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EPITOPE MAPPING OF LYSSAVIRUS STRUCTURAL PROTEINS

A Thesis

Submitted to the School of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in the

Department of Biochemistry, Microbiology, and Immunology

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University of Ottawa

by

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Ottawa, Ontario

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ABSTRACT

Lyssavirus specific monoclonal antibodies (Mabs) were used in competitive ELISAs to topographically map two major structural proteins of representative viruses of each of the six lyssavirus genotypes. This is the first description of topographical mapping of lyssavirus proteins based upon competitive Mab binding. A panel of anti-glycoprotein Mabs, all generated against rabies viruses, was used to map antigenic sites on lyssavirus glycoproteins. These Mabs identified thirteen unique epitopes that define four major antigenic sites and two antigenic sub-sites. Glycoprotein antigenic sites were found to be highly conserved throughout all lyssavirus genotypes. Thirty-four unique epitopes were identified on lyssavirus nucleoproteins using a panel of Mabs comprised of representative Mabs generated against nucleoproteins of all lyssavirus genotypes. The panel of anti-nucleoprotein Mabs used in this study revealed five major antigenic sites that again were largely conserved in all lyssavirus genotypes. The epitope variability of the nucleoprotein was much greater than that of the glycoprotein, confirming previous Mab studies of lyssaviruses. Mabs to several epitopes of both structural proteins studied were lyssavirus specific, while at least one anti-nucleoprotein Mab for each lyssavirus was genotype specific with the exception of genotype 5 (European bat lyssavirus type 1). Most of the epitopes identified on both proteins were conformational, however, certain linear epitopes identified in this study that are lyssavirus specific may be useful for diagnosis and vaccine development. Genotype specific epitopes will be useful epidemiologically. The competitive antigenic analysis of the glycoprotein and nucleoprotein support the published phylogenetic relationships of the lyssavirus genotypes. Two of the African lyssaviruses (Mokola virus and Lagos Bat virus) are the

furthest diverged of the lyssaviruses. The nearly identical topographical maps of the antigenic sites of both the glycoprotein and nucleoprotein strongly suggest that lyssaviruses have evolved from a common rhabdovirus ancestor. Further, this ancestral rhabdovirus was probably a bat virus.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 Lyssavirus taxonomy

Lyssaviruses are members of the virus family *Rhabdoviridae*. Rhabdoviruses are very likely the most widely distributed of viruses in nature, and can infect and cause disease in vertebrates, invertebrates and plants (Wagner 1990). More than 200 different rhabdoviruses are known at present (Tordo et al. 1998). Along with the virus families *Filoviridae* and *Paramyxoviridae*, the *Rhabdoviridae* belong to the virus order *Mononegavirales*. Members of this order all have linear non-segmented genomes of negative polarity surrounded by a helical capsid. A phylogenetic tree describing the Rhabdoviridae is shown in Figure 1.1. The main rhabdoviruses that infect mammals are the vesiculoviruses and the lyssaviruses. The type species of the *Vesiculovirus* genus is vesicular stomatitis virus (VSV) and the type species for the *Lyssavirus* genus is rabies virus. Another rhabdovirus genus that infects mammals is the *Ephemerovirus* genus, of which bovine ephemeral fever virus is the type virus. Tropical haematophagous insects transmit members of this genus to cattle and water buffalo. Members of the Rhabdoviridae family that infect fish belong to the proposed *Piscivirus* genus (Morzunov et al. 1995). More recently, a new virus genus, the *Aquarhabdoviridae* (Bjorklund et al. 1996) has been proposed for the fish rhabdoviruses. Plant rhabdoviruses have so far been placed into one of two genera in the *Rhabdoviridae* family, depending on the cellular location used for replication (Tordo et al. 1998). These genera are the *Cytorhaboviruses* and the *Nucleorhabdoviruses* (Wunner et al. 1995).

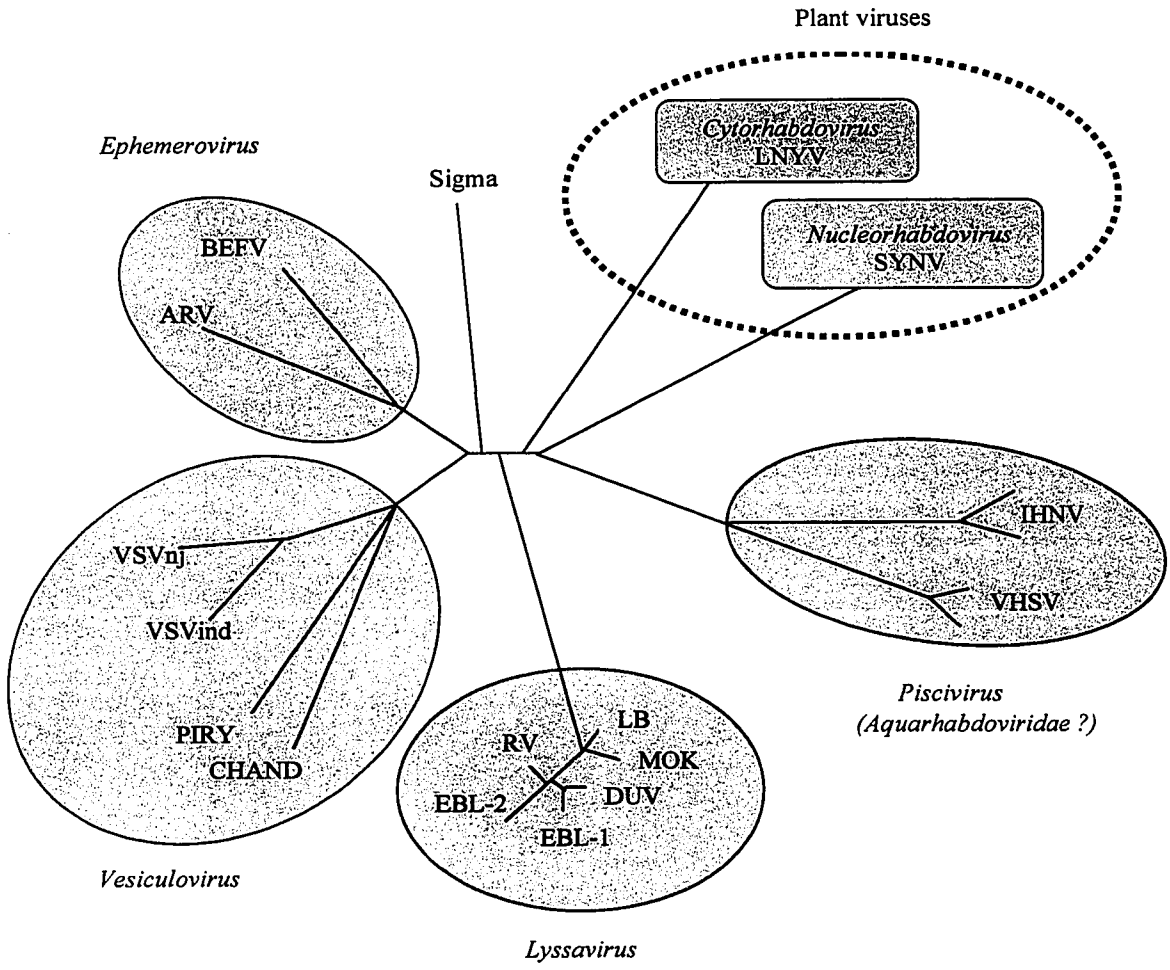


Figure 1.1 Radial phylogenetic tree of the *Rhabdoviridae* family. Grey circles outline the major genera and the dotted line outlines the plant rhabdovirus genera. ARV, Adelaide River virus; BEFV, bovine ephemeral fever virus; CHAND, Chandipura virus; DUV, Duvenhage virus; EBL-1, -2, European bat lyssavirus types 1 and 2; IHNV, infectious haematopoietic necrosis virus; LB, Lagos bat virus; LNYV, lettuce necrotic yellow virus; MOK, Mokola virus; PIRY, piry virus; RV, rabies virus; Sigma, sigma virus; SYNV; sonchus yellow net virus; VHSV, viral haemorrhagic septicaemia virus; VSVind, vesicular stomatitis virus, Indiana; VSVnj, vesicular stomatitis virus, New Jersey. After Tordo et al. (1998).

Before the routine use of monoclonal antibodies and other molecular techniques, all rabies viruses were thought to be the same serologically. The discovery of two new African viruses in the 1960's (Lagos bat and Mokola viruses) changed this view (Shope et al. 1970). Using complement fixation and neutralization tests, it was shown that three distinct sero-groups existed within the rabies and rabies-like viruses. After the discovery of Duvenhage virus (Meredith et al. 1971), the lyssavirus genus was traditionally divided into four serotypes based on serum neutralization and monoclonal antibody studies (Schneider et al. 1973, 1985) . These serotypes included classic rabies virus (serotype 1), Lagos bat virus (serotype 2), Mokola virus (serotype 3), and Duvenhage virus (serotype 4). European bat lyssaviruses were either left unclassified or were grouped together in serotype 4 (Schneider and Cox 1994). The use of more extensive monoclonal antibody panels (King 1993) and molecular biology techniques (Bourhy et al. 1993a, 1993b; Bourhy et al. 1992; Tordo et al. 1993) have since allowed a more precise characterization of lyssaviruses. Phylogenetic analyses of the nucleoprotein and glycoprotein genes have clearly delineated six lyssavirus genotypes, the first four of which match the previously defined serotypes. The two other genotypes are European bat lyssavirus type 1 (EBL-1) (genotype 5) and EBL-2 (genotype 6) (Bourhy et al. 1993a). A seventh lyssavirus genotype may now have to be created for the recently discovered pteropid bat lyssavirus isolated in Australia in 1996 (Tordo et al. 1998). Antigenic and genetic analysis revealed that the Australian bat lyssavirus is closely related to, but distinct from, rabies virus (genotype 1).

