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Firouzeh Fallahi
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Characterization of Epitopes on the Rabies Virus Glycoprotein by Selection and Analysis of Escape Mutants
TITRE DE LA THÈSE / TITLE OF THESIS

Alex Wandeler
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Ken Dimock

Ashok Kumar

Gary W. Slater
LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES /
DEAN OF THE FACULTY OF GRADUATE AND POSTDOCORAL STUDIES

CHARACTERIZATION OF EPITOPES ON THE RABIES VIRUS GLYCOPROTEIN BY SELECTION AND ANALYSIS OF ESCAPE MUTANTS

A thesis Submitted to the
School of Graduate Studies and Research

In Partial Fulfillment of the Requirements
For the Degree of
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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
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by

Firouzeh Fallahi

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ABSTRACT

Rabies virus, a negative sense single-stranded RNA virus, is the type member of the genus *Lyssavirus* of the *Rhabdoviridae*. The glycoprotein (G), which projects from the surface of the lyssavirus particle, is the only protein known to be capable of eliciting the production of neutralizing antibodies and knowledge of the antigenic nature of this protein is therefore important. Five different antigenic sites have been mapped on the G protein. In this study, the isolation of mutants resisting antibody neutralization (escape mutants) was attempted by a selection strategy employing three distinct strains of rabies: Evelyn Rokitnicki Abelseth (ERA), Big Brown Bat (BBB), and Silver Haired Bat (SHB). No escape mutants were generated from BBB and SHB but a total of seven independent ERA mutants were recovered using monoclonal antibodies (Mabs) directed against antigenic sites I and IIIa of the glycoprotein. Antigenic site I mutants, C12 and C8 were isolated using Mab 10EC9 while mutants B9 and G6 were recovered using Mab M725. Antigenic site IIIa mutants, C3 and A11 were isolated using Mab 16AH8 while mutant G10 was isolated using Mab M785. All ERA mutant preparations were more cytopathogenic than the parental virus when propagated in cell culture, an observation that could not be attributed to the presence of adventitious bacterial or viral agents. The cross-neutralization patterns of these mutants, as well as two additional mutants derived from the Western Canada skunk strain (isolated by 10EC9 and 16AH8 in the CFIA laboratory in Lethbridge, Alberta) were used to confirm the location/nature of the G protein epitopes recognized by these Mabs. Nucleotide sequencing of the G gene indicated that those mutants derived using Mabs directed to antigenic site III all contained amino acid substitutions in this site. However, of the four mutants selected with antigenic site I Mabs, two bore mutations within antigenic site II while the remaining two

carried mutations in antigenic site I as expected. Western skunk mutants exhibited mutations at the sites appropriate for the Mabs used in their selection. All the ERA mutant viruses were titrated in mice and their pathogenicity was determined. Three of the ERA escape mutants i.e. C12, C8, and B9 showed a reduction in pathogenicity while the pathogenic properties of all other mutants were comparable to those of the parent virus.

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LIST OF ABBREVIATIONS

BBB	Big Brown Bat
BEFV	Bovine Ephemeral Fever Virus
cDNA	Complementary Deoxyribonucleic Acid
cELISA	Competitive ELISA
CNS	Central Nervous System
CPE	Cytopathic Effect
CTL	Cytotoxic T cell
CVS	Challenge Virus Standard
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERA	Evelyn Rokitnicki Abelseth
FBS	Fetal Bovine Serum
FITC	Flourescein-5-isothiocyanate
G Protein	Glycoprotein
G_s	Soluble G protein
HDCV	Human Diploid cell rabies Vaccine
L Protein	Polymerase Protein
LD50	Lethal Dose
M Protein	Matrix Protein

Mab	Monoclonal Antibody
MEM	Minimum Essential Medium
MNA Cells	Mouse Neuroblastoma Cells
MOI	Multiplicity of Infection
N Protein	Nucleoprotein
nAchR	Nicotinic Acetylcholine Receptor
NCAM	Neuronal Cell Adhesion Molecule
P Protein	Phosphoprotein
PCECV	Purified Chick Embryo cell rabies Vaccine
PCR	Polymerase Chain Reaction
PV	Pasteur Virus
PVRV	Purified Vero Cell rabies vaccine
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RT	Reverse Transcriptase
RT-PCR	Reverse Transciption Polymerase Chain Reaction
SAD	Street Alabama Dufferin
SAG	SAD Attenuee Gif
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHB	Silver Haired Bat
TCID50	Tissue Culture Infectious Dose 50%
VNA	Virus Neutralizing Antibody
VSV	Vesicular Stomatitis Virus

WHO	World Health Organization
WSk	Western Skunk

INTRODUCTION

Rabies is one of the oldest recognized infectious diseases. Despite the historical pre-eminence of smallpox, influenza, and, more recently, AIDS, rabies continues to be known as one of the most notorious infectious diseases because of its inevitable progression into a fatal disease (23) in the absence of post-exposure prophylaxis. Rabies is an acute viral encephalomyelitis that principally affects carnivores and chiroptera, although it can infect the central nervous system (CNS) in any mammal. The disease is caused by a neurotropic virus, which is almost always transmitted through introduction of virus-laden saliva into the tissue, usually by the bite of a clinically rabid animal (134).

Rabies continues to be a worldwide problem. The situation of rabies in the world is constantly evolving and differs greatly from one continent to another. The World Health Organization (WHO) reports over 50,000 human rabies deaths annually (138). Virtually all are caused by dog bites and occur in developing countries where modern post-exposure treatment is often not available or affordable; the dog is the most important vector of rabies in such countries. In contrast, the main reservoirs of the rabies virus in North America are wildlife species.

The indigenous rabies virus strains circulating within Canada are strains of terrestrial hosts. These include: the Western skunk strain (*Mephitis mephitis* host), with a range from Manitoba to Alberta; the Arctic rabies strain [arctic fox (*Alopex lagopus)* and red fox (*Vulpes vulpes*) hosts], which persists to a limited degree in Ontario and extensively in northern regions of the country; and the raccoon strain (*Procyon lotor* host), a recent incursion from the United States which entered Ontario in 1999 (132) and New Brunswick

in 2000. In addition, several rabies virus strains, characterized in detail (97), circulate in particular species of insectivorous bats, including big brown bats (*Eptesicus fuscus*); various *Myotis* species, including the little brown bat (*Myotis lucifugus*); members of the *Lasiurus* genus, including red and hoary bats; and silver-haired bats (*Lasionycteris noctivagans*).

Pasteur's research on rabies is perhaps the most well known historical achievement in the field. In the 1880s he described the involvement of the CNS and salivary glands in the disease, achieved attenuation of the virus by animal passage, and developed the theoretical and practical basis of immunizing injections. Desiccated spinal cords from rabies-infected rabbits became the first rabies vaccine, and they were supposedly safe, although now it is known that the fixed viruses from which the vaccines were derived were not completely apathogenic. In 1885 he reported the landmark first human post-exposure vaccination, and by 1896 he had reported on the treatment of 350 exposed persons, of whom only one developed rabies (10, 23).

Whole killed rabies virus particles are highly immunogenic and form the basis for the vaccines currently used for pre- and post-exposure prophylaxis of rabies in human [e.g. human diploid cell rabies vaccine (HDCV), purified chick embryo cell rabies vaccine (PCECV), purified Vero cell rabies vaccine (PVRV)] and immunization of domestic animals. Live attenuated rabies viruses, which are also highly immunogenic, are used in vaccination of wildlife (ERA and the closely related SAD-19, SAG-2). In particular, the human tissue culture rabies vaccines have an excellent safety record (53), are highly effective in post exposure prophylaxis (53), and include long lasting immunity (120). The protective activity of rabies virus-based vaccines usually correlates with virus neutralizing antibody (VNA) titres, suggesting the importance of such antibodies in immune defence

against rabies virus. Although the major rabies virus antigen responsible for the induction of VNA is the glycoprotein (G) (22), efforts to develop G subunit vaccines have not been very successful. In that context, it was shown that immunization with soluble G (G_s), which contains the intact antigenic domain but lacks the cytoplasmic domain of G, completely failed to confer protective immunity (31). It was proposed that the disparate immunogenicities between intact virus and G_s may be due to differences in the presentation of antigenic sites by either isolated, monomeric G or by virus-associated, multimeric G. In contrast to the monomeric G_s, the antigenic sites of virus-associated G (or G inserted into cell membranes) are presented in a repetitive rigid form, which is known to stimulate B-cells efficiently (2, 152). It has also been suggested that the tip of the virus associated G protein, which is the target for VNA, is the only site exposed on the surface, whereas isolated G exposes sites that are normally buried and not accessible in the intact virus (152). The latter sites will not only compete for antibody production but will also induce non-neutralizing antibodies that are ineffective against rabies virus.

Presently, killed intact rabies virus particles are the safest and most efficacious antigen suitable for immune prophylaxis of rabies, and killed rabies virus vaccines such as HDCV remain the gold standard. The development of better and more cost effective vaccines and antiviral therapeutics remains a major goal for the treatment of human rabies. Moreover, there continues to be a need to develop efficacious vaccines for the control of animal rabies, and particularly for the eradication of the disease in rabies virus reservoirs. Several strategies are currently being investigated, including the use of naked plasmid DNAs that contain sequences encoding rabies virus G protein (3, 87, 101), recombinant viruses that express rabies virus G proteins [e.g. vaccinia virus vector (V-RG), adenovirus vectors] (11,

133, 141), and the use of recombinantly expressed anti-rabies monoclonal antibodies (94). A more extensive knowledge of the antigenic nature of the G protein would facilitate development of more effective rabies vaccines.

1.1 Taxonomy of the rabies virus

Rabies virus is the prototype species of the genus *Lyssavirus*, family *Rhabdoviridae*, and order Mononegavirales (146). The other members of this order are *Filoviridae* and *Paramyxoviridae*. All members of this order have a linear, non-segmented RNA genome of negative polarity surrounded by a helical capsid. The members of the *Rhabdoviridae* family are the simplest of all the RNA viruses classified in the Mononegavirales order and possess a widespread distribution. Members of this family infect vertebrates, invertebrates, as well as some plant species (108).

The *Rhabdoviridae* family is subdivided into six genera including the Vesiculoviruses, Lyssaviruses, and Ephemeroviruses, which infect animals, Novirhabdoviruses that infect aquatic hosts, and the Cytorhabdoviruses and Nucleorhabdoviruses, which infect plants. The type species of the animal rhabdovirus genera are vesicular stomatitis virus (VSV), rabies virus, and bovine ephemeral fever virus (BEFV) respectively.

Prior to the use of monoclonal antibodies and other molecular techniques, all rabies viruses were thought to be the same serologically. Historically, placement of a species as a rabies or rabies-related virus was determined by recognition of antigenic sites of the G protein via virus neutralization tests. The use of more extensive monoclonal antibody (Mab) panels (70) and molecular biology techniques such as gene sequencing (7-9, 125), have allowed a more precise characterization of Lyssaviruses. Phylogenetic analysis of the

nucleoprotein (N), phosphoprotein (P) and the G protein genes has to date clearly delineated seven Lyssavirus genotypes which comprise genotype-1 (classical rabies virus) and six other genotypes (genotypes 2-7) of viruses that are closely related antigenically and genetically and that cause a clinical disease indistinguishable from rabies. Genotype-1 includes the majority of field viruses of global distribution in terrestrial mammals and insectivorous and hematophagous bats of the Western hemisphere, as well as the laboratory and vaccine strains (137). The distribution of non-rabies viruses (genotypes 2-7) is restricted to the old world. They have a narrower geographic distribution than rabies and, although they occasionally infect humans and domestic animals, they seem to preferentially infect specific host species (123). They include genotype 2, Lagos bat virus (isolated from African bats); genotype 3, Mokola virus (isolated from African terrestrial mammals); genotype 4, Duvenhage virus (isolated from African bats); genotypes 5 and 6, European bat lyssaviruses 1 and 2 respectively (isolated from European bats) (123); and genotype 7, Australian bat lyssavirus (isolated from Australian flying foxes) (55). Antigenic and genetic analysis revealed that the Australian bat lyssavirus is closely related to, but distinct from, rabies virus (genotype 1). The non-rabies lyssaviruses are of particular interest since classical rabies vaccines may fail to protect animals against exposure to some of them (73, 80), especially the more divergent Lagos bat and Mokola viruses. Lyssaviruses are serologically distinct from other rhabdoviruses (110).

1.2 Structure of the virus: morphology and physical properties

The rabies virion is a rigid, bullet-shaped, enveloped particle, with one flat end and one rounded end, that measures 75-80 nm in diameter and 180-200 nm in length (59, 130). The virion particle is composed of two structural units: a central cylinder and a lipoprotein

membrane. The central cylinder is composed of a tightly coiled ribonucleoprotein (RNP) with helical symmetry which consists of the single-stranded, non-segmented negative-sense genome RNA encapsidated with N, polymerase (L), and P proteins. The RNP is contained within the second unit, the lipoprotein membrane, generated by the cell membrane during budding, and through which protrudes an array of knobbed G protein spikes 10 nm in length (124) (refer to Fig.1.1).

The virus infectivity is stable in the pH range of 5 to 10, but it is rapidly inactivated at 58°C, by ultraviolet (UV) or x-ray irradiation, or by exposure to lipid solvents and oxidizing agents (23).

The rabies virus RNA genome has a molecular weight of 4.6×10^6 Daltons (112), and is approximately 12,000 nucleotides in length (122). From the 3′ to 5′ end, the viral RNA genome is organized into five genes encoding the N protein, P protein, matrix protein (M), G protein, and L protein (122) (refer to Fig.1.1).

1.3 Pathogenesis

Although all mammals are susceptible to rabies virus infection, only a few species are important in maintaining the disease in nature. With very rare exceptions, rabies virus infection terminates in the death of infected individuals. Rabies viruses have evolved a pathogenesis, both within the individual animal and through the process of animal to animal transmission that facilitates the maintenance of a particular variant in a certain reservoir population (95).

