VNA titers were determined as described in materials and methods.

Table 4

Dependence of interference level on concentration of the vaccine administered

Treatment* (Dose of vaccine in µg)	**Survival†**			
	SO57	**SOJB**	**HRIG**	**Control**
2	10/10	10/10	8/10	8/10
0.4	7/10	6/10	7/10	5/10
0.08	4/10	7/10	8/10	3/10
0.016	7/10	9/10	7/10	3/10

† Groups of ten Swiss Webster mice were injected intraperitoneally with 10 IU per mouse of SO57, SOJB and HRIG antibodies. Control group were mock injected with PBS. Survival was recorded in each group after treatment.

***** After 24 h of antibody administration each group was vaccinated intramuscularly with indicated doses of SPBN-BPL vaccine per mouse. Seven days after primary vaccination all groups were re-vaccinated with same dose as the primary. Seven days after secondary vaccine all groups were challenged intracerebrally (i.c) with 25 $MICLD_{50}$ of CVS-24 virus.

Table 5

Correlation between antibody-dependent interference and reduction of protective immunity

Treatment* (Days of vaccination)	Survival†			
	SO57	**SOJB**	**HRIG**	**Control**
0 and 7	10/10	9/10	9/10	9/10
7 and 14	9/10	9/10	7/10	10/10
14 and 21	9/10	9/10	9/10	10/10
21 and 28	9/10	9/10	9/10	9/10

† Groups of ten Swiss Webster mice were injected intraperitoneally with 10 IU per mouse of SO57, SOJB and HRIG antibodies. Control group were mock injected with PBS. Survival was recorded in each group after treatment.

***** All groups were given two vaccinations intramuscularly on indicated days with 2µg of SPBN-BPL vaccine per mouse. Seven days after secondary vaccine all groups were challenged intra cerebrally (i.c) with 25 MIC LD_{50} of CVS-24 virus.

Table 6

Passive immunity protects against intracerebral challenge

Group*	Treatment§	Survival†
SO57	Mock vaccination	7/8
SOJB	Mock vaccination	7/8
HRIG	Mock vaccinated	6/8
Control	Mock vaccinated	0/8
Vaccine	Vaccinated	8/8

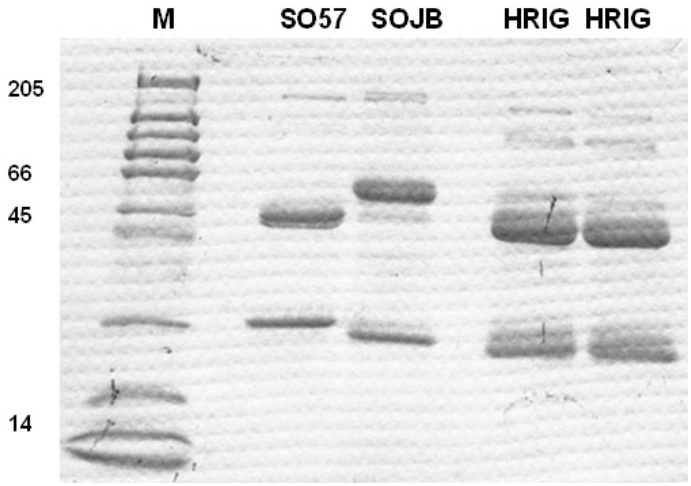
***** Groups of eight Swiss Webster mice were injected intra peritoneally with 10 IU per mouse of SO57, SOJB and HRIG antibodies. Vaccine and control groups were mock injected with PBS.

§ Vaccine group was given two doses of 2µg of SPBN-BPL vaccine per mouse, intra muscularly on 0 and 7 days of antibody treatment. All other groups were mock vaccinated intramuscularly with PBS on same days.

† Seven days after the second vaccine and or mock vaccine all groups were challenged intra cerebrally (i.c) with 25 MIC LD_{50} of CVS-24 virus and survival was recorded.