1.2 Lyssavirus physical characteristics

All rhabdoviruses are bullet shaped enveloped viruses with one flat end and one rounded end. The particles measure 75 – 80 nm in diameter, 180 – 200 nm in length (Hummeler et al. 1967; Vernon et al. 1972), and enclose a helical ribonucleocapsid. The ribonucleocapsid is tightly coiled 30 – 35 times (Tordo and Poch 1988) within the viral envelope to form a complete virion (refer to Figure 1.2).

The genome of lyssaviruses is an unsegmented, negative sense molecule of single stranded RNA, with a molecular weight of 4.6 X 10^6 daltons (Sokol et al. 1969) and approximately 12 kb in length (Tordo et al. 1988). The *Paramyxoviridae* and the *Filoviridae* share this feature of *Rhabdoviridae* genomes, which together make up the order *Mononegavirales* (Tordo and Kouknetzoff 1993). From the 3' to 5' end, the lyssavirus genome encodes the genes for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) (Tordo 1996).

1.3 Lyssavirus proteins

1.3.1 Polymerase

The polymerase (L), a 2142 amino acid, 244 kd molecule (Wunner 1991), accounts for approximately 54 % of the lyssavirus genome yet is the least abundant protein in the virion (Tordo 1996). Only 20-150 copies of L are present in each lyssavirus virion (Tordo 1996), consequently, it has been the least studied protein at both the biological and immunological levels. L is an RNA-dependent RNA polymerase that functions with its cofactor, the phosphoprotein (Emerson and Wagner 1972). Together

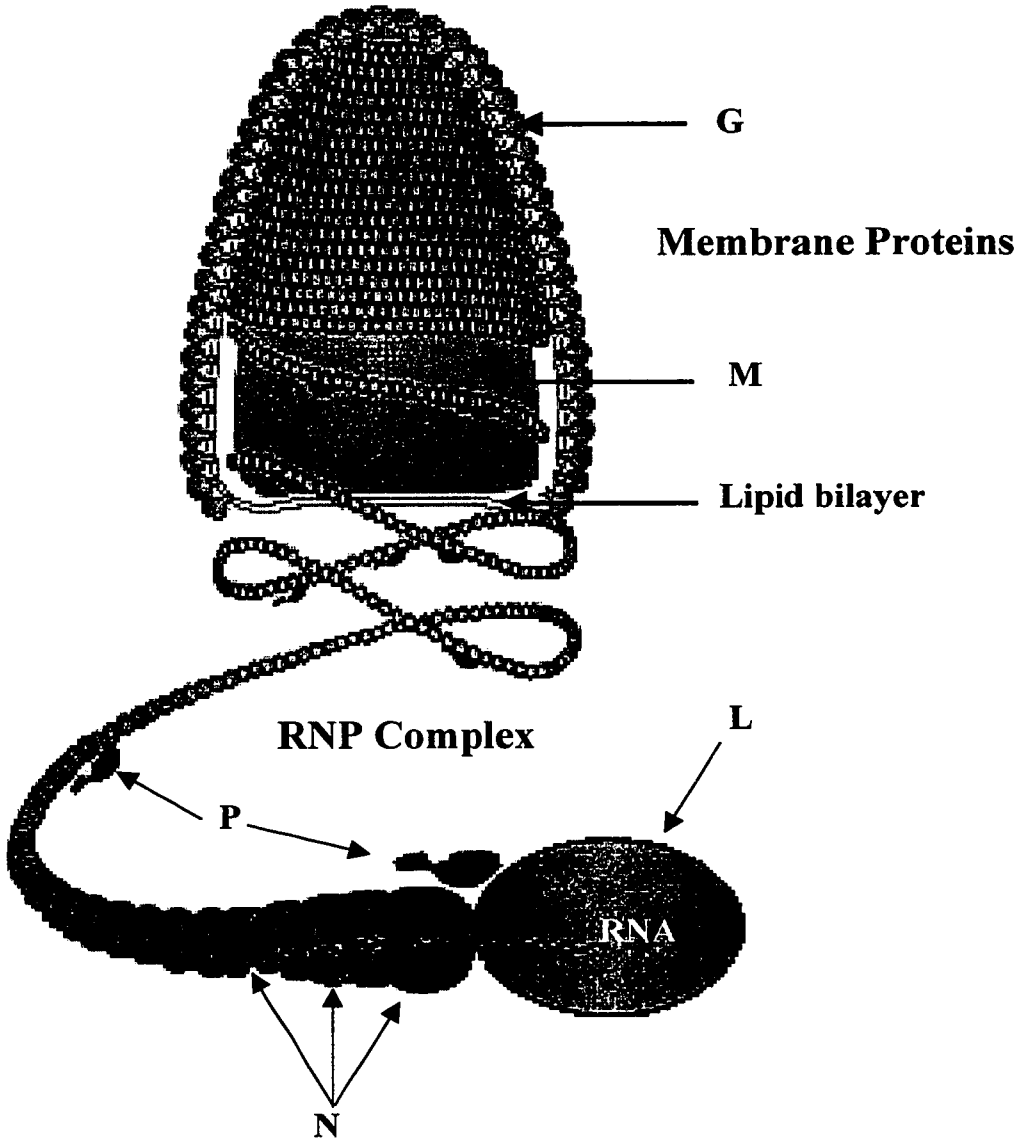


Figure 1.2 Rhabdovirus morphology. G, glycoprotein; M, matrix protein; L, polymerase; P, phosphoprotein; N, nucleoprotein; RNP Complex, ribonucleoprotein complex. After Tordo et al. (1998).

with the nucleoprotein, these core proteins form the ribonucleoprotein (RNP) complex that interacts with the RNA genome. Within the L protein gene is maintained six conserved areas throughout the *Mononegavirales* (Poch et al. 1990) as well as all known viral RNA-dependent RNA and DNA-dependent polymerases (Tordo et al. 1992). Despite its low copy number, the L protein has undergone extensive theoretical analysis and putative functions have been assigned to each domain (Poch et al. 1990). Also, advances in molecular biology have recently allowed detailed analysis of the lyssavirus L genes and proteins (Conzelmann and Schnell 1994). The catalytic centre of the polymerase has been located within the highly conserved amino-terminal half of the L protein (Schnell and Conzelmann 1995) and the carboxy-terminal is required for interaction with the phosphoprotein cofactor (Chenik et al. 1998).

1.3.2 Phosphoprotein

The lyssavirus phosphoprotein (P) is a part of the ribonucleoprotein complex. It is a highly phosphorylated protein with an apparent molecular weight of 40-45 kd when resolved by SDS-polyacrylamide gel electrophoresis, or 33 kd when calculated from the deduced protein sequence (Wunner 1991). This discrepancy in molecular weights is due to varying levels of phosphorylation. The P gene is less well conserved than other lyssavirus genes (Nadin-Davis et al. 1997). Historically, this protein has been called the M1 protein because it was believed that it was a membrane protein (Sokol 1975), however, once it was discovered that it was part of the RNP complex, it was called the NS (non-structural) protein, the name of its counterpart in vesicular stomatitis virus (Cox

et al. 1981). Due to its highly phosphorylated nature, it is now referred to as P, or nominal phosphoprotein, consistent with the naming of other viral phosphoproteins.

The functions of the lyssavirus P are unclear, but since rabies virus and VSV are structurally and chemically similar, their phosphoproteins may have similar properties. The VSV P is a regulatory protein involved in replication and transcription and associates with both the N and L proteins (Emerson and Schubert 1987; Masters and Banerjee 1988; Takacs and Banerjee 1995). Varying levels of phosphorylation leads to different P subspecies that bind with different affinities to the RNP template and have different transcription activities (Barik and Banerjee 1992; Gao and Lenard 1995). The rabies virus P has at least two differently phosphorylated forms (Tuffereau et al. 1985). In cell culture, up to four additional proteins, translated from the P gene due to a leaky scanning mechanism, have been identified with unknown functions (Chenik et al. 1995). As has been reported for VSV (Takacs et al. 1993), two domains of the rabies virus P are involved with binding of N both *in vivo* and *in vitro* (Chenik et al. 1994; Fu et al. 1994). The binding site of the P to the L has been identified as being within the first 19 amino acids of the amino-terminus of P (Chenik et al. 1998).

1.3.3 Nucleoprotein

The nucleoprotein (N) is the most abundant protein in the RNP complex, with twice as much N present as P (Wunner 1991). The sequence of this 450 amino acid protein has been deduced from the nucleotide sequence of several vaccine isolates of rabies virus (Tordo et al. 1986; Wunner et al. 1988; Ertl et al. 1989; Conzelmann et al. 1990) and shows high levels (98-99.6 %) of amino acid sequence conservation among

these strains (Fu et al.1994a). Greater diversity is seen in the nucleotide sequence of the N gene of genotype 1 (rabies) where mainly synonymous substitutions allow for the establishment of phylogenetic lineages within this genotype (Nadin-Davis et al. 1993, 1994; Kissi et al.1995).