There are a number of sequential steps that occur after peripheral inoculation of rabies virus via an animal bite, which is the most common mechanism of transmission. The steps include replication in peripheral tissues, spread along peripheral nerves and the spinal

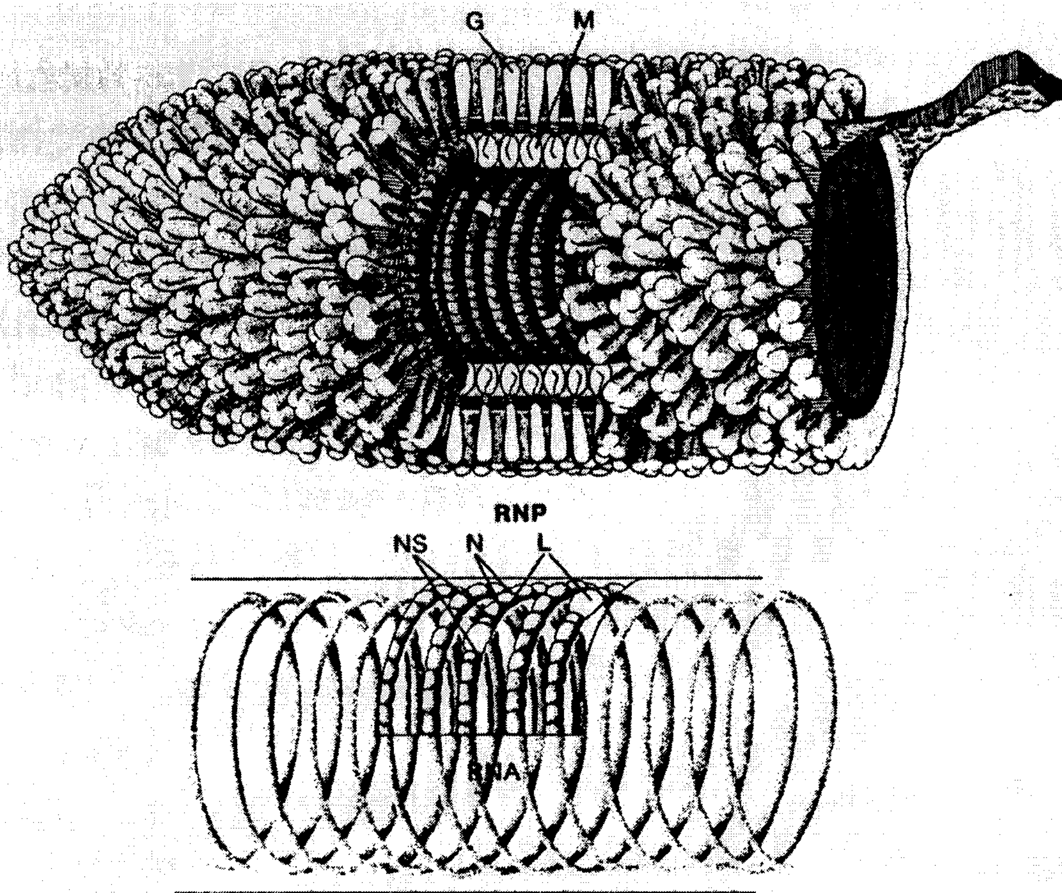

Fig.1.1. Schematic representation of the rabies virion showing the RNP core consisting of single-strand, negative-sense genome RNA encapsidated with N, L, and P proteins. The RNP core, in association with M protein, is condensed into the typical bullet-shaped particle. The RNP-M structure is surrounded by a lipid bi-layer envelope in which the surface trimeric G protein spikes are anchored. (Reproduced from Wunner, W. H., et al (148), used with permission from the University of Chicago Press; refer to Appendix)

cord to brain, distribution within the CNS, and centrifugal spread along nerves to various organs, including the salivary glands (refer to Fig.1.2.). Rabies virus does not generally cause host cell destruction. After the entry of RNP into the cytoplasm of infected cells the viral RNA polymerase must first transcribe the viral RNA to produce positive sense monocistronic mRNA transcripts which are then translated into viral proteins (122). The viral RNA is then replicated, using the newly synthesized N, P, and L proteins, via the formation of positive sense replicative intermediate nucleocapsids. Following replication, further rounds of transcription (secondary transcription) ensue (146). The virus is released by budding through cell membranes (95, 108) and the viral infection can then spread from cell to cell.

When a host animal becomes infected following exposure from the bite of an infected animal, the virus may invade peripheral nerves or nerve endings directly or may first be "amplified" by invasion of striated muscle cells prior to infection of the nerve endings (15, 96). The variable and sometimes long incubation periods noted with this disease may be due to restricted viral replication in the muscle cells before viral entry into the nervous system (134). The early events of viral replication and muscle and nerve cell infection at the site of exposure occur without substantial stimulation of the immune system (23). The virions are highly neurotropic, and once they reach sufficient concentration, they infect motor and sensory nerves by binding to cell receptors, a process mediated by the viral G protein. The nature of the rabies virus receptor may vary with cell type. The putative receptor in muscle cells is the nicotinic acetylcholine receptor (nAchR) (83), but in neurons, other components such as membrane oligosaccharides and lipoproteins may also serve as

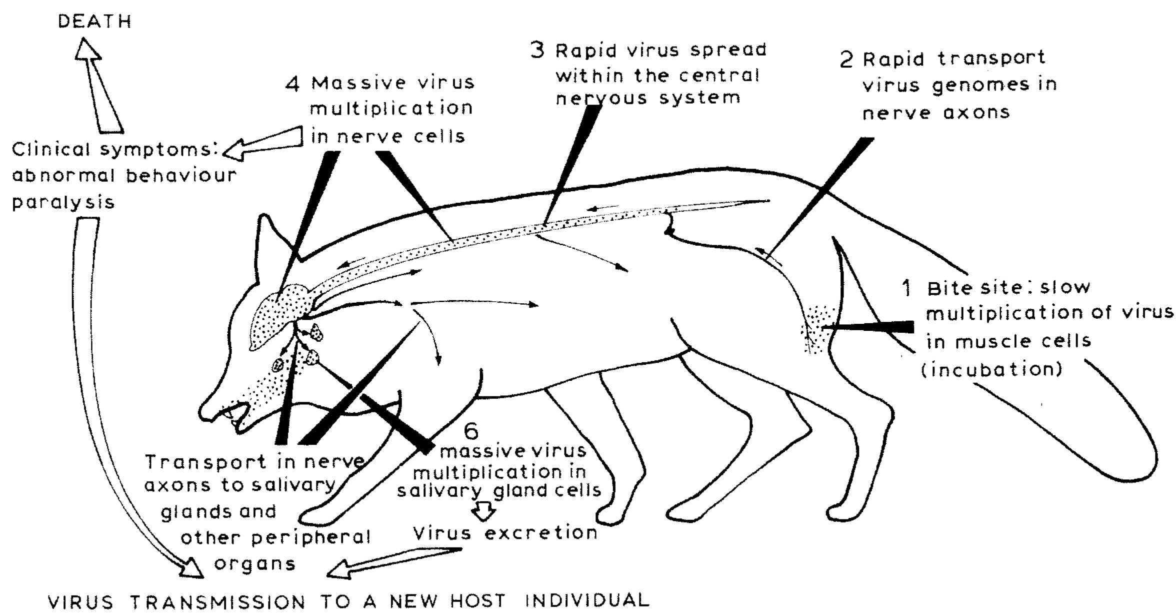

Fig.1.2. Sequential events in rabies pathogenesis. (Reproduced from Wandeler A. I. (131), used with permission from publisher and the author; refer to Appendix.)

rabies virus receptors (127) (see section 1.6.5). The virions enter the cells by endocytosis and are uncoated, releasing nucleocapsid into the cytoplasm where viral replication occurs. The newly replicated virus is assembled on the intracytoplasmic membranes, and maturation involves budding and release from the plasma membranes (108). Although rabies proteins are highly immunogenic, and capable of eliciting a full spectrum of protective immune responses, the virus is not highly cytopathogenic and no cell lysis occurs during replication and maturation. Thus, in the absence of cell lysis, little antigen is presented to the immune system. Neither humoral nor cell-mediated immune responses can be detected during the early stages of movement of virus from the site of the bite to CNS (135).

After entering sensory and motor nerve endings, the rabies genome progresses centripetally transneuronally, only by retrograde axoplasmic flow to the CNS (23, 134). Thereafter, massive replication occurs mainly in neurons, with extensive distribution in the brain and spinal cord. Recognizable clinical signs of rabies generally do not appear until several replication cycles have occurred in the brain (64). From the CNS, the virions spread centrifugally to peripheral nerves and to some non-nervous tissues, including, most importantly, salivary glands where they bud from the plasma membranes of the mucous cells and are released in high concentrations into the saliva, thereby making this secretion highly infectious (23). Although the presence of an infectious dose of virus in the saliva of host vectors is critical for the maintenance of the virus in host populations, virus titres in saliva may be sporadic during and just prior to the clinical period (39).

Maintenance of the virus in the reservoir population by direct host-to-host transmission is dependant on co-incident infection of the brain and salivary glands. The infection of the limbic system induces biting behaviour, and infection of salivary glands

results in the generation of large numbers of viral particles in the saliva that serves as an infectious inoculum for bite transmission (64, 134, 135).

1.4 Disease

The classical rabies pathogenesis described above results in a variable, but long incubation period, which typically is 2-12 weeks, but could be longer. The incubation period is followed by an acute, undelayed progression of encephalitis, culminating in death. The range in length of illness in most species is 1-10 days. Most of the clinical signs of rabies are expressions of neurological dysfunctions which include disorientation, hallucination, nuchal rigidity, aerophobia, pharyngeal spasm, hydrophobia, hypersalivation, dysphagia, focal or generalized seizures, cardiac and respiratory arrhythmias, hypertension and paralysis, leading to coma and death (91). The clinical syndrome has been classified either as dumb (paralytic) or furious (encephalitic) according to the predominance of paralysis or of excitability and biting (123).

1.5 Immune response

The immune responses triggered during rabies virus infection are limited because of the immunoprivileged nature of the nervous system (5), within which T cell migration is restricted and professional antigen presenting cells (APCs) are lacking. In addition, pathogenic strains of rabies virus trigger an immunosuppressive state (78). As a result, host immune response to rabies virus infection is very limited, a reflection of the successful adaptation of the rabies virus to the host.

Virus-neutralizing antibodies (VNA) are important for immune resistance. However, VNA do not become detectable until late in infection when virus has multiplied in the brain and is released, usually in the terminal days of infection. If survival is prolonged, either

naturally or by intensive therapy, VNA in blood, brain, and cerebrospinal fluid (CSF) may reach high titres. This is related to the disappearance of infectious virus, but seldom affects the lethal outcome of the disease (123).

Infection with rabies virus results in the generation of a virus-specific T cell response. The G protein is the only viral antigen that induces VNA (22). Antibody production is T cell dependent and requires CD4+ T cells as well as B cells. These antibodies might be effective in the early stages of infection, before virus entry into peripheral nerves. There is evidence that IgG plays a more important role in protection compared to IgM (77). It has been shown that RNP complex is capable of inducing a virus-specific antibody response, and antibodies directed against RNP can contribute to protection against infection (86, 123). Cytotoxic T cells (CTL) can be induced by rabies G protein, nucleocapsid, N protein, and P protein (77). However, the role of CD8+ T cells in immune defence is unclear. Some studies report clearance of rabies virus after transfer of rabies virus-specific T cells and protection against rabies by a CTL clone, whereas other investigations showed that CTLs are insufficient to protect against challenge, and *in vivo* deletion of CD8+ T cells had no effect on host resistance (68, 102). Besides, CTLs may also be involved in immunopathology and have been implicated in neuritic paralysis (114). By comparison, the induction of CD4+ T cells is an integral part of the protective immune response to rabies (26).

1.5 Rabies virus proteins: structure and function

Three of the viral proteins (N, P, and L proteins) are located in the RNP core. All of these proteins are essential for the RNA polymerase activity of the virion. Both N and P are phosphorylated in rabies virus, unlike other rhabdoviruses, including VSV, in which only P

protein is phosphorylated (23, 111). The remaining two structural proteins of rabies virion, the G and M, are associated with the lipid bi-layer that surrounds the RNP core. The M protein lines the viral envelope, forming an inner leaflet between the envelope and RNP core, whereas the G protein produces the spike-like projections or peplomers on the surface of the viral envelope (143).

1.6.1 Nucleoprotein (N)

The 450 residue N protein is the major protein component of the viral RNP complex (143). The N protein is the most highly conserved of all rabies proteins in terms of amino acid sequence similarity between genotypes. The sequence of this protein, as deduced from the nucleotide sequence of several vaccine isolates of rabies virus (38, 126), shows high levels (98-99.6%) of amino acid conservation among different strains (44). Greater diversity is seen in the N gene of field isolates of genotype-1 (rabies) and between the N genes of other Lyssavirus genotypes (71). The rabies virus N protein is phosphorylated (111) at a serine residue at position 389 (67).

Tightly bound to the viral RNA (113), the N protein protects the RNA from ribonucleases and keeps the RNA in a suitable configuration for transcription and replication (143): indeed, encapsidation of the genome and antigenome occurs concomitantly with, and is essential for, RNA elongation (150). It has been proposed that binding of N protein to the 5′ proximal (encapsidation) sequence of nascent positive- or negative-sense viral RNA species prevents transcription of the negative-sense RNA and, by progressive addition of N, promotes replication of the positive-sense RNA including read through of transcription termination signals (100, 150, 151). Thus, the switch from transcription to replication of the rhabdovirus genome is controlled by the amount of N present in the infected cells (123). At

low levels of N protein, the polymerase stops at the end of the leader RNA and immediately reinitiates transcription at the start signal of the first mRNA, preventing encapsidation. As cellular levels of N protein increase, the simultaneous encapsidation prevents the polymerase from stopping at the end of the leader RNA and replication of the full length, encapsidated RNA takes place (123). Rabies RNP can prime T cells, elicit N-protein specific antibodies, and enhance immune responses (56), thereby protecting animals against a peripheral challenge with rabies virus in the absence of detectable VNA (88, 115, 121). The N protein has been shown to prime the immune system and enhance the production of VNAs following subsequent inoculation of animals with inactivated rabies vaccine (30, 43, 121). Antigenic sites on N protein have been examined extensively by enzyme-linked immunosorbent assay (ELISA) (81). B cell epitopes were further mapped by use of synthetic peptides, resulting in the identification of two linear epitopes (28). The rabies virus N is also a major target antigen for T helper cells (13, 14, 28, 38). T cell epitopes on N protein were identified and mapped using a series of overlapping synthetic peptides (38).

1.6.2 Polymerase (L)

The 2142 amino acid L protein is the largest lyssavirus protein, as the L gene accounts for 54% of the genome, yet it is the least abundant protein in the virion (122). L, the catalytic component of the polymerase complex, is an RNA-dependant-RNA polymerase that functions with its non-catalytic cofactor, the P protein (37). Together with the N protein, these core proteins interact with the genome to form the RNP complex.

L protein possesses all of the virion's enzymatic activities: RNA synthesis (24); mRNA capping and methylation (57); and mRNA polyadenylation (60, 61), but because it is present in minute amounts it is the least well studied lyssavirus protein.

1.6.3 Phosphoprotein (P, M_1, NS)

The 297 amino acid P protein is part of the RNP complex. It is a highly phosphorylated protein (143). The P gene is less well conserved than other lyssavirus genes (98). Historically, this protein was called M_1 protein because it was believed that it was a membrane protein (144). However, once it was discovered that it was part of the RNP complex, it was called the NS (non-structural) protein (21). Due to its highly phosphorylated nature it is now referred to as P protein.

The function of P in rabies virus, while not fully understood, appears to be multifaceted. P protein acts as a cofactor for viral replicase activity by interacting with the L gene product (143). Specific binding of P gene product to the N protein has also been documented (16, 45). P protein acts as a chaperone of soluble nascent N, preventing its polymerization (self-assembly) and non-specific binding to cellular RNA. The P in N-P complexes specifically directs N encapsidation of the viral RNA (16, 51). Therefore it maintains N in a competent form for RNA encapsidation. In addition, the P protein binds to LC8 dynein chain of the host cell (63, 106). Dynein LC8, as a part of cytoplasmic dynein and myosin V, participates in the myosin V complex, which is involved in a wide range of intracellular motile events. The significance of this interaction is yet to be fully determined, but it is speculated that dynein may be involved in the axonal transport of rabies virus components along the microtubules of neurons (63, 106).

1.6.4 Matrix protein (M)

The M protein is the smallest of the structural proteins (202 amino acid) (143). It accounts for 25% of the total rabies virion protein and like the P protein, appears to have multiple roles.