Figure.1

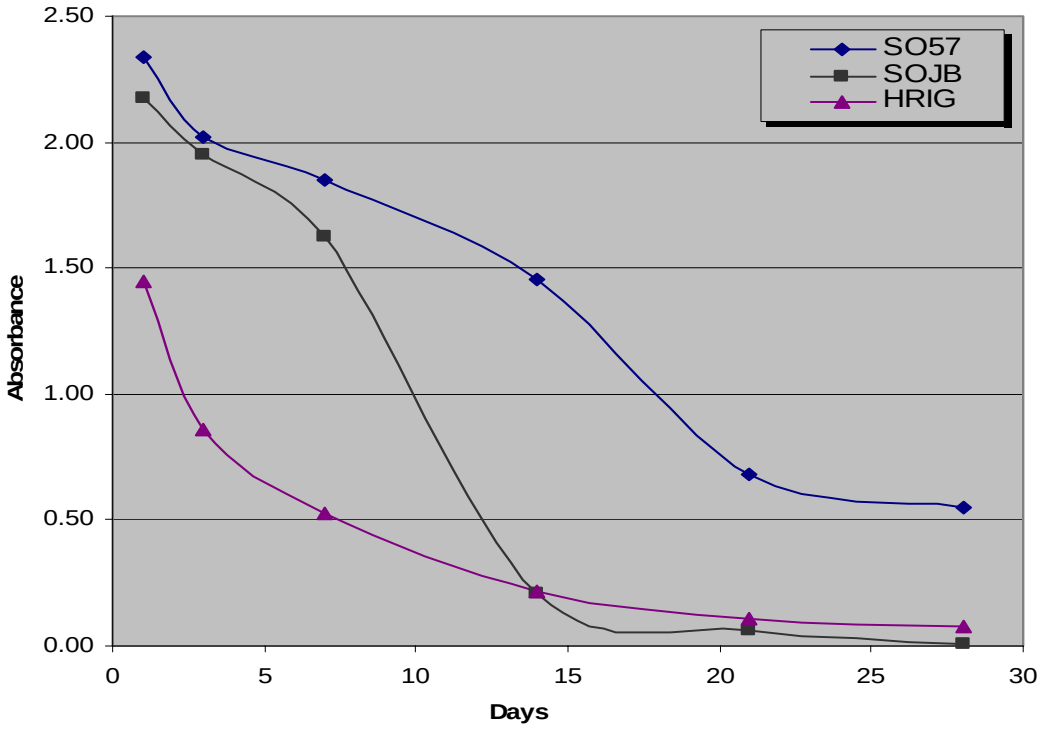
In vitro Characterization of Antibodies



Purity and molecular weight of antibody preparations (IgG) were checked by 10% SDS-PAGE. Molecular weight of IgG heavy chain is 53-70 KD where as light chain is ~25 KD.

Figure 2

Serum Half-life of MAbs and HRIG in mouse



Groups of ten Swiss Webster mice were injected intramuscularly with 50 IU per mouse of each antibody. Serum was collected from mice on 1, 3, 7, 14, 21and 28 days after antibody administration. Levels of antibody present in the serum were determined by ELISA as per the method described in materials and methods. Serum half-lives were found to be 16 days for SO57 (IgG1); 11 days for SOJB (IgG3), and 8 days for HRIG (polyclonal).

CHAPTER 4

CONCLUSIONS

Rabies continues to be a threat to humans apart from animals. Recent estimate says that annually world wide over 70,000 people die of rabies and 10 million people receive post exposure prophylaxis (**1**.WHO 2001; **2**. Ko *et.al*. 2003). To avoid any failures in rabies post exposure prophylaxis (PEP) and to maximize the efficacy of treatment, WHO guidelines recommends the simultaneous use of both vaccine and anti-rabies immunoglobulin (**3**.WHO 1992). Currently used anti-rabies immunoglobulin either from equine (ERIG) or human (HRIG) are associated with problems like being allergic, contaminated, inherently variable between batches and expensive. In the wake of monoclonal antibodies aimed for therapeutic purposes in recent times, a cost-effective and suitable alternative can be the production of rabies- specific human monoclonal antibodies.

Different panels of human anti-rabies monoclonal antibodies were produced, characterized and protective efficacies were tested by different researchers (**4**.Ukei *et.al*.1990, **5**.Dietzschold *et.al*.1990; **6**.Dorfman *et.al*.1994; **7**.Champion *et.al*.2000 and **8**.Hanlon *et.al*.2001; **9**.Morimoto *et.al*. 2001 and **10**.Prosniak *et.al*.2003). From their studies three monoclonal antibodies SO57, SOJB and SOJA proved to be useful for therapeutic purposes. But the protective effect of these monoclonal antibodies, in the possibility of interference, when simultaneously administered along with vaccine need to be given a serious thought. In the present study we evaluated overall prophylactic efficacy of SO57 and SOJB individually, in a mouse model when used in combination with rabies vaccine and compared with conventional HRIG.

Our in vivo studies of SO57 and SOJB in mice proved a protection level higher or comparable to that of HRIG. Our serum half-life estimation study proved SO57 (IgG1) has longer half-life than SOJB (IgG3) (**11**). The human monoclonal antibodies were shown to have different half -lives in heterologous environment like mice compared to the serum half-lives for respective subclass of antibodies.

The suppression of immune response to vaccine when specific antibodies were administered was evident from previous studies (**12**.Murphy et al.1989; **13.** Schumacher et al.1992; **14.** Wiktor et al.1971; **15**.Wiktor et al.1977). Similar to the previous studies the interference of antibodies on vaccine-induced immunity is evident from our studies as groups of mice received both antibody and vaccine developed VNA lower than control group.