Unlike its VSV counterpart, the rabies virus N is phosphorylated (Sokol and Clark 1973; Sokol et al. 1974). Using ^{32}P labeled peptide cleavage fragments of rabies virus N, the phosphorylation site was localized to the carboxy-terminus of the protein (Dietzschold et al. 1987). Further amino acid sequence analysis of the phosphorylated fragment and acid hydrolysis revealed that the N is phosphorylated at a serine residue at position 389.

Tightly bound to the viral RNA (Sokol et al. 1971), the N protects the RNA from ribonucleases and keeps the RNA in a suitable configuration for transcription and replication (Wunner 1991). The switch from transcription to replication of rhabdovirus genomes is controlled by the amount of N present in infected cells (Tordo et al. 1998). To be functional templates, the genome and anti-genome must be encapsidated by the N protein, which is concomitant with RNA elongation. The genome entry site for the N protein is located within nucleotides 20-30 of the 5' leader RNA (Yang et al. 1998). At low levels of N, the viral 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 increase, the concomitant encapsidation prevents the polymerase from stopping at the end of the leader RNA and replication of full length, encapsidated RNA takes place (Tordo et al. 1998). Until recently, the RNA binding site on the N itself was not known. Using full length (450 aa) and truncated forms of the nucleoprotein

along with a synthetic RNA probe corresponding to the 5' leader RNA, it has been shown that the amino terminal portion of the N contained all the determinants necessary for binding (Fu et al. 1994b; Kouznetzoff et al. 1998). Furthermore, a 55 aa peptide (position 298-352), found in the most conserved region of rhabdovirus N proteins, was demonstrated to directly bind the viral RNA (Kouznetzoff et al. 1998).

The antigenic nature of lyssavirus N proteins has to some extent been examined. Lafon and Wiktor (1985), using a panel of 37 monoclonal antibodies delineated three topographical B cell antigenic sites on the rabies virus N. Further characterization of these sites was obtained by generating peptide fragments. Fragments of the N were separated by SDS-PAGE and transferred to nitrocellulose for western blot analysis. This technique revealed that two of the antigenic sites were linear (aa 369-383 and aa 313-337) and one conformational (Dietzschold et al. 1987a, 1988). Analysis of many field virus isolates and vaccine strains of rabies virus using monoclonal antibodies has revealed that rabies viruses vary considerably in their antigenic make up. Identification of lyssavirus isolates can be made by observing these characteristic monoclonal antibody binding patterns (Dietzschold et al. 1987a, 1988; Rupprecht et al. 1991; Flamand et al. 1980a; King 1993; Smith et al. 1990; Smith and King 1996).

Besides being highly antigenic humorally, the rabies virus N protein is also a major target antigen for T helper cells (Ertl et al. 1989; Fu et al. 1994a). T cell epitopes have been identified by screening overlapping synthetic peptides spanning the entire length of the N protein with T lymphocytes from rabies virus immunized mice (Ertl et al. 1989) and by using cloned T cell hybridomas and synthetic peptides (Celis et al. 1989).

At least six T cell epitopes on the rabies virus N protein have been identified (Dietzschold and Ertl 1991).

The rabies virus N plays a role in immunoprotection. To determine the role the N protein plays, mice were immunized with synthetic peptides representing either a B cell epitope, a T helper cell epitope, or B and T helper cell epitopes in tandem (Dietzschold et al. 1989). Only mice that received both the T helper cell epitope and the B cell epitope were protected against a lethal rabies challenge, indicating that the production of protective anti-N antibodies depends on T helper cells. N specific T helper cells may also be responsible for augmenting the virus neutralizing antibody titre of T helper cell epitope or N protein primed mice upon booster immunization with inactivated vaccine (Dietzschold et al. 1989, 1987c; Fu et al. 1991). High levels of non-neutralizing anti-N antibodies may negatively regulate rabies virus replication (Fu et al. 1994b) since it has been shown that in cells scrape-loaded with anti-N monoclonal antibodies, rabies virus replication is inhibited (Lafon and Lafage 1987). In other experiments, mice immunized with a recombinant raccoon poxvirus expressing the rabies nucleoprotein showed production of anti-N antibodies and were protected against a lethal rabies virus infection (Lodmell et al. 1991). Similarly, rabies virus N expressed in and purified from insect cells is efficacious as a vaccine (Fu et al. 1991). Monkeys have also been immunized with rabies virus RNP with promising results (Tollis et al. 1991). RNP alone was able to induce protective immunity against rabies virus infection and was able to prime for the production of virus neutralizing antibodies. Monkeys primed with RNP followed by a single injection of human diploid cell vaccine developed neutralizing titres comparable to

the titres of non-primed monkeys after a second dose of vaccine. These same effects have also been demonstrated in mice with orally administered RNP (Hooper et al. 1994).

One final property of the nucleoprotein is that it behaves as a superantigen. Superantigens, unlike normal peptide antigens, bind outside the peptide groove of MHC class II molecules and cross-link the faces of the MHC molecules with the variable ß (Vß) chains of T cell receptors (Marrack and Kappler 1990). Antigen processing is not required for superantigens and they are recognized almost without MHC restriction. Both rabies virus RNP and purified N induce the proliferation of lymphocytes from unprimed donors when presented by fixed B cells (Lafon et al. 1992). Rabies ribonucleocapsid is able to preferentially activate Vß T cells in mice and humans (Lafon 1993) and can act as an adjuvant for non-related antigens (Astoul et al. 1996). Coinjection of mice with the rabies virus RNP and influenza virus resulted in a rapid and long term increase in specific anti-influenza antibodies, antigen specific proliferation, and lymphokine secretion by lymph node lymphocytes when compared to mice that received influenza virus alone. The rabies virus superantigen may also have a role in rabies pathogenesis through immune system subversion. It has been postulated (Lafon and Galelli 1996) that the rabies virus superantigen may hinder the development of an efficient immune response by activating T and B cell populations with irrelevant specificities.

1.3.4 Matrix protein

The lyssavirus matrix (M, formerly M2) protein is the smallest of the structural proteins with a molecular weight of 21-26 kd (Wunner 1991) and 202 amino acids in

length (Rayssiguier et al. 1986; Tordo et al. 1986; Wunner et al. 1988). It accounts for about 25 % of the total rabies virion protein but is the least understood of the lyssavirus proteins. The M protein and the surface glycoprotein (G) are the only membrane associated proteins in lyssaviruses (Delagneau et al. 1981). Following osmotic shock treatment of intact virions, only M and G are released. This suggested that the M protein might play a role in anchoring the membrane bound glycoprotein to the internal RNP capsid. More recent studies have revealed that rhabdovirus M proteins extend from the inner layer of the viral membrane to the internal core of the RNP complex (Barge et al. 1993). A central 19 amino acid region (aa 89-107) of the rabies virus M protein appears to be sufficiently hydrophobic to anchor the protein to the viral membrane (Tordo et al. 1986). This may be accomplished through palmitoylation of the M protein, however, this site remains to be characterized (Gaudin et al. 1991a). There appears to be a sequence of the cytoplasmic portion of the VSV glycoprotein that interacts with the M protein to stabilize the glycoprotein (Lyles et al. 1992).

The M protein is believed to play an important role in lyssavirus morphogenesis, however, because few studies have been done with lyssavirus M proteins, parallels can only be inferred from studies of its homologue in VSV (Tordo et al.1998). During VSV morphogenesis, the M protein causes condensation of the RNP (Newcomb et al. 1982) and inhibits genome transcription (Clinton et al. 1978; De et al. 1982). In VSV infected cells, rounding occurs as a result of interactions between the cells cytoskeleton (tubulin) and the M protein (Melki et al. 1994). M protein has also been shown to block host cell transcription (Black et al. 1993). As is seen in VSV, the 40 amino terminal residues of the rabies virus M protein could also inhibit transcription and replication before coiling of

the RNP (Poch et al. 1988). This same site appears to be important immunologically since a major antigenic determinant has been mapped to this region (aa 1-72) (Hiramatsu et al. 1992). Using 21 different monoclonal antibodies, six epitopes were discovered within this site.

1.3.5 Glycoprotein

The rabies virus glycoprotein (G) is the best studied of all lyssavirus proteins (Dietzschold et al. 1988; Benmansour et al. 1991; Kawai and Morimoto 1994). The spike-like peplomers that cover the outside surface of the rabies virus membrane are composed of homopolymers of the glycoprotein (Wunner 1991). Gaudin et al (1992) have shown, using electron microscopy and sedimentation analysis, that indeed the surface glycoprotein is a homotrimer of glycoprotein molecules.