1. Structural: The M protein forms a sheath around the RNP core in virion assembly, producing the skeletal structure of the virion. Studies have suggested that M protein might play a role in anchoring the membrane bound G protein to the internal RNP capsid (25), and condensation of helical nucleocapsid cores into tight coils. More recent studies have revealed that M protein extends from the inner layer of the viral membrane to the internal core of the RNP complex (4).

2. Regulatory: M protein can affect the level of cytopathogenesis in virus infected cells (62), possibly via interaction with some cellular components involved in the apoptotic pathway, and it can also down regulate viral RNA transcription (62).

1.6.5 Glycoprotein (G)

The G protein is the only surface protein of the virion and is the best studied of all Lyssavirus proteins (6, 29, 66). The spike-like peplomers that cover the outside surface of the rabies virus membrane are composed of homopolymers of G protein molecules (143). Electron microscopy and sedimentation analysis have shown that the surface glycoprotein forms homotrimeric spikes extending from the virus surface (47).

The rabies virus G protein gene encodes a 524 open reading frame translated from a gene of 1675 nucleotides (126, 143). However, the mature G protein is a 505 amino acid, type I transmembrane protein, because 19 amino acids at the amino-terminal end are a signal sequence. This sequence provides the membrane insertion signal, which transports the nascent protein into the membrane of the rough endoplasmic reticulum (ER)-Golgi-plasma membrane pathway before it is cleaved from the G molecule (122). Each G protein of the spike is anchored in the viral envelope by a 22-amino-acid transmembrane domain located between residues 439 and 461 (47). The transmembrane domain remains anchored in the

membrane due to palmitoylation of a cysteine residue at position 461 (48). The C-terminal portion of G (44 amino acids), constituting the cytoplasmic domain, interacts with the M protein of the skeleton particle to complete the virion assembly. The hydrophilic G protein ectodomain (439 amino acids), extends outward from the virion (144) and is responsible for mediating rabies virus interaction with cellular binding sites (receptors) and is therefore important in viral pathogenesis. It is also critical to the host immune response to rabies virus infection because it is responsible for the induction of VNAs, and is a target for specific helper and cytotoxic T cells (6, 29).

Lyssavirus G proteins have three or four potential N-glycosylation sites depending on the viral strain (1, 93, 126). Glycosylation occurs at asparagine residues, but only two of these sites (247 and 319) are glycosylated in mature rabies virions (147). N-glycosylation at site 319 may be functionally important because it is present in all lyssaviruses sequenced to date (122). As observed for other viruses, N-glycosylation of the rabies virus G is required for intracellular transport. Core glycosylation and palmitoylation usually occur during transport from the rough ER to the Golgi apparatus and the cytoplasmic membrane. The transmembrane and cytoplasmic regions of G protein are not necessary for proper glycosylation to occur (142). No O-linked glycosylation has been identified on the G protein.

The rabies virus G protein plays many important roles in the pathogenesis of rabies. It is the only viral protein that can elicit neutralizing antibodies (22), which relates to its role in binding to cellular receptors (119, 128). The involvement of G protein in virus attachment to specific receptors on the cells is important in determining the tissue tropism of the virus (18, 76). The nature of the cellular receptors employed by the rabies virus is not clearly understood. The nAchR was the first molecule proposed to mediate rabies virus binding

(83). Sequence homology between G protein and snake toxins (α-bungaro toxin and D-tubercurain) that bind to nAchR suggested that these molecules contain a common binding domain (85). This specific interaction (between nAchR and G protein) is inhibited either by natural ligands or by a Mab directed against the receptor (47, 84). However, the observation that many cell culture systems, some of which lack the nAchR, are fully susceptible to rabies virus infection, suggests that rabies virus can employ receptors other than the nAchR (107). Other cellular components, including phospholipids and glycolipids (149), and gangliosides (117), have been suggested as receptor molecules.

The neuronal cell adhesion molecule (NCAM), which is expressed on the surface of many different types of cells (neurons, lymphocytes, and fibroblasts), may serve as a receptor for laboratory strains of rabies virus. It was reported that NCAM is present on all cell lines susceptible to rabies virus infection but not on the surface of resistant cell lines (119). In addition, a neurotrophic receptor (p75NTR), a low affinity nerve-growth factor receptor that is abundantly synthesized in neuronal and non-neuronal tissues of young animals, and which is present at synapses in several categories of neurons, has been proposed to act as a specific neuronal receptor for rabies virus (128).

Subsequent to cellular binding, the virus particle is internalized by endocytosis and released to the cytoplasm following low pH induced membrane fusion in the endosome (50, 92). The G protein mediates this membrane fusion, even in the absence of other structural proteins (136). The rabies virus glycoprotein adopts three different conformations depending on the pH of its surrounding milieu (50, 116). At neutral pH, the G protein does not fuse membranes, but as pH is lowered, two conformational changes occur. The first, at pH 6.4, is responsible for hemagglutination and tight binding of the virus to membranes. The second

change, at pH values less than 6, is responsible for membrane fusion. Increasing the pH can reverse these conformational changes (46). After entry, the G assumes a reversible fusion-inactive conformation (46, 47). The biological function of the fusion-inactive state of G protein appears to be to prevent non-specific fusion during transport of the G protein through the acidic environment of Golgi apparatus (49), after which the G protein acquires its native surface conformation.

1.7 Antigenticity of rabies G protein

The first detailed study on antigenic variation between several rabies and rabies-related viruses performed by Wiktor and Koprowski (139), employed a collection of Mabs that differentiated these viruses (42). The twenty five Mabs available at that time were classified into 14 groups that identified distinct antigenic determinants on the G protein. Eight different lyssaviruses could be differentiated with these Mabs. As more Mabs became available, limited epitope mapping of rabies virus G protein could be undertaken. Using 25 Mabs and 90 monoclonal antibody resistant (Mar) mutants of the Challenge Virus Standard (CVS) strain of rabies virus (140), three antigenic sites were topographically mapped on the CVS G protein (82). Later, using the same approach, five antigenic sites were mapped on the Evelyn Rokitnicki Abelseth (ERA) strain of rabies virus G protein (79). These sites were all found to be conformational in nature and require G protein secondary structure for Mab recognition (149).

Sequencing of Mar mutants has identified the location of most of these antigenic sites with respect to G protein primary structure. Site I, recognized by a single monoclonal antibody, is located in the region of residue 231 (147). Antigenic site II is a discontinuous site that involves two separate stretches of amino acids that are brought into close proximity

by folding of the G protein. Site II Mar mutants map to amino acids 34-42 and 198-200 (145) which are linked by a disulfide bridge between cysteines 35 and 207. Site III maps to amino acids 330-338 (145). Sites IV and V have not been definitively mapped (122).

A sixth, linear antigenic site was found using Mabs produced against the Pitman-Moore (PM) strain of rabies virus (12). Antigenic site VI maps in the region of residue 264 (27). The discovery of this and other linear epitopes has suggested the possible use of G peptide(s) as a rabies vaccine (27).

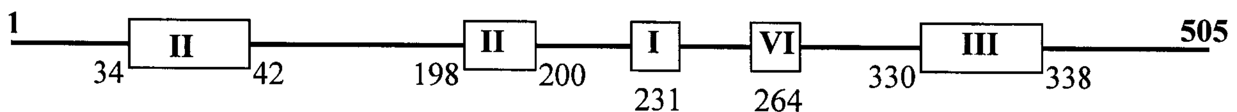

Fig.1.3. Location of the antigenic sites on the rabies virus G protein. The solid line shows the open reading frame of the G protein sequence. The boxed areas represent the position of each antigenic site on the G protein.

As more epitopes and antigenic sites were discovered on lyssaviruses, it was suggested that a new system of nomenclature was needed (6). Since 97% of anti-G monoclonal antibodies tested bound to antigenic sites II and III (79), it was proposed that the term antigenic site be reserved for sites II and III. Other groups of overlapping epitopes would be referred to as minor antigenic sites, and regions of G protein that were recognized by single Mabs would be classified as epitopes (6). This last category of sites would include

those that were previously referred to as antigenic sites I, IV, V, VI. The first minor antigenic site, including six overlapping epitopes, was defined as minor antigenic site "a" at amino acids 342-343 (6). Two more linear minor antigenic sites at amino acids 251 and 264 were defined (75). The site at amino acid 264 includes the former site VI. A later report described a linear epitope at position 263 (99).

Antigenic site III is critical for rabies virus pathogenicity. Mar mutants of rabies (CVS and ERA strain) virus isolated using Mabs directed to site III determinants were avirulent (20, 32). Analyses of tryptic peptides of rabies virus G protein from pathogenic and non-pathogenic viruses were compared and the residue responsible for virulence was found to be amino acid 333 (20, 32). An arginine at 333 is essential for the integrity of this epitope of antigenic site III and for the ability of rabies virus to cause lethal infection of adult mice.

In studies performed previously in our laboratory (36), several Mabs directed to G protein were used in a competitive enzyme-linked immunosorbent assay (cELISA) to reevaluate the antigenic properties of the lyssavirus G protein using representatives of six lyssavirus genotypes. This study identified four major antigenic sites and two subsites (Ia and IIIa) that were for the most part conserved among the G proteins of all the lyssavirus isolates used. This study was successful in identifying thirteen epitopes defining different antigenic sites. Seven of these epitopes appeared to be conserved in all lyssavirus genotypes. Two of the thirteen epitopes were proved to be linear (36).

To date, neutralization-resistant mutants have been used successfully to gain knowledge on the antigenic and structural nature of the proteins of several viruses. These include influenza virus (52, 65, 89), Hantaan virus (69), BEFV (72), and infectious haematopoietic necrosis virus (IHNV) (58), to name a few. The combination of this

technique with the topographical epitope mapping performed previously by cELISA, together with nucleotide sequence analysis, can provide significant insight to the antigenic structure of the G protein of rabies virus. Therefore this study aimed to isolate escape mutants from different rabies virus strains using Mabs directed to antigenic sites I and IIIa (as defined previously by cELISA). Strains employed included ERA, a strain well adapted to cell culture, two strains of rabies associated with two species of bat, the Big Brown Bat (BBB) and Silver Haired Bat (SHB), and the indigenous Western Canadian skunk (WSk) strain. Results of cross-neutralization assays and nucleotide sequence analysis of the isolated escape mutants were compared to the topographical map established by cELISA to investigate the accuracy of this map.

1.8 Hypothesis

Cross reactivity analysis of neutralization-resistant escape mutants will allow further characterization of the neutralization epitopes on rabies virus glycoprotein.

1.9 Objectives

1. To determine the nature of antigenic sites on the G protein of several rabies viruses by:

    a. Generating escape mutants from three different rabies virus strains: Evelyn Rokitnicki Abelseth (ERA), Big Brown Bat (BBB), and Silver Haired Bat (SHB).

    b. Determining the cross-neutralization patterns of a panel of Mabs tested against both parent and escape mutants, such studies include escape mutants generated in this project and those received from Lethbridge Rabies Laboratory.

    c. Determining the sites of the mutation on the G gene of all escape mutants as compared to the parent virus.

2. To relate these findings to the G protein epitope map that has been established previously.

MATERIALS AND METHODS

2.1 Cell line

Mouse Neuroblastoma (MNA) cells [originally obtained from BioWhittaker (Walkersville, MD)], were routinely employed as the cell line of choice in this study. They were routinely grown in 25 or 75 cm^2 plastic flasks (Falcon BD Sciences) using complete minimal essential medium (MEM) which contained Eagle's MEM (Life Technologies Inc., Burlington, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL), 5 % tryptose phosphate broth (TPB) (Sigma Chemical Company, St. Louis, M), and 1% antibiotic/antimycotic (AbAm) solution (100 units/mL streptomycin, 0.25 µg/mL amphotericin B) (Sigma Chemical Company) in a humidified incubator at 37°C and 5% CO_2. Cells were grown to confluent monolayers prior to passage every three days. The monolayers were washed twice with trypsin-EDTA (Gibco-BRL) using 1 mL of trypsin-EDTA per 25 cm^2 flask. The trypsin-EDTA was then decanted and the flasks were incubated in a 5 % CO_2 incubator at 37°C for 2-4 min with 0.5 mL of trypsin. Once the cells were trypsinized, fresh growth medium was added according to a previously determined optimal split ratio and the cell suspension was divided into new 25 cm^2 flasks. The split ratio was determined by the amount of time needed for the formation of a 100% confluent monolayer and the number of the new flasks to be seeded.

2.2 Viruses

A laboratory strain of rabies virus, Evelyn Rokitnicki Abelseth (ERA) virus (ADRI #307) was grown in MNA cells from ERA virus stock (ADRI #302, Supernatant fluid). The Big Brown Bat (BBB) virus (ADRI submission number 2001RAB8445) was originally

isolated from a BBB recovered from Barrie, Ontario, and typed as BBB antigenic type 1. The Silver Haired Bat (SHB) virus (ADRI collection number V077) was originally isolated from a silver haired bat in Saskatchewan in 1988 and passaged in mouse brain once. These viruses are part of the permanent lyssavirus collection of Dr. A. I. Wandeler, Canadian Food Inspection Agency (CFIA). The Western skunk strain virus (WSk), as well as its related escape mutants, were received from CFIA Lethbridge Laboratory, Alberta.

2.3 Monoclonal antibodies (Mab)

A total of eighteen different Mabs (hybridoma supernatants) that specifically bind rabies virus glycoprotein (G protein) were used for neutralization tests and for the production and propagation of escape mutants. These anti-G Mabs were selected from a larger collection of anti-lyssavirus Mabs maintained for the Rabies Centre of Expertise, by the Monoclonal Antibody Unit, both part of CFIA, Ottawa Laboratory, Fallowfield, Ontario. Thirteen of the hybridoma cell lines; i.e. 10EC9, M725, M818, 16EH11, M1089, M778, M1094, 10ED8, M110, M724, 16AH8, M785, M1078 were produced at the University of Bern, Switzerland by Dr. A. I. Wandeler. The other five Mabs used in this study; i.e. W509-6, W101-1, W110-3, W1120-10, and W120-6; were obtained from the Wistar Institute, Philadelphia (courtesy of Dr. Hildegund Ertl).

2.4 Titration of virus in cell culture

Viral titrations are expressed in units of tissue culture infectious dose 50 per mL ($TCID_{50}$/mL). The $TCID_{50}$ is the quantity of virus that will infect 50% of the cell monolayers challenged with the defined inoculum. To determine the virus titre, MNA cells were seeded in 96-well plates (Falcon BD Sciences) at a concentration of $2 X 10^5$ cells/mL, 100 µL/well. The cells were infected with 100 µL of the virus supernatant that had been serially diluted

tenfold (10^{-2}, 10^{-3} to 10^{-9}) in complete MEM. The 96-well plates were then placed in a 37°C, 5% CO_2 incubator for 2-4 days. Thereafter, the cells were fixed with 75% cold acetone for 15 min and stained by a fluorescent antibody staining technique. Briefly, the fixed monolayer was incubated with a predetermined optimal dilution (e.g. 1:100) of an in-house anti-rabies RNP antibody conjugated to fluorescein-5-isothiocyanate (FITC) for 1 hr at 37°C, in a 5% CO_2 incubator. The antibody was diluted in FA-buffer [50 mM Tris (Sigma Chemical Company), 200 mM NaCl (Sigma), and 0.5% Tween20 (Sigma)]. Following the incubation, the monolayers were washed with FA-buffer for 10 min. After counter staining with 0.5% Evan's blue (Sigma Chemical Company) diluted in FA buffer, the presence of fluorescent inclusions in each well was scored under a Leica UV-fluorescent microscope. Virus titres were determined using the Spearman-Karber method tables (40), and are reported as the geometric mean of triplicate determinations.