In order to study more whether the interference on vaccine-induced immunity will seriously lower the protective efficacy of the antibodies, we administered both vaccine and antibody to mice, in different doses and with a different time gap, and then challenged mice intra cerebrally (i.c). We selected intracerebral route because as shown in previous studies for mouse monoclonal antibodies (**16**.Schumacher et al. 1989; **17.** Schumacher et al.1992), that only actively immunized, not passive immunized animals are protected against an i.c challenge. So i.c challenge is considered a way to differentiate between passive and active components of the immunity and the protection from i.c challenge is an indicator of vaccine-mediated (active) immunity. We did not find a direct effect of interference that could lower the protection level in the groups of mice that were administered both antibody and vaccine. In turn surprisingly a higher level of protection above the control group was observed. We thought that the reason for this observation could be partly because of the protection offered by passively administered

antibodies. A separate experiment by us, including all controls, proved that the human anti-rabies monoclonal antibodies (SO57, SOJB) and HRIG protected mice against i.c challenge. This observation is different from Schumacher et al (**16**; **17**) observations may be for difference in time gap between antibody administration and challenge and other unexplained reasons.

Overall our study showed that SO57 and SOJB have prophylactic efficacy comparable to that of HRIG and have potential to serve as an alternative to HRIG in rabies post exposure prophylaxis.

References:

1. World Health Organization. Rabies. Fact Sheet. 2001; 99: 1-3.

2. Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A. 2003; 100(13): 8013-8018.

3. World Health Organization. Expert Committee on rabies. Eighth report.WHO Tech Rep Ser 824. 1992; 1-84.

4. Ueki Y, Goldfarb IS, Harindranath N, Gore M, Koprowski H, Notkins AL, Casali P. Clonal analysis of a human antibody response. Quantitation of precursors of antibody-producing cells and generation and characterization of monoclonal IgM, IgG, and IgA to rabies virus. J Exp Med.1990; 171(1): 19-34.

5. Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, Koprowski H. Biological characterization of human monoclonal antibodies to rabies virus. J Virol.1990; 64(6): 3087-3090.

6. Dorfman N, Dietzschold B, Kajiyama W, Fu ZF, Koprowski H, Notkins AL. Development of human monoclonal antibodies to rabies. Hybridoma.1994;13(5): 397-402.

7.Champion JM, Kean RB, Rupprecht CE, Notkins AL, Koprowski H, Dietzschold B, Hooper DC. The development of monoclonal human rabies virus-neutralizing antibodies as a substitute

for pooled human immune globulin in the prophylactic treatment of rabies virus exposure. J Immunol Methods.2000; 235(1-2): 81-90.

8. Hanlon CA, DeMattos CA, DeMattos CC, Niezgoda M, Hooper DC, Koprowski H, Notkins A, Rupprecht CE. Experimental utility of rabies virus-neutralizing human monoclonal antibodies in post-exposure prophylaxis. Vaccine.2001; 19(28-29): 3834-3842.

9. Morimoto K, Schnell MJ, Pulmanausahakul R, McGettigan JP, Foley HD, Faber M, Hooper DC, Dietzschold B. High level expression of a human rabies virus-neutralizing monoclonal antibody by a rhabdovirus-based vector. J Immunol Methods.2001; 252(1-2): 199-206.

10. Prosniak M, Faber M, Hanlon CA, Rupprecht CE, Hooper DC, Dietzschold B. Development of a cocktail of recombinant-expressed human rabies virus-neutralizing monoclonal antibodies for postexposure prophylaxis of rabies. J Infect Dis.2003; 188(1): 53-56.

11. Chapter 6- Antibodies and their Receptors In: Riott I, Brostoff J and Male D editors, Immunology (5th ed.) Mosby International Ltd, London, UK, 1998: 71-82.

12. Murphy BR, Collins PL, Lawrence L, Zubak J, Chanock RM, Prince GA. Immunosuppression of the antibody response to respiratory syncytial virus (RSV) by pre-existing serum antibodies: partial prevention by topical infection of the respiratory tract with vaccinia virus-RSV recombinants. J Gen Virol.1989; 70(8): 2185-2190.

13. Schumacher CL, Ertl HC, Koprowski H, Dietzschold B. Inhibition of immune responses against rabies virus by monoclonal antibodies directed against rabies virus antigens.Vaccine.1992; 10(11): 754-760.

14.Wiktor TJ, Lerner RA, Koprowski H. Inhibitory effect of passive antibody on active immunity induced against rabies by vaccination. Bull World Health Organ. 1971; 45(6): 747-753.

15. Wiktor TJ, Doherty PC, Koprowski H. In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus. Proc Natl Acad Sci U S A. 1977; 74(1): 334-338.

16. Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H.Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest.1989; 84(3): 971-975.

17. Schumacher CL, Ertl HC, Koprowski H, Dietzschold B. Inhibition of immune responses against rabies virus by monoclonal antibodies directed against rabies virus antigens.Vaccine.1992; 10(11): 754-760.