The rabies virus glycoprotein is a 524 amino acid long protein translated from a gene of 1675 nucleotides (Wunner 1991; Tordo et al. 1986). The mature lyssavirus glycoprotein, a type I transmembrane protein, contains only 505 amino acids however, because the amino-terminal 19 amino acids are a signal sequence that is cleaved from the mature glycoprotein after translocation through the rough endoplasmic reticulum (Tordo 1996). Following the signal sequence is a stretch of 439 amino acids that make up the hydrophilic ectodomain of the glycoprotein, which ends at the transmembrane domain (aa 440-461). The transmembrane domain remains anchored in the membrane due to the palmitoylation of a cysteine residue at position 461 (Gaudin et al. 1991a). Extending from the transmembrane domain into the viral particle is the 44 amino acid cytoplasmic carboxy-terminal domain of the glycoprotein.

Lyssavirus glycoproteins have three or four potential N-linked glycosylation sites, depending on the virus (Anilionis et al. 1981; Tordo et al. 1986; Morimoto et al. 1989; Conzelmann et al. 1990). Glycosylation occurs at asparagine residues, but only two of these sites (247 and 319) are glycosylated in mature rabies virions (Wunner et al. 1985b) and O-linked glycosylation has not been detected. Glycosylation at site 319 is presumed to be important because it is present in all lyssaviruses sequenced to date (Tordo 1996) and is the only region that shows homology to the VSV glycoprotein (Rose et al. 1982). As seen with other viruses, glycosylation of the rabies virus G is required for intracellular transport. Treatment of rabies virus infected cells with tunicamycin results in no surface expression of the glycoprotein (Burger et al. 1991; Shakin-Eshleman et al. 1992). Core glycosylation and palmitoylation usually occur co-translationally during transport from the rough endoplasmic reticulum to the Golgi apparatus and the cytoplasmic membranes. The transmembrane and cytoplasmic regions of the glycoprotein are not necessary for proper glycosylation to occur (Wojczyk et al. 1995).

The rabies virus glycoprotein plays many roles in the pathogenesis of rabies. It is the only lyssavirus protein that can elicit neutralizing antibodies, which relates to its role in binding to cellular receptors. The glycoprotein also has a role in fusion of biological membranes, and in virus maturation.

Elucidation of the cellular receptor(s) for rabies virus is very complex issue and is not clearly understood. Evidence exists that suggests that the nicotinic acetylcholine receptor (nAChR) is the rabies virus receptor (Baer et al. 1990; Gastka et al. 1996; Lentz 1990; Lentz et al. 1982). Sequence homologies exist between rabies virus glycoprotein and the snake venom curaremimnetic neurotoxins alpha-bugarotoxin and d-tubocurarine

(Lentz et al. 1984), both of which are known to have high binding affinities for nAChR. Synthetic peptides of the neurotoxin–like region of the rabies virus glycoprotein, covering amino acids 189-214 (Kawai and Morimoto 1994), were able to compete with the snake neurotoxins (Lentz et al. 1987) for binding to the nAChR. Antibodies raised against these peptides were also cross reactive to the native glycoprotein and the neurotoxins (Donnelly-Roberts and Lentz 1991), indicating that the rabies virus glycoprotein has amino acid sequences that are capable of binding the nAChR. Another example of molecular similarity is between the gp120 glycoprotein of human immunodeficiency virus and rabies virus glycoprotein. HIV gp120 also binds to the nAChR and immunization against rabies can induce production of anti-gp120 in humans (Bracci et al. 1997).

There is also evidence that suggests that rabies virus must have receptors other than the acetylcholine receptor. Many cell culture systems, some of which lack the nAChR, are fully susceptible to rabies virus infection (Reagan and Wunner 1985). Different cellular components have been suggested as receptor molecules, including phospholipids and glycolipids (Wunner et al. 1984), as well as gangliosides (Superti et al. 1986). Conti et al. (1988) could not demonstrate a role for proteins in cellular binding of rabies virus but recently, it has been shown that a membrane protein or protein complex is involved in rabies binding to BHK-21 cells (Broughan and Wunner 1995).

Following cellular binding of rabies virus, the next step in the infection process is internalization of the virus particle by endocytosis and low pH membrane fusion in the endosome. Cell cultures infected with rabies virus undergo massive giant cell formation when exposed to acidic conditions (Mifune et al. 1982; Gaudin et al. 1991b). The

glycoprotein has the ability to cause this observed membrane fusion, even in the absence of other lyssavirus structural proteins (Whitt et al. 1991). HeLa cells transfected with rabies glycoprotein cDNA showed syncytia formation when the cells were exposed to slightly acidic conditions. The rabies virus glycoprotein adopts three different conformations depending on the pH of its surrounding milieu (Gaudin et al. 1991b). At neutral pH, the glycoprotein does not fuse membranes, but as the 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's less than 6, is responsible for membrane fusion. Increasing the pH can reverse these conformational changes. The structural transitions that the glycoprotein undergoes for fusion activities have been described (Gaudin et al. 1993). The biological function of the fusion-inactive state of the glycoprotein appears to be to prevent non-specific fusion during transport of the glycoprotein through the acidic environment of the Golgi apparatus (Gaudin et al. 1995), after which the glycoprotein acquires its native surface conformation. Amino acids responsible for controlling these conformational changes have recently been identified (Gaudin et al. 1996) using monoclonal antibodies that recognize only the acidic configuration of the glycoprotein (Raux et al. 1995). Mutants that were able to escape neutralization by these antibodies were sequenced to determine the residues involved in conformational changes. Photolabeling experiments have now identified the fusion domain of the glycoprotein to be in residues 59-221, and this domain may play a role similar to fusion peptides in other viral fusion proteins (Durrer et al. 1995).

The study of antigenic variation among rabies viruses and other lyssaviruses began with the advent of hybridoma technology in the late 1970's. Wiktor and

Koprowski (1978) were the first to produce and use monoclonal antibodies (Mabs) for detection of antigenic variants of rabies virus. These Mabs were used to examine the antigenic profiles of several rabies and rabies-related viruses, and to differentiate these viruses (Flamand et al. 1980b). At the time, 25 different Mabs were available and these were classified into 14 groups that identified antigenic determinants on the glycoprotein. Eight different lyssaviruses could be differentiated with these Mabs. As more Mabs became available, limited epitope mapping of rabies virus glycoproteins was begun. Using 25 monoclonal antibodies and 90 monoclonal antibody resistant (Mar) mutants of the Challenge Virus Standard (CVS) strain of rabies virus (Wiktor and Koprowski 1980), three antigenic sites were topographically mapped on the CVS glycoprotein (Lafon et al. 1983). Later, following the same methodologies, five antigenic sites were mapped to the Evelyn Rokitnicki Abelseth (ERA) strain of rabies virus glycoprotein (Lafon et al. 1984). These sites were all found to be conformational in nature and required glycoprotein secondary structure for monoclonal antibody recognition (Wunner et al. 1985a).

Sequencing of Mar mutants has identified the locations of most of these sites on the glycoprotein primary structure. Site I, recognized by a single monoclonal antibody, is located at residue 231 (Wunner et al. 1985b). Antigenic site II is a site that is dependent on folding of the glycoprotein. Site II Mar mutants map to amino acids 34-42 and 198-200 (Wunner et al. 1985a) which are linked by a disulfide bridge between cysteines 35 and 207. Site III is at amino acids 330-338 (Wunner et al. 1985a). Sites IV and V have not been definitively mapped (Tordo 1996).

A sixth, linear antigenic site was found after monoclonal antibodies were produced against the Pitman-Moore (PM) strain of rabies virus (Bunschoten et al. 1989).

Antigenic site VI maps to amino acid 264 (Dietzschold et al. 1990) and has been analysed by Pepscan analysis to determine the exact amino acid sequence of the epitope (van der Heijden et al. 1993). The discovery of this and other linear epitopes (identified below) has suggested the possible use of synthetic peptide(s) as vaccines (Dietzschold et al. 1990).

With the continued discovery of epitopes and antigenic sites on lyssaviruses, it was suggested that a new system of nomenclature was needed (Benmansour et al. 1991). Since 97% of anti-G monoclonal antibodies tested belong to antigenic sites II and III as defined by Lafon et al. (1984), 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 the glycoprotein that are recognized by single monoclonal antibodies would be simply epitopes (Benmansour et al. 1991). This last category would include what were previously referred to as antigenic sites I, IV, V, and VI. The first minor antigenic site, including six overlapping epitopes, was defined by Benmansour (1991) as minor antigenic site "a" at amino acids 342-343. Lafay et al. (1996) defined two more linear minor antigenic sites at amino acids 251 and 264. The site at amino acid 264 includes the former antigenic site VI. Two final linear epitopes have been described at positions 263 (Ni et al. 1995) and at 251 (Luo et al. 1997).

Antigenic site III is critical for rabies virus pathogenicity. Avirulent Mar mutants of rabies virus were isolated using monoclonal antibodies to site III determinants (Coulon et al. 1982). Analyses of tryptic peptides of rabies virus glycoprotein from pathogenic and non-pathogenic viruses were compared and the residue responsible for virulence was found to be amino acid 333 (Coulon et al. 1983; Dietzschold et al. 1983). 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.

1.4 Lyssavirus epidemiology and evolution

Virus epidemiology is the behavior of a specific virus in host populations. This makes the epidemiology of lyssaviruses very complex because all lyssaviruses have their own very defined and specific host ranges. Before the use of monoclonal antibodies, all serotype I (rabies) viruses were considered to be sufficiently identical on the basis of standard immunological tests and were either sylvatic or urban in nature. Because clear lines cannot be easily drawn between wildlife and urban rabies, it may be better to classify lyssaviruses as circulating in domestic animals or in wildlife (Cherkassky 1989).