2.5 Titration of the escape mutants

The escape mutants were titrated in a slightly modified way to facilitate the maintenance of 1:1 complete MEM:Mab ratio. The 96-well plates were seeded with cells 24 hr prior to the addition of the virus. 100 μL of diluted virus was added to a 70% confluent monolayer and plates were incubated at 37°C, 5% CO_2 for 1 hr to allow viral adsorption. After the incubation, the inocula were decanted and 200 μL complete MEM was added into each well. Each escape mutant was titrated both in the presence and absence of the corresponding Mab. Therefore, in one plate the inoculum was replaced by 200 μL complete MEM. At the same time an identical plate was treated with 200 μL of 1:1 complete MEM:Mab solution. The plates were incubated at 37°C, 5% CO_2 for 4 days, fixed and stained as previously described (section 2.4). The parent virus was titrated with this method

and identical results were obtained as for the method described in section 2.4.

2.6 Neutralization tests

This test was performed to determine the titre at which each monoclonal antibody was capable of neutralizing the virus. Each monoclonal antibody to be tested was serially diluted four fold (1:4, 1:16, 1:64, etc.) in a 96-well plate (the test was done in quadruplicate for each Mab) and incubated with an equal volume of virus suspension added into each well (60 µL each). The virus was diluted to contain 100 $TCID_{50}$/60µL. The plates were incubated for 1 hr at 37°C, 5% CO_2. Thereafter, 100 µL of cell suspension containing 2 X 10^5 cells/mL was added into each well and the plates were incubated for 48 hr at 37°C. At the end of incubation time, the plates were fixed and stained with FITC conjugated anti-Rabies RNP antibody as previously described (section 2.4). The plates were scored for fluorescent inclusions after counter staining with Evans's blue and the titre of each Mab preparation was calculated using the Spearman-Karber method.

2.7 Selection of neutralization resistant mutants (escape mutants)

To select the neutralization resistant escape mutants, three ten fold dilutions of the parent virus were propagated in the presence of each of the neutralizing Mabs, at a dilution selected using the results of the neutralization test. The Mab-virus mixture, containing an equal volume of virus suspension and neutralizing Mab solution (250 µL each), was incubated in a CO_2 incubator for 1 hr at 37°C. Afterwards, 250 µL of the incubated mixture was added into a 12.5 cm^2 flask (Falcon BD Sciences) containing 2 mL MNA cells at the concentration of 2 X 10^5 cells/mL. In order to prevent the growth of viruses other than the ones that escaped the neutralization effects of the antibody, the cells were maintained in the presence of the corresponding Mab (1:1, complete MEM:Mab solution). The flasks were

incubated for 3 days at 37°C and 5% CO_2. As rabies virus does not cause a rapid cytopathic effect (CPE), the presence of virus in each flask was monitored by seeding 10 μL of cell-virus suspension from each flask at the start of the incubation into the well of a 96-mini well Terasaki plate (Robbins Scientific Corp. Sunnyvale, CA). These monitor plates were incubated in a humidified chamber concurrent with the tissue culture flasks, fixed and stained as previously described (section 2.4). The supernatant of the flasks showing viral growth on the monitor plate was replaced by 1:1 complete MEM:Mab (corresponding Mab). These flasks were incubated for an additional 24 hr at 37°C, and 5% CO_2. These flasks were frozen at -80°C the following day. The cells in those flasks which did not show any detectable viral growth on the monitor plate were passaged and incubated for an additional 3 days. The negative cells were passaged up to four times. If no viral growth was observed after four passages, the entire selection procedure was repeated.

To harvest the escape mutant viruses, the flasks were frozen and thawed three times to disrupt the cells and release virus. The disrupted cells were centrifuged at 1200 g, for 10 min (MSE Mistral 3000*i* refrigerated bench centrifuge with swing-out rotor, Sanyo) to remove cellular debris. The supernatant was collected and stored at -80 °C.

2.8 Cloning the escape mutants

The isolated escape mutants were first titrated in the presence and absence of the corresponding Mab as described (section 2.5). The virus was then cloned in a 96-well plate. A 70% confluent monolayers of MNA cells in a 96-well plate were infected with 100 μL/well of a virus (escape mutant) dilution containing 1 $TCID_{50}$/100μL. The plate was incubated for 1 hr in a CO_2 incubator at 37°C. The virus inoculum was then removed and the culture was replenished with 200 μL of 1:1 complete MEM:Mab solution and incubated for

4 days in a CO_2 incubator at 37°C. Thereafter, tissue culture supernatants, identified according to origin, were harvested by transferring with a multi-channel pipettor (Biohit 1200) to a new 96-well plate and were kept frozen at -80°C for later use. Wells containing a single focus of infection, assumed to have been infected by a single virion, were identified by fixing and staining the monolayers as described previously (section 2.4). Supernatant from these wells were passaged individually in MNA cells to produce sufficient material for storage and characterization.

2.9 Propagation of the escape mutant clones

Preparation of the virus stocks and propagation of each escape mutant was first carried out in 24-well plates (Falcon BD Company). 100 μL of the chosen virus clone supernatant (from the wells exhibiting a single focus formation) were added to 1 mL of suspended MNA cells (2 X 10^5 cells/mL) in 1:1 complete MEM:Mab in a 24-well plate. A monitor plate was prepared in order to detect the level of virus antigen present in the cell monolayer before passaging. After three days of incubation at 37°C, the monitor was stained to detect the viral growth. If sufficient virus was detectable, the cells were incubated for an additional 24 hr. Thereafter, cells were trypsinized and transferred into a 12.5 cm^2 tissue culture flask (Falcon BD). Monitor plates were once again made to monitor infection in the flasks. After 3 days incubation at 37°C, the cells were trypsinized and transferred into 25 cm^2 flasks. Infection was again monitored. At this stage of propagation, because the ERA escape mutant clones appeared to be cytopathogenic for the cells, instead of transferring the cells into a bigger flask, the infection level was kept under 25%. Briefly, as soon as the infection level reached 25% (as determined in monitor plates), the cells were trypsinized and diluted with fresh, uninfected cells. This procedure was carried out until there was a sufficient

amount of virus for harvesting. Virus-laden supernatants were eventually prepared by centrifuging the cell suspension at 1200 g, 4°C, for 10 min to remove cell debris. The virus suspensions were stored at -80°C until further use.

2.10 Preparing slides for detection of rabies virus

Infected cells were harvested by centrifugation at 1200 g, 4°C for 10 min. Cell smears were prepared on glass slides. The slides were fixed with 100% acetone for 15-20 min at room temperature and incubated with 200 μL of a previously determined optimal concentration of FITC-conjugated anti-rabies RNP antibody for 50 min in a humidified chamber at 37°C. The slides were washed with FA-buffer for 10 min. After counter staining with Evan's blue for 2 min, coverslips were placed on the slides and the slides were viewed for the presence of rabies virus under the fluorescent microscope.

2.11 Preparation of slides using the Giemsa stain

Giemsa stain is excellent for detecting bacteria and fungi. The cells were spun at 1200 g, 4°C for 10 min, and the supernatant was discarded. Cell smears were prepared on glass slides. The slides were fixed in 100% methanol for 15-20 min, dried and stained with 200 μL 1:50 Giemsa stain (diluted in dH_2O and filtered) for 20 min. The slides were then rinsed with FA-buffer and differentiated with 0.5% acetic acid, rinsed with FA-buffer, and viewed under the light microscope for the presence of any bacterial or fungal infection. A positive test would generate dark blue/purple staining.

2.12 Electron microscopy (EM)

The infected MNA cells were centrifuged at 1200 g, 4°C for 10 min, and the supernatant was discarded. The cells were fixed for 1 hr at room temperature in 1-1.5 mL glutaraldehyde solution (25% glutaldehyde in 0.1 M phosphate buffer, pH 7.4). Samples

were submitted to the EM Unit at CFIA where they were processed according to an established procedure for fixation and embedding of tissue for transmission electron microscopy (CFIA SOP# EM-PR001.00). Briefly, the cells were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr, stained *en bloc* in 3% uranyl acetate for 2 hr and then placed in low melting temperature agarose, dehydrated in ethanol and embedded in Jembed 812 resin (Epon). Blocks were trimmed on an LKB 11800 Piramytome and sectioned on a Reichert Ultracut Ultramicrotome. Sections were placed on 600 mesh copper grids, stained with 0.5% lead citrate for 10 min followed by 3% uranyl acetate for 20 min. Grids were viewed on an Transmission Electron Microscope (Hitachi H-7000) at 10, 20, and 40 thousand times magnification.

2.13 Cross-neutralization test

Each of the escape mutant viruses were tested in a previously described neutralization assay (section 2.7) using the eighteen monoclonal antibodies used in this study (section 2.3). The results of these tests were compared to the neutralization pattern of the parent virus from which each mutant had been derived.

2.14 Determination of escape mutant growth rates

Since, unlike ERA itself, the escape mutants isolated from ERA appeared to be cytopathogenic for the MNA cells, this experiment was performed to compare the growth curves of the ERA escape mutants with that of the parental virus and thereby determine whether this cytopathogenic phenomenon was due to elevated growth rates of these viruses.

MNA cells (10^6 in 5 mL complete MEM) were infected separately with each of the ERA escape mutants and with the parent virus at a multiplicity of infection (MOI) of 0.1. A total of 3 flasks were used for each of the viruses. A monitor plate was made from each

flask. After 4 hr of incubation in a CO_2 incubator, the inoculum was removed and the culture was replenished with 5 mL of 1:1 complete MEM:Mab; the Mab corresponded to that used in the selection process for each mutant. The culture containing the parent virus was replenished with complete MEM alone. The cultures were incubated for 24, 36, and 48 hr from the initial inoculation. After the designated incubation time, the monitor cultures were fixed and stained as described previously (section 2.7), as a confirmation of viral growth in the cells. The flasks were frozen at -80°C for later use. The virus from each flask was harvested after three cycles of freeze-thawing. The material was spun at 1200 g, 4°C, for 10 min. The supernatant was then collected and titrated for determination of virus production (refer to section 2.5).

2.15 Titration of the escape mutants in mice

All of the ERA escape mutants as well as the parent virus were titrated in mice to determine the pathogenicity of the viruses. A total of 300 female CD-1 mice (3-4 weeks old) were obtained from Charles River. For each virus, a sufficient number of ten fold serial dilutions were made in MEM containing 2% FBS to yield a lowest virus concentration of approximately 10^{-1} $TCID_{50}$/mL (see Table 2.1). Mice were anaesthetized with isofluorane through an inhalation system and received 0.03 mL of the virus suspension intracerebrally using tuberculin syringes. Remaining virus suspensions were titrated to re-confirm the titre of the viruses inoculated. Animals were checked daily and once clinical signs of rabies were observed, the mice were euthanized with a mixture of oxygen and carbon dioxide. The brains of these animals were removed, used for diagnosis of rabies infection by the fluorescent antibody test (FAT) (as described in section 2.16), and stored at -80°C. After one month, the serum was collected by cardiac puncture from the survivors of each group and analyzed for

the presence of anti-rabies antibodies (as described in section 2.17). The survivors were sedated and humanely euthanized. The LD_{50}/mL was determined using the Spearman-Karber method (40). The protocols for experiments in mice were reviewed by the CFIA/OLF Animal Care Committee and were approved under ACC#175 (refer to appendix).

Table 2.1. Initial titre and the number of dilutions of each virus used for intracerebral inoculation of mice

Virus	ERA	C12	C8	B9	G6	C3	A11	G10
Initial Titre ($TCID_{50}$/mL)	$10^{6.7}$	$10^{5.9}$	$10^{4.6}$	$10^{5.6}$	$10^{5.9}$	$10^{5.5}$	10^{8}	$10^{7.35}$
Dilutions used	10^{-1}-10^{-8}	10^{-1}-10^{-7}	10^{-1}-10^{-6}	10^{-1}-10^{-7}	10^{-1}-10^{-7}	10^{-1}-10^{-7}	10^{-1}-10^{-9}	10^{-1}-10^{-9}

2.16 Fluorescent Antibody Test (FAT)

This test was used to confirm presence of rabies antigen in brains of mice that were euthanized after developing clinical signs of rabies. Brain smears were prepared on glass. The slides were treated with UV light for 2 min to deactivate the virus, and then fixed in 100% acetone at 4°C for 15 min. The slides were then incubated for 50 min in a humidified chamber with an optimal dilution of FITC-conjugated goat anti-rabies RNP, provided by the Rabies Diagnostic Laboratory (CFIA). After washing with FA buffer, the slides were viewed under a fluorescent microscope for the presence of fluorescent inclusion bodies.

2.17 Competitive Enzyme-linked Immunosorbent Assay (cELISA)

The presence of rabies G protein-specific antibodies in sera collected from mice that

survived the *in vivo* titration experiment was detected by cELISA, according to established procedures (Elmgren and Wandeler, CFIA TD-SOP# Elmgren L –PR007.01). Briefly, flat-bottomed, 96-well microtiter plates, previously coated with an optimized suspension of whole ERA virus, were thawed at room temperature and washed with phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST). Plates were blocked with 200 μL PBST containing 5% bovine-equine serum (BES) for 2 hr at 28°C. The plates were washed with PBST and then 50 μL of test sera, diluted 1:10 in PBST + 3% BES, were added to the wells. Each serum sample was tested in duplicate. Immediately after addition of the test serum, 50 μL of a previously-determined optimal concentration of horseradish peroxidase-labelled, rabies G protein-specific Mab 10EC9 was added to each well. The plates were incubated for 1 hr at 28°C and then washed with PBST to remove unbound antibodies. 100 μL of substrate solution containing 1 mM hydrogen peroxide and 4 mM chromogen [2', 2-azin-bis (3-ethylbenzthiazol-6-sulfonic acid)] (ABTS) in 0.05M citrate buffer, pH 4.0, were added to each well and color allowed to develop for 10 min while shaking at 28°C. Absorbance at 414 nm was read using a Multiscan RC Plate Reader (EL 403 microplate reader, Multiscan MCC 1340, Labsystems Multiscan RC). Results are expressed as mean percent inhibition compared to control wells containing diluent in the place of serum, calculated using the formula:

Percent Inhibition = (1-Absorbance of Sample ÷ Absorbance of Buffer Control) × 100.

20% inhibition was considered positive for the presence of specific antibody.