The use of monoclonal antibodies has revealed that antigenic and phenotypic differences do indeed exist between rabies virus isolates. Monoclonal antibody reactivity patterns have allowed a clear differentiation to be made between rabies vaccine strains (Wiktor and Koprowski 1978; Flamand et al. 1980b), rabies virus isolates from different geographical regions (Sureau et al. 1983), and rabies viruses circulating in different host populations (Smith 1988; Smith and Baer 1988; Smith et al. 1984). Molecular biology techniques have gone even further in revealing strain differences between rabies viruses that monoclonal antibodies cannot reveal. These mostly involve unique nucleotide substitutions that do not change the amino acid sequence of the protein, as seen in the N protein of rabies viruses circulating in Ontario (Nadin-Davis et al. 1993, 1994).

Host species of lyssavirus epizootics vary considerably, however most are of the orders *Carnivora* and *Chiroptera.* Carnivore hosts are small (0.4-20 kg) omnivores that

scavenge on small vertebrates and invertebrates, fruit, and human refuse. These adaptive animals reach high population densities near human settlements and have very high intrinsic population growth rates. This allows them to recover rapidly from decimation by persecution or disease, and to support initial epidemics of high case density. After recovery, oscillating disease prevalence is seen for many years (Wandeler 1991). Chiropteran hosts are quite different from carnivore hosts. Bats are small, long lived, and have low intrinsic population growth rates (Tordo et al. 1998). Much about these ecological specialists remains unknown.

Rabies viruses (now classified as genotype 1) that circulate in wild carnivore hosts have been documented in several parts of the world. In the northeastern and subarctic parts of North America, eastern and central Europe, and some areas of Asia, the red fox (*Vulpes vulpes*) is the principle rabies host (Blancou et al. 1991). The arctic fox (*Alopex lagopus*) is the principle rabies virus host in arctic regions of North America and Asia (Crandall 1991) while the striped skunk (*Mephitis mephitis*) is the principle host in the midwest regions of North America (Charlton et al. 1991). Other carnivore rabies hosts in North America include the raccoon (*Procyon lotor*) along the eastern seaboard of the United States (Winkler and Jenkins 1991), and the grey fox (*Urocyon cinereoargenteus*) and coyote (*Canis latrans*) in Texas (Clark et al. 1994; Rohde et al. 1997). Although genotype 1 virus circulates in wild populations of coyotes and grey foxes in Texas, these are believed to have originated from domestic canine rabies in Mexico (Clark et al. 1994). In southern parts of Africa, wildlife rabies hosts include the jackal (*Canis sp.*) (Bingham and Foggin 1993), yellow mongoose (*Cynictis penicillata*) and the bat-eared fox (*Otocyon megalotis*) (King et al. 1994). In the Caribbean,

mongooses (*Herpestes auropunctatus*), which were introduced by colonists, have become the main rabies host (Everard and Everard 1985). Numerous bat species in North America also serve as hosts for rabies viruses (Baer 1991; Baer and Smith 1991). Monoclonal antibodies have revealed at least 12 distinct rabies virus variants circulating in bats in Canada (Tordo et al. 1998). In South America, parts of Africa, and large parts of Asia, the dog (*Canis familiaris*) is the main host for rabies of domestic animals. Dogs make up 95 % or more of all diagnosed cases of rabies in these regions (Tordo et al. 1998).

The hosts of the remaining five genotypes of lyssaviruses are not as precisely defined as they are for the genotype 1 rabies virus. Genotype 2 (Lagos bat virus) was first isolated from fruit bats (*Eidolon helvum*) in Nigeria (Boulger and Porterfield 1958) and has since been isolated several times from fruit bats in southern Africa. Lagos bat virus has never been implicated in disease of humans (King 1989), but has been isolated from cats (Schneider et al. 1985) and dogs (Mebatsion et al. 1992). The Lagos bat isolates from cats were originally reported as Mokola virus (King et al. 1994). Mokola virus (genotype 3) was first isolated from shrews (*Crocidura sp*) in 1970 (Shope et al. 1970) and has also been isolated from domestic cats and dogs (Foggin 1983; Mebatsion et al. 1992). Unlike Lagos bat virus, Mokola virus is fatally infectious in humans (Kemp et al. 1972). In contrast to Lagos bat virus, Duvenhage virus (genotype 4) probably uses insectivorous bats as hosts. Although the first Duvenhage virus was isolated from a fatal human case (Meredith et al. 1971), it has since been isolated from a bat (King et al. 1994). The European bat lyssaviruses have insectivorous bats as their primary hosts. EBL-1 virus (genotype 5) is isolated primarily from *Eptesicus serotinus* bats and EBL-2

virus (genotype 6) is isolated primarily from *Myotis sp* bats (WHO 1994). Both types of European bat lyssaviruses have been responsible for deaths in humans (Bourhy et al. 1992).

In lyssavirus epidemiology, the principle host is not always the only species involved in the epizootic, however, involvement of other animal species may not be significant to the maintenance of the epizootic. Each principle host species has its own biological and social patterns that allow it to be uniquely able to support an epizootic of a specific lyassavirus. Host qualities determine which virus variants are able to survive because survival of the virus depends on the ability of the host to transmit the virus to enough susceptible individuals during the relatively short period of virus excretion (Wandeler 1991). For lyssaviruses to have survived, they must have adapted to the social characteristics and the unique biology of their hosts (Bacon 1985; Wandeler 1991; Wandeler et al. 1994). Each lyssavirus host has its own virus variant that has a uniquely adapted pathogenicity that allows for viral persistence in the environment. This serves to explain why all isolates from the area of a specific epizootic show very little antigenic variability, regardless of the species involved, even if the species has its own variant in another geographic location. Through the use of monoclonal antibodies and molecular biology techniques it has been very clearly shown that distinct variants of lyssaviruses showing phylogenetic relationships circulate among different host species populations (Rupprecht et al. 1991; Benmansour et al. 1992; Sacramento et al. 1992; Smith et al. 1992; Bourhy et al. 1993a, 1993b; Kissi et al. 1995; Nadin-Davis et al. 1993, 1994; Nel et al. 1993; Smith et al. 1993; Tordo et al. 1993).

Despite the antigenic differences noted among different lyssavirus variants, all lyssaviruses must have evolved from a common ancestor (Wandeler 1991). The very large degree of similarity between the genomes (Tordo and Poch 1988) and some epitopes (King and Crick 1988) clearly shows an evolutionary relationship among lyssaviruses. The nature of the ancestral lyssavirus can only be speculated upon. It has been proposed that carnivore rabies may have originated from bat rabies (Wandeler 1991), which may also be an explanation for the emergence of raccoon rabies in the United States. A new rabies epizootic of Florida raccoons in the 1950's may have originated from a bat virus (Winkler and Jenkins 1991).

Evolution of RNA virus genomes is probably due to point mutations and recombination events (Steinhauer and Holland 1987). In the *Rhabdoviridae*, no evidence exists for recombination of viral genomes in street virus isolates, therefore lyssavirus evolution must be driven by point mutations (Wandeler et al. 1994). Mutations in viral RNA genomes occur at a high rate when compared to DNA genomes. Virus RNA genomes mutate from a thousand to a million times faster than their DNA virus counterparts (Holland et al. 1982), which is likely due to the absence of intrinsic proofreading mechanisms associated with RNA polymerases (Steinhauer and Holland 1987). Direct measurements of the error rate of lyssavirus RNA polymerases have not been made but they are probably close to those of VSV, which have been measured at 1-4 x 10^{-4} per base incorporated (Steinhauer and Holland 1987). This level of mutations in a virus population is evidence that RNA virus genomes exist as very heterogeneous populations or as quasispecies. The term quasispecies was first used to describe a population of self-replicating RNA molecules (Eigen and Schuster 1977) having a high

mutation rate (Eigen and Biebricher 1988). Quasispecies are then a highly heterogeneous population of mutated RNAs held together in a dynamic equilibrium as a result of mutation and selection (Domingo et al. 1985).

Such high levels of RNA heterogeneity have been observed in rabies virus populations. Upon adaptation of a human rabies virus to growth on cell culture, five percent divergence was seen from the original canine virus (Benmansour et al. 1992). This emphasizes the importance of performing genetic analyses on virus containing material derived from natural hosts as opposed to cell culture adapted variants (Wandeler et al. 1994). More recently, a population of rabies virus was shown to contain conserved minor populations exhibiting different biological properties that became dominant in different environments (Morimoto et al. 1998). In a constant environment, virus quasispecies maintain a dominant virus species within a pool of viruses with extremely heterogeneous genomes (Domingo and Holland 1988; Domingo 1992). This complexity affords RNA viruses the capacity to adapt to changing host populations and may explain the emergence of new viral strains (Novella et al. 1995). For lyssaviruses, this may explain the emergence of both raccoon rabies (Winkler and Jenkins 1991) and silver-haired bat rabies (Rupprecht et al. 1995; Morimoto et al. 1996).

Variability within wild lyssavirus genomes has been studied at the level of the non-coding pseudogene, the N gene and the G gene. In an analysis of the pseudogene of twelve wild rabies virus isolates from France, Sacramento et al. (1992) found that divergence among these isolates was only 2 %, however, when compared to laboratory adapted strains, this divergence increased to almost 15 %. The amount of divergence varied along the sequence of the pseudogene as well, with the central portion showing up

to 44 % divergence. Because the pseudogene is a non-coding region of the genome it should be relatively unaffected by selective pressures. This makes the pseudogene a very sensitive molecular clock for the study of lyssavirus evolution (Sacramento et al. 1991, 1992). These high rates of variation have not been explained, but similar observations have been made for several Canadian rabies virus isolates (Nadin-Davis et al. 1994).

Studies of the N gene from the same twelve French rabies virus isolates showed a divergence of 7.3 % compared to the Pasteur virus (PV) strain (Sacramento et al. 1992). In a similar study, sequence divergence of the N genes of rabies virus isolates from Ontario gave an 11 % variability from the PV strain (Nadin-Davis et al. 1993). Similar results were obtained when lyssaviruses from all genotypes were analyzed (Bourhy et al. 1993a). Smith et al. (1992) in another study examined 87 dog rabies virus isolates from around the world with similar results. No insertions or deletions were found in the N gene, and most mutations produced transitions that resulted in synonymous codons.

While studies on the G gene have shown that this gene is more variable than the N gene, it is still a well-conserved gene. Ontario rabies virus isolates diverged up to 2 % among themselves but up to 13 % from the PV strain, with obvious differential variability in discreet protein domains (Wandeler et al. 1994). The Ontario strains differed from the PV strain by 16 % in the leader sequence, 5 % in the ectodomain, and 33 % in the transmembrane and cytoplasmic domains. The same results were obtained in two French studies (Sacramento et al. 1992; Benmansour et al. 1992). Selective pressure is evident favoring the transmembrane and cytoplasmic portions of the glycoprotein, possibly because these regions are only responsible for anchoring the glycoprotein in the

membrane and to the RNP core. Obviously high levels of mutation can be tolerated in this region.

Though variability exists between the genes of different wild rabies virus isolates, overall high levels of conservation are observed. This indicates that significant selective pressures do operate to constrain lyssavirus variability (Wandeler et al. 1994). Variability in virus isolates is greatest at epizootic wavefronts (Nadin-Davis et al. 1994), where it is believed that viruses evolve most rapidly as a consequence of being placed in a potentially new environment (Domingo and Holland 1988). After a few generations or passages, the most fit virus population becomes the stabilized, or dominant population of the quasispecies (Holland et al. 1991; Morimoto et al. 1998). All the above indicate that Darwin's concept of evolution by natural selection of the fittest is valid for rabies virus, its hosts, and interacting organisms (Wandeler et al. 1994).

1.5 Hypothesis

Variation exists in the antigenic nature of lyssaviruses although all lyssaviruses have evolved from a common ancestor. A comprehensive knowledge of the antigenic nature of all lyssavirus genotypes will help in understanding the evolutionary relationship between lyssaviruses. Clues to help unravel the mystery of lyssavirus host species specificity will be obtained from a comprehensive understanding of lyssavirus antigenic structure.

1.6 Objectives

Though the antigenic nature of the N and G proteins of vaccine strains of rabies virus is fairly well described (Lafon and Wiktor 1985; Lafon et al. 1983; Benmansour et al. 1991; Lafay et al. 1996; Dietzschold et al. 1987, 1988), little is known about the antigenic nature of other lyssaviruses. Epitopes, or antigenic sites, have not been comprehensively mapped on wild rabies virus isolates or on representatives of the other lyssavirus genotypes. To better understand lyssaviruses in general, and rabies virus in particular, a comprehensive analysis of the antigenic nature of all lyssaviruses should be undertaken. New advances in vaccine development might be achieved if a greater understanding of the rabies-like viruses was appreciated. Some rabies virologists believe that currently available rabies vaccines offer imperfect protection against genotypes 2 (Tignor and Smith 1972), 4 (Tignor et al. 1977), and 5 (Lafon et al. 1988) while they fail to protect against Mokola virus (genotype 3) at all (Koprowski et al. 1985). Whether this is true or not remains to be discovered, however, the involvement of rabies-like viruses in several epizootics make them dangerous and potential public health threats (Bourhy et al.

1990; Foggin 1983; King and Crick 1988) that must be addressed. The main objectives of this work then are:

1. To describe the antigenic profiles of the glycoproteins from representatives of all six lyssavirus genotypes.
2. To describe the antigenic profiles of the nucleoproteins from representatives of all six lyssavirus genotypes.
3. To correlate the findings from points 1 and 2 to achieve a better understanding of lyssavirus antigenic structure.

It is hoped that by achieving the above objectives, certain antigenic sites will be found to be more or less variable than others. Heavy constraints on variability may be placed on some antigenic sites, which may prove useful in identifying epitope functions. Alternatively, high variability in other sites may be related to a virus's adaptation to a particular host species. If a linkage can be established between differential variability and host specificity and virulence, then important clues for further investigations on functional morphology of rabies virus proteins may be obtained. Our current knowledge of lyssavirus epidemiology will be furthered by the results of this study.

1.7 Experimental approach

The approach used to achieve the above objectives was an immunological analysis. Competitive enzyme immunoassays (ELISAs) were used to map two of the major structural proteins of lyssaviruses; the glycoprotein and the nucleoprotein. Three different isolates of genotype 1 and one isolate each of genotypes 2-6 were analyzed. Of the rabies viruses examined, one was a vaccine strain and the other two were canine

strains isolated from opposite parts of the world. This selection is considered to be a good representation of all known lyssaviruses, except for the newly discovered Australian lyssavirus.

Monoclonal antibodies are of prime importance to this research. To achieve a meaningful analysis of any protein, large numbers of monoclonal antibodies are necessary to detect as many epitopes as possible. Also, one should use monoclonal antibodies that have been generated against all virus variants being tested. These two points were met in this study. Monoclonal antibodies directed against all 6 lyssavirus genotypes were used, with the exception being that only one monoclonal antibody was available for EBL-1 virus (refer to Appendix 1). Over 300 monoclonal antibodies were available for use in these studies and all were tested for their usefulness in defining antigenic variability.

Topographical analysis of the epitopes of the G and N proteins was achieved as mentioned by competitive ELISA. All of the monoclonal antibodies were labeled with biotin for use in competitions with unlabeled monoclonal antibodies. To more fully understand the relationships between epitopes, all competitions were carried out reciprocally. The binding affinities of all monoclonal antibodies were measured on all antigens tested by competitive ELISA to be aware of, if not eliminate, potential anomalies created by extremely different affinities. These combined data are then used to elucidate the epitope topography of the N and G proteins of the lyssaviruses.

2.0 MATERIALS AND METHODS

2.1 Cells and viruses

Baby hamster kidney (BHK-21-C13) cells (American Type Culture Collection, Manassas, Virginia) were propagated in Eagle's minimal essential medium (MEM) (Canadian Life Technologies Inc., Burlington, Ontario) supplemented with 10 % fetal bovine serum (Canadian Life Technologies) in a CO_2 incubator at 37° C and at 5% CO_2.

Eight lyssavirus isolates were used in this study to represent the six lyssavirus genotypes as shown in Table 2.1.

Table 2.1 Lyssavirus isolates used in this study

Genotype Number	Genotype Name	Species	Location Isolated	Year	Reference
1	Rabies	Canine (ERA[1] vaccine)	USA	1964	Abelseth, 1964
1	Rabies	Canine	Peru		Wandeler, personal communication
1	Rabies	Canine	Sri Lanka		Wandeler, personal communication
2	Lagos Bat	frugivorous bats	Nigeria	1956	Boulger and Porterfield, 1958
3	Mokola	shrew	Nigeria	1968	Shope et al. 1970
4	Duvenhage	human	South Africa	1970	Meredith et al. 1971
5	EBL-1	insectivorous bat	Germany	1970	Schneider, 1982
6	EBL-2	human	Finland	1985	Lumio et al. 1986

[1] Evelyn Rokitniki Abelseth

All the above viruses are part of the permanent lyssavirus collection of Dr. A.I. Wandeler, Canadian Food Inspection Agency. The African viruses were gifts received from Dr. A.A. King, formerly of the Central Veterinary Laboratory, UK.

2.2 Preparation of lyssavirus antigens for ELISA

Large volumes of lyssavirus infected cell culture supernatants were required for producing sufficient quantities of G and N ELISA antigens. By passaging lyssavirus infected cells, this task was made easier. Initially, a 25 cm^2 flask containing a near confluent monolayer of BHK-21-C13 cells was infected with the lyssavirus to be produced at a multiplicity of infection (moi) of 0.01-0.1. Cells were incubated until 50-100 % of the cells were infected. The level of infectivity was determined by examining monitor plates, which were set up at the same time as the initial flask, by indirect immunofluorescense using a fluorescein labeled goat anti-rabies polyclonal antibody conjugate. When the desired level of infectivity was obtained, the cells were disassociated with trypsin and reseeded equally into two 162 cm^2 cell culture flasks (Corning Costar Corporation, Cambridge, Ma) with fresh media. Once again, cells were allowed to incubate until 50-100 % of the cells were infected. For the final cell expansion, lyssavirus infected cells were mixed with uninfected cells at a ratio of 1:1 to 1:10, depending on the condition of the infected cells, and seeded into forty 162 cm^2 cell culture flasks. Cells were incubated at 37° C until all cells were infected (as determined by immunofluorescense); usually 5-8 days. When it was time to harvest the virus, cellular debris was removed from the collected cell culture supernatants by low speed centrifugation at 500 x g using a Sanyo Mistral 3000i table top centrifuge (VWR Canlab,

Mississauga, Ontario). Clarified supernatants were pooled and stored at -70° C until needed.