2.18 RNA extraction

Total RNA was extracted from virus infected cell supernatants using TRIzol LS

reagent (Gibco BRL). 1 mL TRIzol LS reagent was added to 250 μL of virus in a microfuge tube and mixed by vortexing (vortex, VWR Canlab). Since small amounts of RNA are normally obtained when isolating RNA from supernatant, 10 μg of molecular biology grade glycogen solution (1 mg/mL) (Gibco BRL) was added to act as a carrier for RNA precipitation. After mixing, 200 μL of molecular biology grade chloroform was added. Tubes were capped and shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2-3 min. Samples were then centrifuged at 10,000 g for 15 min at 4°C (Eppendorf Microcentrifuge, Brinkmann Instruments, Westury, NY). Following centrifugation, the top aqueous layer was removed, and transferred to a new microfuge tube. RNA was precipitated from the aqueous phase by addition of 500 μL of molecular biology grade isopropyl alcohol, followed by incubation at room temperature for 10 min. Samples were then centrifuged for 10 min at 10,000 g and 4°C. The supernatant was removed and RNA pellet was washed with 1 mL 75% ethanol, re-centrifuged briefly and the liquid was decanted. The pellet was then dried in a Speedvac (Savant Oligoprep OP120) for 15-30 min and the RNA was dissolved in 100 μL of RNase-free water prepared by treatment with diethylpyro carbonate (DEPC). RNA was stored at -80°C.

2.19 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to amplify the G gene of the extracted RNA samples. The primers used in this study were provided through the courtesy of Dr. Susan Nadin-Davis, CFIA-OLF and are described in Table 2.2. The reverse transcription of RNA to cDNA was performed in a 20 μL volume containing 2 μL of RNA, 1 μL of 50 μM primer (number 452For), 4 μL of 5X RT buffer (supplied with enzyme), 2 μL of 100 mM dithiothreitol (DTT), 2 μL of 10 mM dNTPs and 1 μL Moloney murine leukemia virus reverse transcriptase (M-MLV,

200U/μL) (Gibco-BRL) at 37°C for 2 hr (98). An aliquot (5 μL) of the RT reaction (cDNA) was subjected to PCR amplification in a 50 μL volume containing 1X Expand buffer with $MgCl_2$, (supplied with Expand enzyme), 1.25 μL of 25 μM primer (number 453Rev), 1.25 μL of 10 mM dNTPs, and 0.75 μL of 3.5 U/μL Expand High Fidelity^{plus} enzyme (Roche Molecular Biochemicals). The Expand^{plus} enzyme is a thermo-stable DNA polymerase mix that yields high copy fidelity. The Gene Amp PCR system 9600/9700 (PE Biosystems, CA) was used for thermal cycling. Amplification of the G amplicon was performed with the following thermocycling profile: 2 min of denaturation at 93°C, followed by 40 cycles of denaturation at 92°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 2 min. The expected amplicon size was 1.6 Kb. Analysis of the PCR products was carried out by DNA gel electrophoresis through 1% agarose. DNA was visualized under UV light with ethidium bromide stain. Amplicon sizes were determined using a DNA molecular weight standard (1Kb ladder, Invitrogen). The PCR products were stored at -20°C.

Table 2.2. Oligonucleotides used for RT-PCR amplification of the G gene

Primer	Sequence	Position (PV strain)
452For	5′-CCG GGA TCC TTT G(AG) GCC TCT TGG ATG TGA-3′	3260-3279
453Rev	5′-AAG GAG AGT TGA G(AG) TTG TAG TCA GAG TTC C-3′	4989-5058

For = Forward
Rev = Reverse
PV strain = Pasteur virus strain (GENBank accession number = NC-001542)

2.20 DNA purification

The PCR products were purified using the Wizard PCR Preps Purification system according to the manufacturer's instructions (Promega Corp., Madison, Wisconsin). 100 μL of direct purification buffer (Promega Corp.) was added to the PCR product (46 μL) and mixed by vortexing. One mL Wizard PCR Preps Purification resin was added, and the suspension was mixed by vortexing three times over a 1 min period. The sample was then transferred to a Wizard Minicolumn attached to a syringe barrel. The column was assembled on a vacuum manifold and the vacuum was applied to draw the solution through the minicolumn. The column was then washed with 80% isopropanol and vacuum was reapplied to draw the liquid through the column. The minicolumn was transferred to a microcentrifuge tube and centrifuged at 10,000 g for 2 min to remove residual wash buffer. The column was then transferred to a new microfuge tube and 50 μL H_2O, pre-warmed to 50°C, was added to the column. After 2 min the DNA was eluted from the column by centrifuging at 10,000 g for 20 seconds. Purified DNA was stored at -20°C.

2.21 Sequence analysis

Using purified PCR products as templates, the complete coding region of the G gene was sequenced using a LI-COR 4200L automated sequencer (Lincoln, NE). The primers used were provided by Dr. Susan Nadin-Davis and they were labelled with either IR700, a pentamethine carboxy dye, or IR800, a heptamethine cyanine dye, for forward and reverse primers respectively (refer to Table 2.3). The sequencing reaction was carried out using a Thermosequence cycle sequencing kit (Amersham Biosciences). Briefly, the template was mixed with dye-labelled primer in a 13 μL volume and then four 3 μL aliquots of this mixture were mixed with 3 μL each of the A, C, G, and T dd/dNTP reagents. Sequencing

was achieved by 30 cycles of thermocycling thus: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min. Four µL of IR^2 stop solution (Li-Cor) was added to each reaction. Samples were heated at 95°C for 3 min and then electrophoresed on a 66 cm (0.25 mm) denaturing acrylamide gel [prepared using KB plus 3% (LiCor)] for 10 hr. Base calling was performed automatically using the eSeqv2 program (LiCor) followed by manual review. Overlapping segments of sequence were assembled using AlignIR software (LiCor) and exported in FASTA format for alignment using CLUSTALX (118). The nucleotide sequence of each complete G gene was translated into its predicted protein sequence using DNASIS software (Hitachi).

Table 2.3. Oligonucleotides used for sequencing the G gene

Primer	Sequence	Position (ERA)
ADRG 2For	5′-GAA GAG TCT CTA CAC AAT CCG TAC-3′	382-405
ADRG3For	5′-GAG TGT CTG GAT GCA CTA GAG TC-3′	901-923
ADRG5Rev	5′-GGA TTA AGA CAT TGG CGTV CAG GTC-3′	1154-1177
ADRG6Rev	5′-GTA GAA GAG ACC GCT ACT CCT GAG-3′	534-557

For = Forward
Rev = Reverse
The positions are given using the first base of ERA G gene open reading frame as position 1.

RESULTS

3.1 Neutralization tests on three different rabies virus strains

Eighteen different anti-G monoclonal antibodies were tested by the neutralization test to determine if they neutralized the three rabies virus strains: Evelyn Rokitnicki Abelseth (ERA), big brown bat (BBB), and silver hair bat (SHB). ERA was chosen as a well adapted tissue culture strain, while the BBB and SHB viruses are representatives of street rabies virus strains. The results of these tests, presented in Table 3.1, indicate the titres at which each Mab is still capable of neutralizing the virus (100% inhibition of viral growth). A higher titre indicated that the hybridoma supernatant used for the test contained more Mab and therefore could be subjected to more dilutions and still retain its neutralizing ability. Titres of ≤ 8 were considered non-neutralizing. Based on these results, the neutralizing antibodies to be used for the production of escape mutants from each of the three strains of rabies virus were selected. Neutralizing Mabs that target antigenic sites I and IIIa (a subsite from antigenic site III) were chosen, in part, because these antigenic sites have been assessed previously by a topographical mapping approach using cELISA (36). In addition, these specific antigenic sites are the target of most of the neutralizing antibodies that were available. 10EC9, M725, 16AH8, and M785 were selected to be used in isolation of escape mutants from ERA while 10EC9, 16AH8, and M785 were chosen for use with the BBB and SHB viruses. Three of the chosen Mabs (10EC9, 16AH8, and M785) neutralize all of the viruses. We chose M725 as the second antigenic site I Mab to be used with ERA although it did not neutralize the other two strains of rabies virus. The reason for this was the strong physical connection that was proposed between 10EC9 and M725 epitopes in previous studies done by cELISA (36).

Antigenic sites	Mab	ERA	BBB	SHB
I	10EC9	512	128	128
I	M725	724	2	2
I	W509-6	256	2	2
I	M818	3	11	8
I	16EH11	8192	3	512
I a	W101-1	362	2	2
I a	M1089	3	2	2
II	M778	8	8	8
II	M1094	2	6	2
II a	W110-3	362	2	2
III	10ED8	3	6	6
III	W1120-10	32	45	23
III	M1100	3	32	2
III a	M724	181	64	11
III a	W120-6	128	91	8
III a	16AH8	2048	512	128
III a	M785	2048	512	512
IV	M1078	3	3	2

Table 3.1. Neutralization results for the three different strains of rabies virus

Eighteen different anti-G monoclonal antibodies were tested for their neutralizing ability in a serum neutralization test. The numbers in the table represent the titres at which the monoclonal antibodies are still neutralizing the virus. The highlighted areas show the Mabs that were chosen to be used for escape mutant production. The Roman numbers on the left side of the table show the antigenic site targeted by each Mab as previously determined by cELISA (36).

3.2 Selection of escape mutants

3.2.1 ERA strain

Mabs directed to antigenic sites I (10EC9, M725), and IIIa (16AH8, M785) were used for the selection of escape mutants from the ERA strain of rabies virus. Three 10-fold dilutions of ERA (10^0, 10^{-1}, and 10^{-2}) were propagated in the continuous presence of each of the selected Mabs. A total of seven escape mutants were collected from ERA (see Table 3.2). Unlike the parent virus, all ERA escape mutants appeared to be highly cytopathogenic and caused cell-death a few passages after infection. Consequently, the propagation of the virus was not achievable by the regular method of infection of the cell culture by a selected clone followed by repeated passaging of the cells. However, by adding new cells and keeping the infection level below 25% until the last passage sufficient virus stocks could be prepared for the subsequent assays (refer to section 2.9).

3.2.2 BBB and SHB strains

10EC9, 16AH8 and M785 were used for the selection of escape mutants from the BBB and SHB viruses. All attempts to select escape mutants from these two strains of rabies were unsuccessful. The process was repeated four times with each of these strains. However, none of these attempts resulted in the growth of any escape mutant virus.

3.2.3 WSk strain

The Western skunk (WSk) strain of rabies virus, together with two escape mutants which were produced from this isolate using 10EC9 and 16AH8, were received from CFIA laboratory in Lethbridge, Alberta and used in this study. These escape mutants were produced using the same method used in this study and are called WSk10EC9 and WSk16AH8 respectively.

Mutant ID	Selecting Mab	Initial ERA dilution used
C12	10EC9	10^0
C8	10EC9	10^{-1}
B9	M725	10^0
G6	M725	10^{-1}
C3	16AH8	10^0
A11	16AH8	10^{-1}
G10	M785	10^0

Table 3.2. Escape mutants isolated from ERA virus

The MNA cells were infected with serial dilutions of ERA in the presence of selecting Mab. The viral growth was monitored after 3 days of incubation. The cells were passaged up to 4 times if there was no growth. The escape mutant was harvested from the flasks showing viral growth.

3.3 Cross-neutralization assays

3.3.1 ERA

The ERA virus and the ERA escape mutants were tested in cross-neutralization assays against all eighteen Mabs described in Table 3.1 to determine how reactivity to each of the monoclonal antibodies has changed for each individual escape mutant. It was rationalized that if a mutant had lost reactivity to a number of Mabs simultaneously, the epitopes of those Mabs would be functionally linked, whereas if the mutant had lost reactivity only to the Mab that was used in its isolation, the epitope of that Mab is

independent of those of the other Mabs. A virus was considered resistant to neutralization only if the neutralizing titre was ≤ 8. The results of this assay are presented in Table 3.3. All escape mutants were specifically resistant to neutralization by the Mab with which they were selected. Several mutants lost reactivity to additional Mabs. For example, mutants C12 and C8, which were produced with 10EC9, lost their reactivity to W101-1 as well as to 10EC9. This indicates a possible linkage between the epitopes of these two Mabs. At the same time there could be a relationship between the epitopes of 10EC9, W101-1 and M725 since B9, produced with M725, has lost reactivity to 10EC9 and W101-1 as well as M725. This however, is not the case for the other M725 escape mutant (G6). This escape mutant has not lost the reactivity to 10EC9 but it is exhibiting a reduction in reactivity. Both of the escape mutants produced with M725 have lost their reactivity to W509-6. There is a significant drop in neutralizing titres of some of the Mabs with regards to some of the mutants. For example there is a reduction in the neutralizing titre of M725 and W509-6 for C12 and C8 or W101-1 for G6. In antigenic site IIIa, it can be interpreted from the results that epitopes of 16AH8 and M785 are closely related because escape mutants produced with each of these Mabs (C3, A11, G10) have also lost reactivity to the other Mab.

There are some Mabs that have gained the ability to react with a variant. For example Mabs M778 and M1094, which are directed to antigenic site II, react to all antigenic site III mutants as well as some of the antigenic site I mutants (see Table 3.2).

3.3.2 WSk

The WSk strain of rabies and its escape mutants were tested by cross-neutralization assays in a manner similar to that employed for the ERA mutants. As shown in Table 3.4,

Table 3.3. Cross neutralization Assay comparing ERA virus to its escape mutants

Neutralization-resistant escape mutants, selected from ERA virus using specific monoclonal antibodies, were then tested for susceptibility or resistance to all 18 monoclonal antibodies of the rabies G protein panel. The results presented in this table show the titre of the monoclonal antibody that is neutralizing the virus. The results for each of the escape mutants are compared to the parent virus (ERA). The light grey areas represent the loss of neutralizing ability of a specific Mab. The darker grey areas represent the change from non-neutralizing to neutralizing activity by a specific Mab. The Mabs having titres ≤ 8 are considered to be non-neutralizing.

Variant/selective Mab	Monoclonal antibody																
	10EC9	M725	W509-6	M818	16EH11	W101-1	M1089	M778	M1094	W110-3	10ED8	W1120-10	M 1100	M724	W120-6	16AH8	M785	M1078
C12 (10EC9)	2	64	128	2	1024	2	2	23	16	128	16	181	2	256	362	1024	2048	2
C8 (10EC9)	2	32	23	2	91	2	2	23	8	23	6	91	2	64	181	512	1448	2
B9 (M725)	8	2	2	2	128	8	2	6	2	11	2	23	2	23	64	91	64	2
G6 (M725)	32	2	2	2	181	16	2	3	3	23	2	11	2	32	32	256	181	2
C3 (16AH8)	362	256	181	2	1448	256	2	91	32	256	32	362	2	256	512	2	2	2
A11 (16AH8)	256	181	128	2	1024	256	2	91	32	11	8	11	2	256	362	2	2	2
G10 (M785)	512	181	128	2	512	256	2	91	45	362	45	256	2	512	512	2	2	2
Parent Virus-ERA	512	724	256	3	8192	362	3	8	2	362	3	32	3	181	128	2048	2048	3
Antigenic site	I							II		IIa	III			IIIa				IV

Variant	Monoclonal antibody																	
	10EC9	M725	W509-6	M818	16EH11	W101-1	M1089	M778	M1094	W110-3	10ED8	W1120-10	M 1100	M724	W120-6	16AH8	M785	M1078
WSk10EC9	2	45	45	2	23	2	2	9	2	2	2	11	2	32	45	64	181	2
WSkunk16AH8	96	2896	8192	23	256	256	2	11	2	32	8	16	8	64	45	2	2	2
Parent virus-WSk	362	2896	4096	91	362	181	2	32	3	11	8	8192	8	91	256	4096	1448	2

Table 3.4. Cross-neutralization assay comparing WSk virus to its escape mutants

Neutralization-resistant escape mutants were selected from the western skunk virus using the monoclonal antibodies indicated, and then tested for susceptibility or resistance to the same monoclonal antibody panel. Numbers show the titre of the Mab that is capable of neutralizing the virus. The light grey areas represent the loss of neutralizing ability of a specific Mab. The Mabs having titres ≤8 are considered to be non-neutralizing.

mutant WSk10EC9 was resistant to neutralization by 10EC9, M818, and W101-1, indicating a linkage among the epitopes defined by these Mabs. This is consistent with the antigenic map produced based on cELISA results, and the results obtained with ERA mutants with 10EC9. In addition, the relationship between 16AH8 and M785 in antigenic site IIIa (identified by C3, A11, and G6) is confirmed by these results because the mutant isolated with 16AH8 (WSk16AH8) could no longer be neutralized by either Mab, unlike the parent virus.