Virus antigens for ELISAs were prepared by partially purifying virus from lyssavirus infected, clarified cell culture supernatants. Using a Beckman L-70 Ultracentrifuge and a SW28 rotor (Beckman Instruments, Mississauga, Ontario), virus was pelleted from clarified supernatants at 70,000 x g for 2.5 h at 4° C. Pelleted virus was resuspended in phosphate buffered saline (PBS) (0.01 M phosphate, pH 7.2, 0.15 M NaCl) to 1/100 of the original volume and stored at -70° C until needed. No additional steps were taken to expose viral proteins. A sufficient quantity of each virus antigen was prepared in one lot such that the same lot of antigen was used for all experiments throughout the study. Individual viral proteins were not purified, rather, whole virus preparations as described were used as ELISA antigens.

2.3 Monoclonal antibodies

All monoclonal antibodies used in this study were drawn from a large collection of anti-lyssavirus monoclonal antibodies maintained for the Centre of Expertise for Rabies, Animal Diseases Research Institute (ADRI), by the Monoclonal Antibody Unit, ADRI, both part of the Canadian Food Inspection Agency (CFIA). Many of the hybridoma cell lines were produced at the University of Bern, Switzerland by Dr. A.I. Wandeler, as indicated in Appendix 1. All other hybridomas that were used in this study were produced under the direction of Dr. A.I. Wandeler at ADRI (Nepean). Hybridoma secreted monoclonal antibodies were used both as cell culture supernatants and as ascites

fluids. Anti-glycoprotein monoclonal antibodies are listed in Appendix 1 and anti-nucleoprotein monoclonal antibodies are listed in Appendix 2.

None of monoclonal antibodies used in these experiments showed any recognizable cross-reactivity to other viral or cellular antigens. This was determined using the Mabs in indirect immunofluorescent staining. Western blotting and radioimmunoprecipitation results confirmed Mab specificities and lack of cross-reactivities.

Stocks of most monoclonal antibodies (supernatants and ascites) were stored at -20° C within the Centre of Expertise for Rabies. Personnel of the Monoclonal Antibody Unit, ADRI, performed all *in vitro* and *in vivo* culturing of hybridoma cell lines. Requests for monoclonal antibodies, both as cell culture supernatants and ascites fluids, were submitted to the Monoclonal Antibody Unit as required.

2.4 Monoclonal antibody purification

Two different methods were used for monoclonal antibody purification; a liquid polymer, differential precipitation method and an affinity chromatography method.

2.4.1 E-Z SEP antibody purification kit

The method of choice for purifying monoclonal antibodies was to use an E-Z Sep Antibody Purification kit (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec). The E-Z Sep polymer solution allows for differential partitioning of gamma globulins into a gel-like pellet, excluding other proteins and lipids. The manufacturer's instructions were followed with some modifications, as follows. Prior to using E-Z Sep, ascites fluids were

clarified by centrifugation at 13,000 x g through a 0.45 μm Spin-X filter (Corning Costar Corporation) in an Eppendorf Model 5415C microcentrifuge (VWR Canlab) for approximately 30 min. The pH of all clarified ascites fluids was adjusted to between 7.3-7.7 using 0.1 N HCl and pH test strips (Sigma Chemical Company, St. Louis, Mo.). The volume of ascites to be purified was then mixed with a volume of reagent 1 equal to three times the starting volume of the ascites and allowed to mix at room temperature for 30 min before being subjected to centrifugation at 2,000 x g for 30 min at 15° C. Following centrifugation, the resulting supernatant was decanted and the pellet resuspended in a volume of deionized water equal to the starting volume of the ascites fluid. After a 10 min incubation at room temperature, a volume of reagent 2 equal to the starting volume of the ascites fluid was added to the sample and allowed to mix for 30 min at room temperature. The sample was again subjected to centrifugation at 2,000 x g for 30 min. After decanting and discarding the supernatant, the pellet was resuspended in a volume of 0.01 M carbonate/bicarbonate buffer, pH 9.0-9.5, equal to the starting volume of the ascites. The purified monoclonal antibody was then buffer exchanged into PBS using a Centriprep 30 (Amicon Canada Ltd., Oakville, Ontario) which was subjected to centrifugation at 2,000 x g.

2.4.2 Protein A affinity chromatography

Monoclonal antibodies that could not be purified using E-Z Sep reagents were purified by MAPS II protein A affinity chromatography (Bio-Rad Laboratories, Mississauga, Ontario). Briefly, ascites were clarified as for E-Z Sep purification before beginning any chromatography. Using a 10 ml syringe, a 1 ml protein A cartridge, with

luer fittings, was equilibrated with 10 bed volumes of MAPS II Protein A Binding Buffer before the application of the ascites sample to be purified. A 1 ml sample of ascites was diluted with MAPS II Protein A Binding Buffer to a final volume of 5 ml for application to the affinity cartridge with a syringe. After applying the ascites sample, the cartridge was washed with 15 bed volumes of MAPS II Protein A Binding Buffer. Bound monoclonal antibody was eluted from the column with 5 bed volumes of MAPS II Protein A Elution Buffer directly into a Centriprep 30 tube containing 3 ml of 1 M TRIS, pH 9.0 for neutralization of the elution buffer and for buffer exchange into PBS. The purified monoclonal antibody was then ready for further analysis.

2.5 Biotinylation of monoclonal antibodies

For competitive binding assays, purified monoclonal antibodies were labeled with biotin following standard procedures (Goding, 1986). Briefly, purified monoclonal antibody at 1 mg/ml was dialyzed overnight against 0.1 M $NaHCO_3$, pH 8.0-8.3 and removed to a clean glass vial in the morning. Immediately prior to labeling the monoclonal antibody, N-hydroxysuccinimidobiotin was dissolved in dimethyl sulfoxide at 1.0 mg/ml. For each mg of protein to be labeled, 120 µl of the biotinylation solution was added for optimal conjugation. The labeling mixture was held at room temperature for 1-2 hours, by which time the reaction had gone to completion. Finally, the biotin labeled monoclonal antibody was dialyzed overnight against PBS to remove any unbound biotin and for buffer exchange. For long term storage, biotinylated monoclonal antibodies were diluted 1:2 with glycerol and kept at -20° C.

2.6 Lyssavirus ELISA

For all lyssaviruses studied, it was necessary to optimize ELISA parameters defining virus (antigen preparation), antibody, and conjugate dilutions required for each lyssavirus preparation. The same general procedure was followed for each protein (N and G) and is described here. First, a checkerboard titration of each virus and 1 or 2 representative monoclonal antibodies was performed. Various dilutions of virus, generally from 1:300-1:1000, were made in coating buffer (0.5 M $NaHCO_3/Na_2CO_3$, pH 9.0-9.5) and applied to the wells of Nunc 96 well polystyrene enzyme immunoassay (EIA) plates (Canadian Life Technologies Inc.), one row per dilution, in a volume of 100 µl. Plates were then incubated, without stacking, overnight at 4° C. The next day, titration plates were washed five times with wash buffer (PBS + 0.05 % Tween 20) (PBST) using a Bio-Tek Automated Microplate Washer, Model EL403 (Mandel Scientific, Guelph, Ontario) before adding various dilutions of monoclonal antibody to the plates in a volume of 100 µl, generally doubling dilutions from 1:100, one dilution per column. Each monoclonal antibody being titrated required a virus coated titration plate. Following the addition of the antibody, plates were incubated at 28° C for 1.5 h and again washed five times with PBST using a plate washer. Streptavidin-horse radish peroxidase (HRP) conjugate (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD) , used at a dilution of 1:5000 as previously determined by checkerboard titration, was added to all wells before incubating the plates at 28° C for 1.5 h. Following the incubation with streptavidin-HRP, the plates were washed five times with PBST using an automatic plate washer. 100 µl per well of substrate solution containing 1 mM peroxide and 4 mM chromogen {2',2-azino-bis (3-ethylbenzthiazol-6-sulfonic acid)} (ABTS) in

0.05 M citrate buffer, pH 4.0, was added and colour allowed to develop for 10 min. After 10 min, absorbances at 414 nm were read using a Labsystems Multiscan RC plate reader (Labsystems, Needham Heights, Ma). The optimal dilutions of both antibody and antigen that gave an A_{414} of 1.0 after 10 min were determined. Large batches of EIA plates (usually 60) were then coated with this dilution of antigen at 4° C overnight followed by storage at -70° C until needed, with the coating buffer in the wells.

After EIA plates were prepared, the optimal amount required of the biotin labeled monoclonal antibodies had to be determined on each antigen. Twofold serial dilutions, starting at 1:100, of the biotinylated monoclonal antibodies were made in PBST + 3% horse serum (PBST-H) and added to rows of lyssavirus coated plates. The dilution that gave an A_{414} of 1.0 after 10 min was selected as the dilution of monoclonal antibody to use in competitive ELISAs.