3.4 Sequence analysis

3.4.1 ERA

The complete coding region of the glycoprotein gene was amplified from ERA and from all seven ERA escape mutants. The nucleotide sequence of the complete open reading frame was determined for all eight amplicons. The location of critical amino acid changes involved in the binding region in each of the related antigenic sites (sites I and IIIa) was then determined (refer to Fig.3.1). The sequence of Street Alabama Dufferin (SAD) strain was also included for comparison. The observed non-synonymous mutations are summarized in Table 3.5. Each mutant had multiple amino acid substitutions; however certain changes were associated with particular Mabs. All of the escape mutants produced with Mabs to antigenic site IIIa, i.e. A11, C3 (isolated using 16AH8), and G10 (isolated with M785) had a mutation which resulted in substitution of asparagine with lysine at amino acid position 336 located within antigenic site III. The escape mutants isolated with Mabs to antigenic site I however, presented two different patterns. C12 and C8, which were selected by 10EC9, both had mutations which resulted in non-synonymous changes at amino acid position 40 located

within antigenic site II. Glycine was replaced by glutamate in C12 and by arginine in C8. Interestingly, these mutants did not have any mutations in antigenic site I, despite being selected with a site I specific Mab. In contrast, the other two site I escape mutants, B9 and G6, showed a mutation within antigenic site I at amino acid residue 231 where a leucine was replaced with a proline. There are some other mutations that might be important since they occur in more than one escape mutant, although they are located outside of any known antigenic site. For example, histidine replaces arginine at the position 120 in all of the escape mutants except C12. Methionine replaces thereonine at position 452 in all of the mutants. Since methionine is also present in this position in the SAD strain of rabies virus, this specific mutation might not be significant. Additional mutations, causing coding changes located outside any known antigenic sites, were present in only one of the escape mutants; for example, as represented by changes at position 99 in A11 and at position 160 in C12. These substitutions may be unrelated to the selective pressure applied by selecting Mab.

The total number of nucleotide mutations in all of the ERA escape mutant G genes have been calculated (see Table 3.6). Out of 37 mutations, 28 were transitions including a high proportion of second base changes. Two of these were located within the portion of the gene encoding the antigenic site under selection. Transversions which are generally rarer in occurrence were indeed less abundant but three of them occurred in the portion of the gene under selective pressure and were non-synonymous in nature. So altogether transversions were rarer, more of them contributed to changes at the site of selection than did transitions.

3.4.2 WSk

The G gene of the WSk virus and the two escape mutants isolated from this strain of rabies was amplified and sequenced. From these nucleotide sequences the predicted protein

Table 3.5. **A summary of the non-synonymous mutations present in the ERA escape mutants**

The location and nature of the nucleotide changes together with the resulting changes in encoding amino acids has been shown for each escape mutant using ERA as reference sequence. At top of the table, the Mabs used for the selection of the mutants are indicated.

	10EC9		M725		16AH8		M785
Ag site	**C12**	**C8**	**B9**	**G6**	**C3**	**A11**	**G10**
Site II	Gly-40 to Glu GGG toAGG	Gly-40 to Arg GGG to GAG					
						Asn-99 to Thr AAC to ACC	
		Arg-120 to His CGC to CAC	Arg-120 to His CGC to CAC	Arg-120 to His CGC to CAC	Arg-120 to His CGC to CAC	Arg-120 to His CGC to CAC	Arg-120 to His CGC to CAC
	Ser-160 to Thr TCA to ACA						
Site I			Leu-231 to Pro CTA to CCA	Leu-231 to Pro CTA to CCA			
			Leu-287 to Pro CTA to CCA				
Site III					Asn-336 to Lys AAT to AAG	Asn-336 to Lys AAT to AAG	Asn-336 to Lys AAT to AAG
	His-352 to Tyr CAT to TAT						
					Gly-366 to Ala GGA to GCA		
							Ala-400 to Gly GCA to GGA
			414 Asp to Asn GAT to AAT				
	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG	Thr-452 to Met ACG to ATG
						Ile-490 to Leu ATC to CTC	

Nature of Change	Number of times the change occurs	Position in codon	Importance
Transition	28	3 on the first base 17 on the second base 8 on the last base	Non-synonymous Non-synonymous Synonymous
Transversion	9	2 on the first base 3 on the second base 4 on the last base	Non-synonymous Non-synonymous 3 non-synonymous

Table 3.6. Nature of the mutations and their frequency in the G gene coding region of ERA escape mutants

All nucleotide substitutions found in escape mutants using ERA parent as reference sequence were summarized according to their nature as transitions (G to A, A to G and C to T and T to C) or transversions (C/T to A/G and A/G to C/T). The effect of each change on the amino acid was determined as is represented in the final column.

Fig.3.1. **Glycoprotein amino acid sequence for the ERA virus and seven escape mutants isolated from ERA**

The G gene of the parent ERA virus and the ERA escape mutants was sequenced. The amino acid sequence was predicted from the nucleotide sequence in each case. These protein sequences were aligned using the sequence of the parent virus. The sequence of closely related Street Alabama Dufferin (SAD) strain was included for comparison. Dots represent identity with the parent virus. Note that the residue numbering includes the 19 amino acid signal peptide and the first amino acid of the mature protein is designated as residue 1.

Fig.3.2. Glycoprotein amino acid sequence for the WSk strain of rabies virus and two escape mutants isolated from WSk

The G gene of the Western Skunk virus and the two escape mutants isolated from this strain was sequenced. The glycoprotein amino acid sequence of each strain was predicted from the nucleotide sequence. Amino acid sequences were aligned, using the WSk parent as reference, to directly compare these proteins. Residues identical to the parent virus are indicated by dots and only those residues different from the parent virus are noted for the escape mutants. Residue numbering is as for Fig.3.1.

sequences were generated and aligned as shown in Fig.3.2. The sequence of the mutant selected with 10EC9 showed a glycine to arginine substitution in antigenic site II at position 40, a mutation similar to that present in the ERA escape mutant isolated with 10EC9 (C8). The WSk escape mutant isolated with 16AH8 has an amino acid substitution in position 336 in the antigenic site III where aspartic acid replaces asparagine. An amino acid replacement occurred in the same position in the ERA mutants isolated with 16AH8, although in these cases asparagine is replaced by aspartic acid. In addition, WSk16AH8 has a substitution at amino acid 186, which results in a change from glycine to tryptophan; such a change was not present in the ERA mutants selected with 16AH8.

3.5 Examining the cells infected with ERA escape mutants for signs of infection with agents other than rabies virus

All of the escape mutants isolated from ERA appeared to be highly cytopathogenic for the cell culture (refer to section 3.2). This was first observed when the clones from the isolated mutants were used to infect the MNA cells as part of the propagation process. The cells started to die after the second passage. In contrast, the parent virus causes little if any CPE after multiple passages. To ensure that this effect was due to the escape mutants, and not some other factor, cell cultures infected with each of the escape mutants or the parent virus were examined in parallel as follows. First, the cells were subjected to staining with anti-rabies FITC conjugated antibody to detect the rabies infection. As shown in Fig.3.3, the cells infected with parent virus (ERA) exhibit a normal appearance despite being infected with rabies virus whereas the cells infected with the escape mutant are dead but exhibit a more intense condensed rabies virus staining, suggesting that higher levels of rabies virus antigen are present in the cells infected with escape mutant. The cell cultures infected with

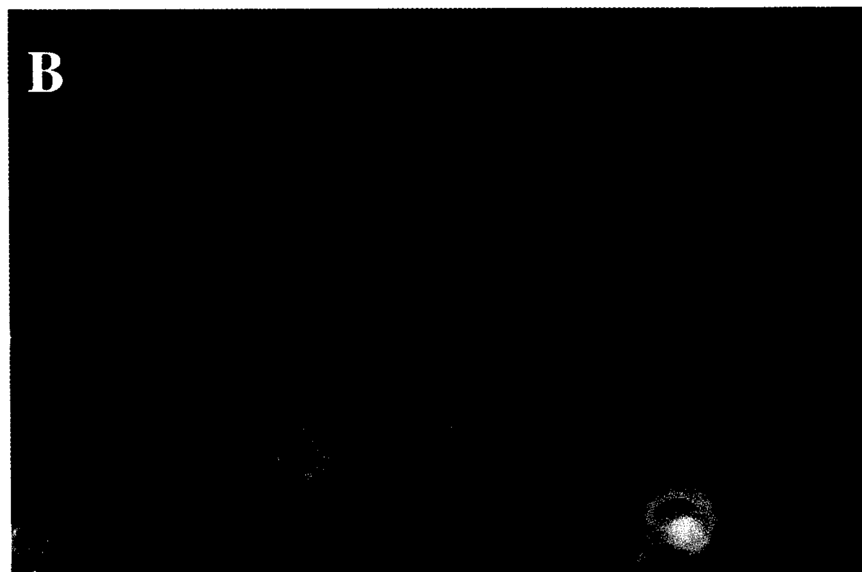

Fig.3.3. Staining the cells infected with anti-rabies FITC conjugated antibody

Cells infected with parent ERA virus (A) and the escape mutant B9 were stained with anti-rabies antibody to detect the rabies infection

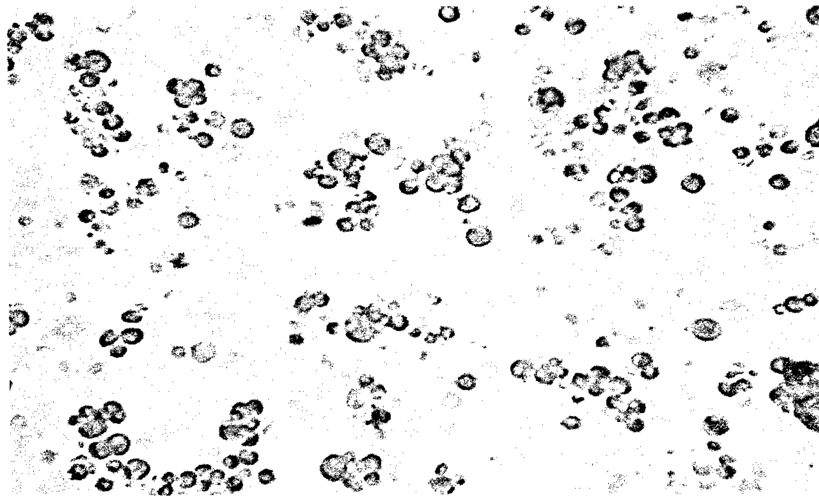

Fig.3.4. Staining the cells infected with ERA escape mutants with Giemsa

The cells infected with ERA escape mutant B9 were stained with giemsa to detect any possible bacterial or fungal infection. There was not any infection detectable.

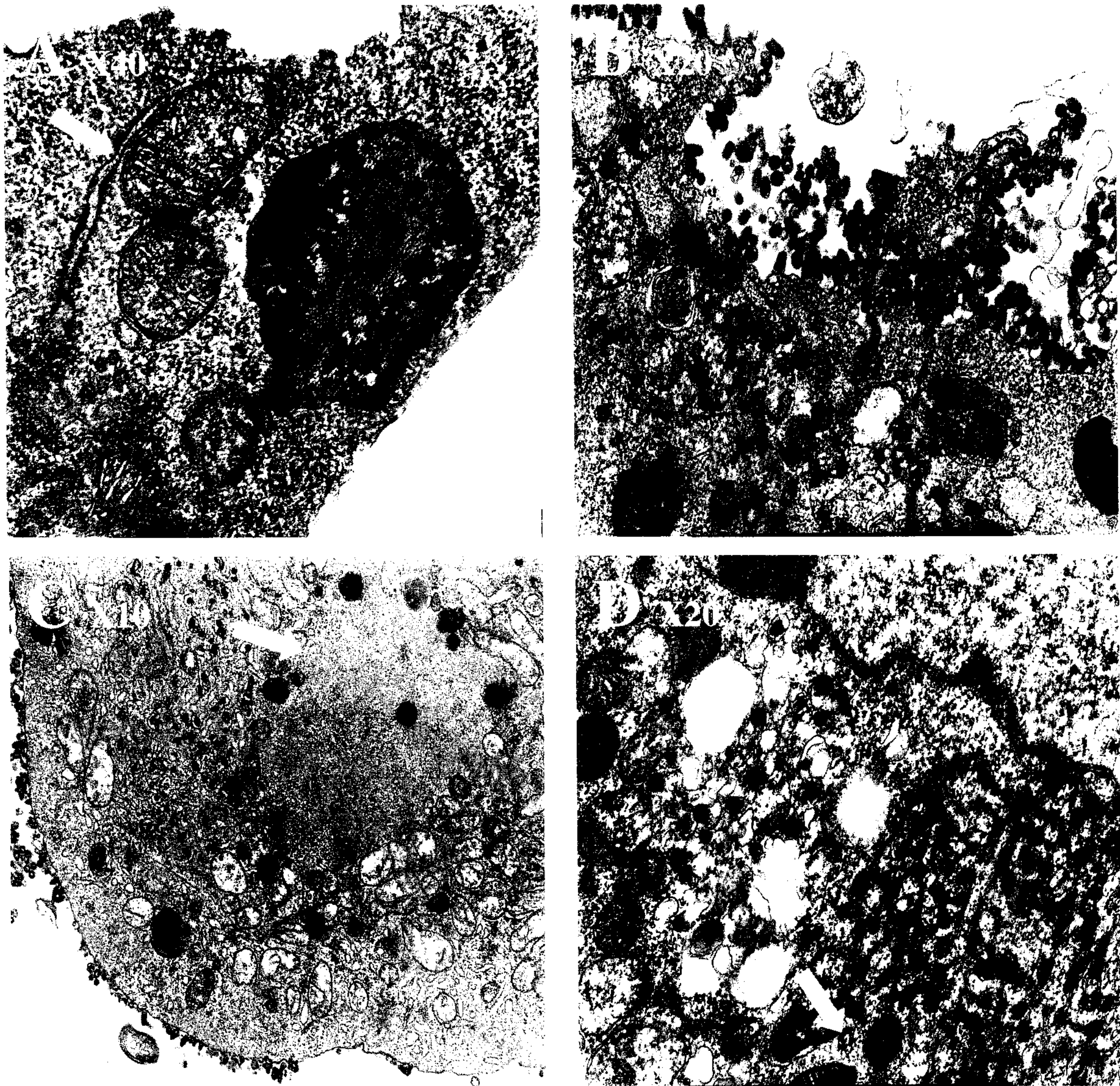

Fig. 3.5. Electron microscopy of the cells infected with escape mutants

The non infected MNA cells (A) as well as cells infected with escape mutants B9 and G10 (B, C, and D) were analyzed with electron microscopy. Rabies virus particles are present in the infected cells (B, arrow). There is a RNP deposition in the infected cells pointed in (C, arrow). The mitochondrial damage is noticeable in infected cells (D, arrow) compared to the mitochondria in negative controls (A, arrow).

escape mutants were also stained with giemsa, for the detection of bacterial or fungal contamination. As shown in Fig.3.4, there was no sign of any bacterial or fungal infection in the cells. Subsequently cell cultures infected with escape mutants were examined by electron microscopy to look for evidence of any other possible viral infection. As it can be observed in Fig.3.5C, the cells are highly infected with rabies virus but no other viral particles specific to the cell culture infected with escape mutants were present. Some retrovirus-like particles were present in both rabies-infected and uninfected cells. Since retroviruses are commonly encountered in mice, it is not surprising to find them in this MNA cell line. Since these viruses are present in control cells not infected with rabies and these cells are dividing normally, it is unlikely that these retroviruses are the cause of cell damage. There were no other virus particles detectable in the electron micrographs of either infected cells or negative controls. However it was observed in the EM pictures that mitochondria in the cells infected with the escape mutants but not controls seem to have under-gone a degenerative process (see Fig 3.5.E). In addition the mitochondria were less abundant in the infected cells compared to negative controls. This observation may have some bearing on the cytopathogenic effects of the escape mutants.

3.6 Investigation of the growth rates of ERA escape mutants

This experiment was performed in order to determine whether the observed cytopathogenicity was the result of an increased growth rate for ERA escape mutants compared to the parent virus (see section 2.14). As indicated in Table 3.7, four of the escape mutants, B9, G6, C3, and A11, showed a more rapid growth after 24 hr compared to the parent virus. B9, G6 and A11 continued to grow faster after 36 hr. In addition C8 showed a more rapid growth between 24 and 36 hr. All the other escape mutants were either growing

at a similar or lower rate compared to ERA. The data do not clearly suggest that the observed pathogenicity is related to the growth rate of the mutant viruses.

Virus	**Fold increase in Virus Titre compared to the previous time point**		
	24h	**36h**	**48h**
ERA	0.45	1.77	4.64
C12	0.08	7.9	10
C8	0.2	22.38	2.23
B9	0.8	31.6	2.5
G6	3.5	12.88	3.54
C3	3.2	1.8	3.98
A11	1.4	177.8	4.38
G10	0.14	3.16	5.62

Table 3.7. Growth rate of ERA and escape mutants isolated from ERA

MNA cells were infected, at an MOI of 0.1, with ERA and all seven escape mutants isolated from ERA in separate cultures. The fold increase in titres compared to the previous time point after 24, 36, and 48 hr was calculated by dividing the titre at the end of the time point by the titre at the end of the previous time point.

3.7 Pathogenic properties of ERA and the ERA escape mutants in mice

The pathogenic properties in mice of the seven escape mutants isolated from ERA were compared to those of the ERA parent virus. Serial dilutions of each virus were inoculated intracerebrally into groups of mice, using five mice per group. Animals were monitored for clinical signs of rabies over a period of 30 days. Viral titres determined in tissue culture ($TCID_{50}$/mL) and those determined *in vivo* (LD_{50}/mL) were compared as shown in Fig.3.6. Overall, all the viruses that fall on the diagonal line on this chart have maintained the same level of pathogenicity in mice as compared to tissue culture. The parent virus demonstrated comparable titres *in vitro* and *in vivo*. For three escape mutants

pathogenicity in mice was reduced compared to that in tissue culture. These escape mutants are C12, B9, and G6, all of which were isolated using Mabs to antigenic site I. In contrast, the C8 variant, produced with a Mab directed to antigenic site I (10EC9), is the only escape mutant of this group that does not show any change in pathogenicity. All the escape mutants isolated with Mabs to antigenic site IIIa (C3, A11, G10) have retained similar titre as $TCID_{50}$ *in vitro* and as LD_{50} *in vivo* and therefore have remained at the same level of pathogenicity as the parent virus.

The brain smears from all of the mice which developed signs of rabies were analysed for rabies antigen by the FA test (see section 2.16). All of these mice were positive for rabies. Serum was collected from all of the survivors at the end of 30 days and examined by cELISA to determine if these animals have developed any antibody against rabies virus. It was important to differentiate the animals who have survived the infection from those which have not been infected at all. The results of this testing are presented in Fig.3.7 and Fig.3.8. Except for the animals that received the three escape mutants that were less pathogenic in mice (C12, B9, and G6), all the other animals that were challenged with the parent ERA virus or each of the other four escape mutants did not survive the infection. Most of the survivors of these groups did not produce antibody against rabies indicating that they did not receive a sufficient dose of virus to develop an infection.

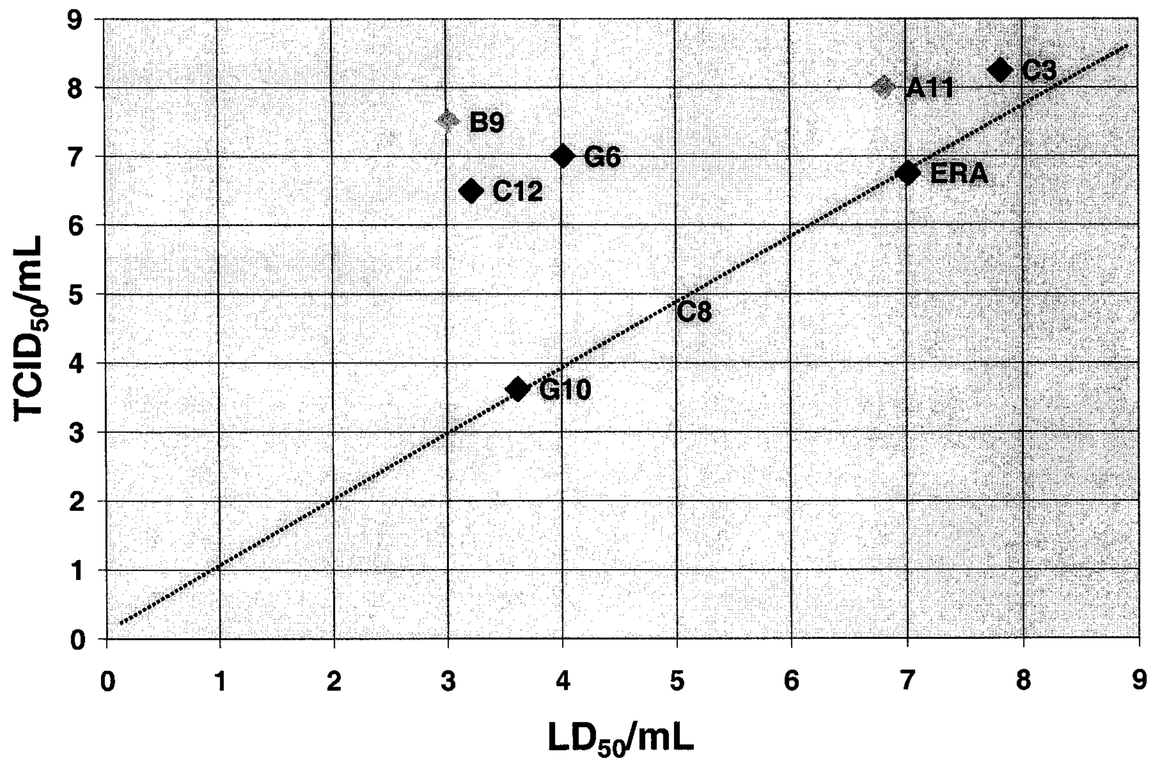

Fig.3.6. Comparison of the pathogenicity of the escape mutants isolated from ERA to the parent virus

Mortality of the mice infected by the intracerebral (i.c.) route was investigated. Serial dilutions of each virus were used to inoculate groups of 5 mice and the survival of all mice was checked over a period of 30 days. The lethal dose 50 (LD_{50}/mL) was calculated and compared to tissue culture infectious dose ($TCID_{50}$/mL).

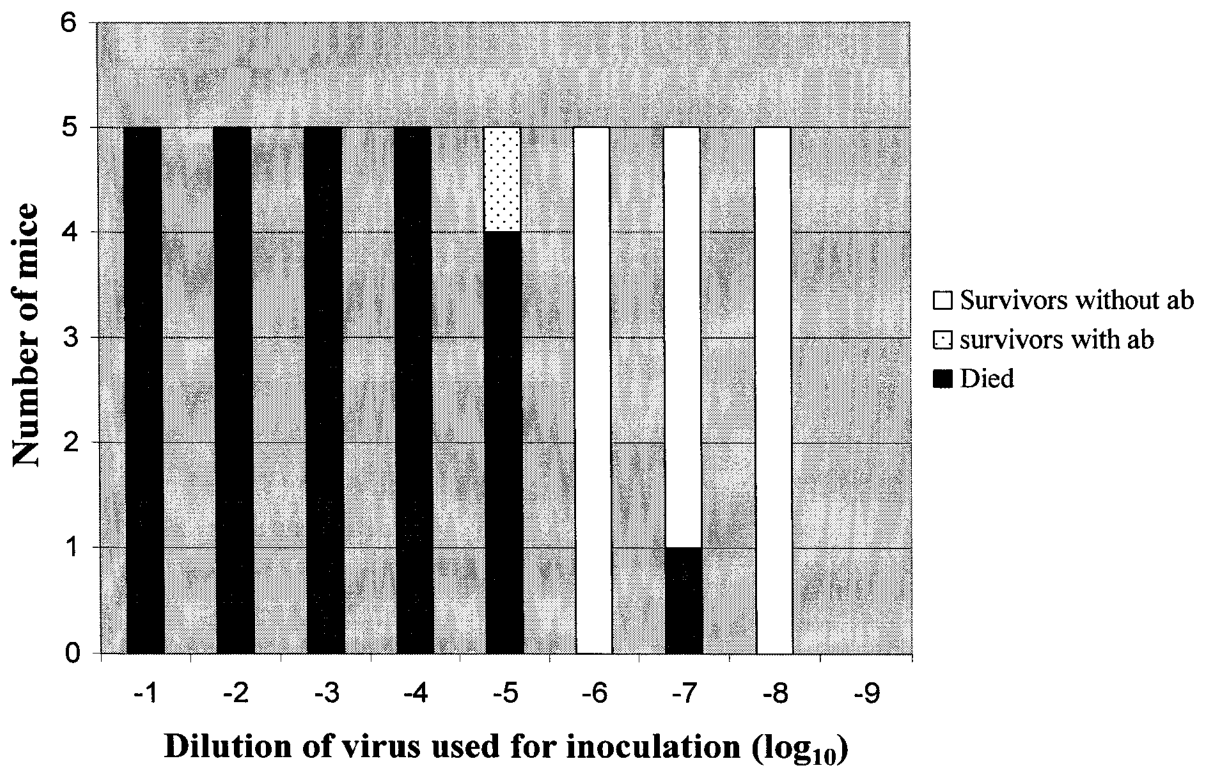

Fig.3.7. **Mortality and serology in Mice infected with ERA**

Mice were inoculated with 10 fold dilutions of ERA and monitored for 30 days. Sera, collected from mice that survived 30 days, were analyzed by cELISA to determine if they have developed any reasonable anti-rabies antibody. Numbers of mice that did or did not seroconvert are shown with the mortality data.

Fig. 3.8. Mortality and serology in Mice inoculated with ERA escape mutants

Mice were inoculated i.c. with 10 fold dilutions of each of the escape mutants isolated from ERA. The sera collected from the survivors at 30 days were detected for the presence of anti-rabies antibody by cELISA. The black areas represent the dead animals, the dotted areas show the animals which survived with seroconversion, and the white columns represent the animals that survived without any antibody production.

DISCUSSION

The glycoprotein of rabies virus has been studied extensively for many years because of its importance in antigenicity for protective immunity and its involvement in determining the neurovirulent nature of the virus (17, 66). Extensive studies of G protein have employed anti-G monoclonal antibodies (Mabs), for detailed structural analysis of the G protein and specifically for: mapping the epitopes involved in virus neutralization by recovery and characterization of viral mutants resistant to neutralization; for comparison of antigenic properties of viral strains to further knowledge of rabies virus diversity; and currently for exploring Mab utility for post-exposure treatments (19, 20, 27, 29, 32, 41, 42, 82, 103, 109).

Previous studies have used ERA and CVS strains of rabies virus to study the antigenic structure of the rabies virus glycoprotein. Epitope mapping has been done using Mabs and cross-neutralization tests. Epitopes have also been mapped using neutralization resistant escape mutants followed by gene sequencing (140). These studies have identified two major conformational epitope sites, sites II and III (27, 82, 103), one minor site (Site a) (6), and several isolated epitopes, including linear epitopes (12, 75, 99, 105) on the ectodomain of G protein. Maps obtained from cross-neutralization tests were confirmed by a few competitive binding assay studies (79, 82).

In 1991, it was suggested (6) that the term antigenic site be reserved for regions on the glycoprotein that are defined by several Mabs arising from different fusions. Regions of the protein that are defined by a single monoclonal antibody would be referred to as an epitope. Accordingly, the authors reserved the term "antigenic site" for sites II and III. Whole previously described antigenic sites I, IV, V, and VI were now referred to as epitopes. Site I is located in the region of residue 231. Antigenic site II is a discontinuous site that

involves two separate stretches of amino acids at residues 34-42 and 198-200 which are linked by disulfide bridges between cysteines 35 and 207. Site III is located at amino acids 330-338; sites IV and V have not been definitively mapped. Site VI maps to the region of amino acid 264. Escape mutants from non-neutralizable monoclonal antibodies have not been detected. However, it might be possible to select escape mutants from non-neutralizing Mabs using complement. In addition, non-neutralizable epitopes can be mapped by ELISA using peptides or polypeptides as the antigen. However, these studies have not yet been done.

Studies performed previously in our laboratory by Elmgren (36), used a competitive binding assay (cELISA) to generate topographical maps of the lyssavirus G protein. Elmgren defined antigenic sites as non-overlapping regions of the glycoprotein. Where overlap occurred, the regions were called minor sites and described by the suffix "a". This study identified four major antigenic sites and two sub-sites that were conserved in the G protein of all lyssaviruses. Most of these epitopes proved to be conformational and only two epitopes defined by Mabs 10EC9 and M818, appeared to be linear. In this study we approached the epitope mapping of G protein by isolation of escape mutants using Mabs directed to antigenic sites I and IIIa (a subsite of site III). These two sites were chosen based on the fact that most of the Mabs available in the Mab collection at CFIA/OLF are specific for these sites. Although the cELISA studies were able to identify some antigenic sites, the present study aimed at more precise characterization of the epitopes contained within these sites. Escape mutants often exhibit a single amino acid substitution, a modification that can have an effect on epitopes containing that amino acid, but may not necessarily have any detectable effect on nearby epitopes unless a conformational change takes place. Thus, the

detection of more sites by escape mutants than by competition assays is possible. The results of this study were compared to the results of the competitive binding assay to determine the accuracy of the antigenic map established previously by this method.

The escape mutants isolated from the ERA and Western Skunk viruses were tested for neutralization by a panel of antibodies and it was shown that these variants escaped neutralization not only by the Mab used for selection but also by other Mabs. The epitopes of these Mabs were therefore assumed to be functionally linked. The cross-neutralization test results of the ERA escape mutants showed a clear functional relationship between some of the epitopes (refer to Table 3.2). In antigenic site I the epitopes of Mabs 10EC9 and W101-1 appear functionally linked since both of the escape mutants isolated with 10EC9 (C12 and C8) had lost reactivity to W101-1. Similarly, the epitopes of M725 and W509-6 also appear to be linked because both escape mutants selected with M725 (B9 and G6) had lost reactivity to W509-6. On the other hand, there are some other epitopes in antigenic site I that might have a partial functional linkage with each other. 10EC9 and M725 might bind to partially overlapping regions of the G protein since one of the mutants (B9) produced with M725 no longer reacts with 10EC9. This effect however is not found in the other mutant selected with M725 (G6) or the mutant isolated with 10EC9. Therefore these two epitopes might be physically close with a very small overlap. Similarly the M725 site may be located close to the W101-1 site, with partial overlap since only one of the M725 escape mutants has reactivity to this Mab (W101-1). Fig.4.1 demonstrates a possible relationship between epitopes in antigenic site I.

In antigenic site IIIa, a close relationship between 16AH8 and M785 is evidenced by the cross-neutralization results. The mutants selected with 16AH8 are not neutralized by

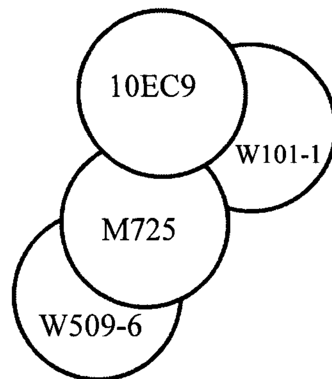

Fig.4.1. **Proposed relationship between the epitopes in the antigenic site I**

Sites for 10EC9 and W101-1 have a large overlap which results in their close functional relationship. The same relationship exists for sites targeted by M725 and W509-6. The M 725 site has a partial overlap with sites for each of the Mabs 10EC9 and W101-1.

M785 and vice versa. Another interesting aspect of the cross-neutralization results occurred where the escape mutants were neutralized with Mabs that were not neutralizing the parent virus. This effect however is mostly observed in antigenic site II of ERA mutants where M778 and M1094 gain neutralizing ability for four of the escape mutants isolated: C12 (isolated with 10EC9), C3, A11 (isolated with 16AH8), and G10 (selected with M785). Therefore there could be a possible relationship between the epitopes of M778 and M1094. In addition, these results are consistent with those of the previous study with cELISA (36), which suggested a possible linkage between each of the antigenic sites I and III with

antigenic site II. This linkage might explain the change from non-neutralizing to neutralizing activity that is seen in the Mabs of the antigenic site II in parallel with the loss of neutralizing ability of Mabs in antigenic sites I and IIIa. A change in either antigenic site I or III could change the conformation of the G protein thereby resulting in a change in conformation of antigenic site II and exposing a region that increases the binding of M779 and M1094. This relationship is evident for all of the site IIIa mutants whereas it is seen in only one of the site I mutants (C12), thus the change in antigenic site IIIa seems to have a stronger effect.

The cross-neutralization results of the escape mutants isolated from the WSk strain of rabies virus (refer to Table 3.3), confirm the relationship between 10EC9 and W101-1 in antigenic site I and the linkage between 16AH8 and M785 in antigenic site IIIa as observed with the ERA mutants. Additionally, these results indicate a relationship between 10EC9 and M818 in antigenic site I, since mutant WSk10EC9 had lost reactivity to both of these Mabs. A similar observation was not possible for ERA strain and its mutants since M818 does not neutralize these viruses. A difference in amino acid at residue 234 was noted upon comparing the ERA and WSk parental virus glycoproteins (see Appendix); in ERA this residue is arginine-234 and in WSk it is lysine-234. This difference in close proximity to antigenic site I may explain the differential binding of M818 to these two viral strains.

Sequence analysis of ERA escape mutants isolated by the Mabs to antigenic site I shows two different mutation patterns. B9 and G6, mutants which were isolated with M725, have a substitution at position 231 where a leucine is replaced by proline. This amino acid has been identified as a critical residue in the antigenic site I by previous studies of neutralization resistant mutants (32, 147). It is likely that Leu-231 is required for binding of

M725. Since results from the cross-neutralization studies indicate that the epitopes of M725 and W509-6 are closely linked, this residue also likely forms part of the attachment site of W509-6. This is in agreement with the studies performed previously with Mab W509-6 where residue 231 was identified to be the critical residue for binding of W509-6 (32). The other two antigenic site I mutants which were produced by 10EC9 exhibit changes in antigenic site II. At position 40, glycine is replaced by arginine in C8 and by glutamate in C12. As mentioned before, results from competitive binding assays suggest that sites I and II may be interrelated (36); it is thus proposed that amino acid substitution in site II may cause a conformational change in site I that hides the binding region of 10EC9 or W101-1. Confirmation of this hypothesis would require resolution of the tertiary structure of rabies G protein. The sequence analysis of the antigenic site III mutants identifies a change of amino acid residue 336 where asparagine is replaced with lysine (antigenic site III). This change clearly affects the binding region of both Mabs 16AH8 and M785, which therefore appear to be closely related.

In the ERA escape mutants there are some other changes that might be important since they occur in more than one escape mutant, although they are located outside of any previously described antigenic site. For example, histidine replaces arginine at position 120 in all of the escape mutants except C12. However, the importance of this replacement is not clear. Additionally, methionine replaces thereonine at position 452. A methionine is also present in this position in the SAD strain of rabies virus, a strain closely related to ERA and having very similar properties with respect to its pathogenic nature. This specific mutation therefore might not be of functional significance. Single nucleotide mutations were observed in a number of escape mutants (data not shown). Many of these were silent mutations that

did not affect the protein sequence, whereas others resulted in changes in amino acids located outside of any known antigenic site, some of these changes were present in only one of the escape mutants. The role these additional amino acid substitutions might play in the neutralization resistant phenotype or altered pathogenesis of the mutants is not known.

The sequence analysis of the WSk escape mutants confirms the relationship between the antigenic site II mutation and the binding region of 10EC9 and W101-1. The escape mutant isolated from the WSk strain in the presence of 10EC9 (WSk10EC9) showed amino acid substitution at position 40 in antigenic site II resulting in replacement of glycine with arginine. This amino acid replacement is similar to the substitution present in C8. Since C8 was the only ERA escape mutant produced with 10EC9 that did not show a decrease in pathogenicity *in vivo*, this specific mutation likely does not have a role in pathogenesis. The WSk escape mutant selected with 16AH8 (WSk16AH8) contained a change at amino acid residue 336 (antigenic site III), where aspargine is replaced with aspartic acid. An amino acid substitution at the same position but to a lysine residue was present in the ERA escape mutants isolated with 16AH8. Therefore results from both the ERA and WSk mutants confirm that asparagine-336 is likely a key residue in the epitopes recognized by 16AH8 and M785. This result is consistent with previous studies which mapped antigenic site III to amino acids 330-338 of ERA and CVS strains of rabies (109, 145, 147).

One of the interesting observations in this study was the cytopathogenic effect of the escape mutants isolated from ERA. In all of the studies performed to date, there has been no report linking increased cytopathogenicity with the emergence of neutralization resistant escape mutants. All attempts to identify any adventitious agents in the viral preparations, (either by histology or electron microscopy) failed, so it is concluded that this increased

cytopathogenicity is a property of the mutant viruses. At this time there is no clear explanation for why these mutants cause a level of cell death not observed with the parent virus. It may be speculated that the abnormal mitochondria observed in mutant-infected cells might contribute to the observed CPE (refer to Fig.3.5.E). Additionally, the mitochondria were appeared to be less abundant in the infected cells. In a growth rate study which was performed to determine if this cytopathic effect was due to change in the growth rate of these viruses, again results were not clear cut. Four of the escape mutants (B9, G6, C3, and A11) showed a higher growth rate after 24 hr compared to ERA. B9, G6 and A11 continued to grow more rapidly between 24 and 36 hr. C8 showed a more rapid growth than the ERA parent over this same time point. However the other three escape mutants (C12, C8, and G10 in after 24 hr, and C12, C3, G10 after 48 hr), did not show such rapid growth rate and yet still caused similar cell death. Between 36 and 48 hr, the growth rate for C8, B9, G6, and A11 decreased, perhaps because the cells' energy resources were depleted from the previous rapid viral growth. Over all there is no clear relationship between the viral growth rate and the cytopathogenicity of these viruses. The WSk escape mutants did not cause a CPE comparable to that of the ERA mutants.

Previous studies have recognized that specific substitutions in the G protein reduce or abolish neuroinvasiveness without imparing the ability of the virus to multiply in cell culture. All avirulent mutants isolated from different rabies virus strains carry a single substitution in the glycoprotein, where arginine 333 is replaced by cysteine, glutamine, glycine, leucine, methionine, or serine in CVS (109, 129), by isoleucine in ERA (32), and by glutamate or serine in SAD (74). Two avirulent mutants of SAD, SAD Avirulent Gif (SAG1, with a serine substitution at residue 333) and SAG2 (with a glutamate substitution at residue

333), differ in their stability. The non-pathogenic status of SAG1 can readily revert back to the pathogenic levels typical of SAD by a single base change whereas this has not been observed for SAG2 in which the mutated codon differs by two nucleotides from the SAD arginine codon (74). It is notable that there was no mutation at position 333 in any of the escape mutants. In addition, all of the mutations observed for the ERA and WSk escape mutants generated in this study are single nucleotide changes which might be readily reversible in the absence of the selective Mab.

Three of the seven escape mutants used for i.c. inoculation of mice showed a decrease in pathogenicity compared to parent virus (ERA). These escape mutants were C12, B9, and G6 which were selected with the Mabs to antigenic site I. In contrast, the C8 variant, produced with a Mab to antigenic site I (10EC9), is the only escape mutant of this group that did not show any reduction in pathogenicity. As mentioned before, B9 and G6 mutants which were isolated with M725, have a proline substitution in place of leucine at position 231. This leucine residue could be the site of attachment for M725 and might also be responsible for the reduced of pathogenicity observed for these mutants. This mutation is not present in the other less pathogenic mutant (C12). The other two antigenic site I mutants which were produced with 10EC9 (C12 and C8), exhibit a substitution in antigenic site II where glycine at position 40 is replaced by arginine in C8 and by glutamate in C12. The distinct physical properties of these two amino acids may explain the difference in pathogenicity between C12 and C8. Since the replacement of glycine by arginine is present in the WSk10EC9 mutant, further testing of this mutant's pathogenicity in mice might help to demonstrate whether this change is responsible for the maintenance of pathogenicity in C8 as compared to C12.

All mice inoculated with either the parent virus (ERA), or the escape mutants (C8, C3, A11, or G10), either died or survived without producing any anti-rabies antibody. One possible explanation for this phenomenon is that the amount of virus inoculated was not enough to initiate infection in the survivors. Where there were enough virus particles to elicit infection, the disease spread so quickly that there was insufficient time for the animal to produce antibody levels capable of limiting the infection. The mice inoculated with G10 showed an interesting pattern. All of the animals that received 10^{-1} dilution of the virus survived, with the production of neutralizing antibody, while the 10^{-2} and 10^{-3} dilutions were fatal. This could be because the high virus load in the first dilution was capable of triggering the immune system rapidly and causing a rapid and effective production of neutralizing antibodies, whereas the more diluted inocula were able to establish a productive infection in the absence of a strong humoral immune response. The three less pathogenic escape mutants; C12, B9, and G6, either caused a fatal disease or triggered the immune system to produce an effective neutralizing antibody to suppress the infection, thus leading to the survival of the animal. Wherever there was no anti-rabies antibody detected in the survivors, these animals may not have encountered enough viral particles to initiate the infection. These attenuated mutants might be able to protect the animals against a challenge with wild type strain.

We were able to isolate neutralization resistant mutants from ERA, but not from BBB or SHB strains of rabies virus. Because of the limited replication fidelity of the viral polymerase activity, caused by an absence of proof reading and post-replicative error correction, it is often reported that RNA virus populations exhibit a heterogeneous population structure within single individuals, known as quasispecies (34, 35, 54). It has

been demonstrated that in a constant environment, quasispecies contain a stable, dominant viral species within a pool of viruses with related, but often extremely heterogeneous, genomes (33). This complexity allows RNA viruses to adapt to changing host environments. Antibody escape mutants could occur from naturally existing quasispecies present in the virus inoculum (90). Previous studies have shown that in wild isolates a relative genetic stability and a limited process of genetic radiation around a prototype sequence exists (71, 104). The reason that we were not able to select any escape mutants from two of the wild type strains (SHB and BBB) might lie in the fact that these viruses are less polymorphic compared to ERA which is highly tissue culture adapted and which therefore might contain a more variable range of quasispecies in its population; this more variable population might make it easier to select mutants with a Mab. Conversely, if there is not a large range of variants available in a wild type population, the appropriate mutant might not exist at all or it might not be in a large enough concentration to be selected under the conditions used in this study. Clearly such a situation did not exist for the WSk variant, as two escape mutants were able to be selected from this street virus.

CONCLUSIONS

This study aimed to further investigate the relationships between the epitopes of two previously defined antigenic sites of the rabies G protein. In agreement with the results of a previous study that employed competitive binding assays, the positions of the epitopes for the Mabs specific for antigenic site I and IIIa were confirmed. Close relationships between the epitopes of the 10EC9 and W101-1 Mabs, those of Mabs M725 and W509-6 in antigenic site I and those of Mabs 16AH8 and M785 in antigenic site IIIa were detected in both of the rabies strains examined (ERA and WSk). In antigenic site I, a partial relationship between the site for M725 and the sites for 10EC9 and W101-1 was observed in the ERA strain. Furthermore, the data supports a relationship between the sites bound by Mabs M818 and 10EC9 in the WSk G protein.

The sequence analysis suggest a possible relationship between antigenic site II and the epitope of the 10EC9 in antigenic site I. Position 231 in antigenic site I is likely a key residue in the site of both M725 and W509-6. Position 336 in antigenic site III appears to be critical residue in the binding site for both 16AH8 and M785. There were no mutations in position 333 in antigenic site III considered to be important for viral pathogenicity, suggesting that this amino acid is not a critical part of the epitopes recognized by 16AH8 and M785.

All of the escape mutants isolated from ERA were more cytopathogenic than the parent virus, as they caused cell death soon after infection. The cell death appears to be due to the rabies variant infection since we were not able to detect any other bacterial or viral infection in the cells. This effect did not correlate well with a more rapid growth of the

mutants since some of the more slowly growing mutants were also highly cytopathogenic.

An extension to this study would be the investigation of the mutants isolated with the neutralizing Mabs to the other antigenic sites. The data from these studies would further determine the positions of the epitopes on the G protein of the rabies virus and would clarify the antigenic map that is presently available. It would be beneficial to examine the pathogenicity of the escape mutants isolated from the WSk virus in mice for comparison to the results obtained from similar tests done with ERA escape mutants. It would also be interesting if the isolation of escape mutants using non-neutralizing Mabs could be achieved. This would provide further insight into the location of the epitopes of the G protein of the rabies virus, the key protein involved in the binding of virus envelope with biological membranes and the elicitation of neutralizing antibodies (144). Identification of the important G protein epitopes would contribute to our understanding of these processes which are so critical to rabies virus pathogenesis.

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APPENDIX