2.7 Competitive ELISA

The procedure for competitive (c)-ELISAs (Elmgren and Wandeler, 1996) followed the basic procedure outlined in the preceding section with the addition of one step. Following the removal from the freezer and washing of a lyssavirus coated plate, 50 µl of unlabeled monoclonal antibody cell culture supernatants diluted 1:5 with PBST-H were added to wells in duplicate. After all monoclonal antibody supernatants were added to a plate, 50 µl of an optimally diluted biotin labeled monoclonal antibody was added to every well on the plate, thus setting up competitive binding for antigen. After stationary incubation of the plates for 1.5 h at 28° C, they were washed and streptavidin-HRP, diluted in PBST-H, was added to each well at a predetermined concentration. The

plates were again incubated for 1.5 h followed by five washes. Chromagen (ABTS) and substrate (peroxide) in citrate buffer were added as previously described and the plates read at 414 nm using a Multiscan RC Plate Reader. Results were expressed as mean per cent inhibition compared to control wells containing diluent in the place of a monoclonal antibody cell culture supernatant. Percent inhibition is calculated using the formula:

$$\frac{A_{414}\text{ (labeled Mab)} - A_{414}\text{ (labeled Mab + unlabeled Mab)}}{A_{414}\text{ labeled Mab}} \quad X\ 100$$

where A_{414} is the absorbance of a sample at 414 nm. Control wells containing irrelevant antibodies, including an anti-adenovirus monoclonal antibody and an anti-cytoskeleton monoclonal antibody, were included on all plates.

2.8 Measurement of relative antibody affinity

The relative affinities of monoclonal antibodies were measured by ELISA using a thiocyanate elution method (Macdonald et al. 1988). Briefly, ELISAs were done as described in section 2.6 above. Following the wash step after the primary antibody incubation, ammonium thiocyanate (NH_4SCN) in 0.1 M phosphate buffer, pH 6, was added to duplicate control and test wells at concentrations of 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 M. The plates were sealed and incubated at room temperature for 15 min on a plate shaker before washing and addition of HRP conjugated streptavidin. ELISAs were completed as in 2.6. Data obtained from these experiments were fitted to graphs of $\log_{10}$ percent binding versus molarity of NH_4SCN and the affinity index, represented by the molarity of thiocyanate required to reduce the A_{414} by 50 percent, was estimated from graphs.

2.9 Western blotting

Lyssavirus preparations (whole virus) were mixed 1:1 with 2X Laemmli reducing electrophoresis sample buffer (Bio-Rad) (containing 5% ß-mercaptoethanol) and boiled for 5 min before being subjected to electrophoresis through denaturing 10 % polyacrylamide preparative gels (Laemmli, 1970), one antigen per gel, using a preparative comb and a Mini Protean II apparatus (Bio-Rad). Electrophoresis was at 100 V for approximately 60 min. Following electrophoresis, gels, and nitrocellulose membranes (Bio-Rad) cut to the same size as the gels, were equilibrated in cold Towbin buffer (25 mM TRIS, 192 mM glycine, 20 % methanol, pH 8.3) for 15 min prior to electrophoretic transfer using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) following the manufacturers instructions. Transfer conditions of 12 V for 24 min were used.

Nitrocellulose blots for western analysis were blocked in a solution of 5 % horse serum (HS) in 50 mM TRIS, 150 mM NaCl, pH 7.2 (TBS) + 0.5 % Tween 20 (TBST) for 1 hour. Following blocking, blots were cut into 4 mm strips, numbered and marked for final alignment of bands, and placed into individual chambers of disposable incubation trays (Corning Costar Inc.) for all incubation and wash steps. Monoclonal antibodies were used as cell culture supernatants diluted 1:5 in TBST + 3 % HS and polyclonal serum was diluted 1:1000 in TBST + 3 % HS. Incubations with the primary antibodies were for 1 hr at room temperature with gentle agitation. Blots were then washed three times with TBST before incubation for 1 hr at room temperature with either goat anti-mouse (KPL) or goat anti-rabbit antibodies (KPL) conjugated with alkaline phosphatase at dilutions recommended by the manufacturer. After 3-5 washes with TBST , the blots

were developed using a BCIP/NBT alkaline phosphatase substrate kit (KPL) following the manufacturer's instructions.

3.0 Results

3.1 Glycoprotein

3.1.1 Selection of monoclonal antibodies for mapping lyssaviral glycoproteins

From a collection of 131 monoclonal antibodies generated against various lyssavirus genotype 1 isolates, 38 were chosen for inclusion in a glycoprotein mapping panel. These 38 monoclonal antibodies were chosen on the basis of their binding to acetone fixed antigen in lyssavirus infected cells as visualized by immunofluorescence. Dr. A.I. Wandeler provided these immunofluorescent staining results which were obtained for all lyssavirus genotypes. Monoclonal antibody staining profiles were developed for each lyssavirus genotype on the basis of whether they stained or not, and by their staining patterns, which were cytoplasmic, diffuse, or inclusion specific. Groups were established containing monoclonal antibodies that showed similar staining profiles. One or more representatives of each group were used to make up the 38 monoclonal antibody mapping panel (refer to Appendix I). Also shown in Appendix I are the isotypes of all the anti-glycoprotein monoclonal antibodies used in this work, which included 15 IgG1, 14 IgG2a, 8 IgG2b, and 1 IgG3. Seventeen of the 38 anti-G Mabs were also neutralizing.

3.1.2 Competitive ELISAs with monoclonal antibody panel on ERA virus antigen

Two way competitive ELISAs, using the ERA vaccine strain of rabies virus and the 38 anti-glycoprotein monoclonal antibodies, were used to examine the epitope topography of the rabies virus glycoprotein. Since all of the monoclonal antibodies used

for the glycoprotein mapping panel were produced against genotype 1 (rabies virus) isolates (refer to Appendix I), all competitive ELISAs were done using ERA virus as the ELISA antigen. ELISA plates were coated with a concentration of virus antigen, which was determined previously by checkerboard titration. A concentration of antigen was used that yielded an absorbance at 414 nm of approximately 1.0 with a homologous antibody, after a ten minute incubation with substrate. The antibody concentration was also previously determined by the same checkerboard titration. The heterogeneous nature of the antigen preparations made it impossible to coat plates with a defined amount of protein and so this qualitative measurement of antigen was made. All 38 monoclonal antibodies were labeled with biotin and tested in competition with each of the non-labeled monoclonal antibodies to establish competitive binding patterns. These results placed the monoclonal antibodies into groups based on identical or very similar competitive binding patterns. This resulted in the establishment of 13 different groups each defining a particular epitope. One monoclonal antibody from each group (highlighted in bold in Appendix 1) was then chosen for use in mapping experiments. Competitive ELISA results for the final mapping panel are given in Table 3.1. While each monoclonal antibody represents a unique glycoprotein epitope, as established above, some epitopes are part of common or overlapping antigenic sites.

Four major non-overlapping antigenic sites can be elucidated from the competitive ELISA results given in Table 3.1. The monoclonal antibodies were grouped together in the table according to the magnitude of their reciprocal inhibition values. Strong inhibition (greater than 80%, highlighted in bold) of one monoclonal antibody by another indicates that the two antibodies are either directed against the same epitope, or

Table 3.1 Competitive ELISA results using the anti-rabies virus glycoprotein monoclonal antibody panel on the ERA vaccine strain of rabies virus (genotype 1)

Biotin labeled Mab

Unlabeled Mab	10EC9	M725	M818	16EH11	M1089	M778	M1094	10ED8	M724	M1100	16AH8	M785	M1078	Antigenic Site
10EC9	**99**	**85**	**49**	**36**	46	0	60	5	0	54	37	9	18	I, Ia
M 725	**99**	**99**	**94**	0	0	10	65	16	8	0	0	0	0	
M 818	**27**	**57**	**71**	0	43	23	61	25	10	0	31	0	47	
16EH11	**99**	7	11	**89**	60	35	76	40	24	0	0	32	0	
M 1089	7	13	0	2	**65**	10	3	1	6	0	0	3	0	
M 778	17	50	35	43	30			10	0	29	11	11	0	II
M 1094	34	30	35	10	0			4	28	30	25	14	0	
10ED8	4	17	37	26	20	22	11	**[illegible]**	5	**[illegible]**	20	20	0	III
M 724	22	29	41	28	0	0	61	0	**[illegible]**	**[illegible]**	53	20	0	
M 1100	5	10	0	0	2	8	6	10	9	**[illegible]**	6	2	0	
16AH8	1	10	31	0	41	3	48	2	0	**[illegible]**	**[illegible]**	29	0	IIIa
M 785	5	1	20	0	64	21	55	27	12	**[illegible]**	**[illegible]**	**[illegible]**	51	
M 1078	4	11	0	0	9	2	0	0	7	0	0	1	**78**	IV

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab. See Methods section 2.7 for details of calculations. Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

that the epitopes are spatially close to each other, within the same antigenic site. Stearic hindrance is indicated by high inhibition values. Higher inhibition values indicate that the two epitopes are spatially closer. Areas of interaction between antigenic sites are indicated in Table 3.1 as boxed unshaded areas. Because all 13 of the monoclonal antibodies used for mapping the glycoprotein show unique inhibition patterns, they all represent unique epitopes.

3.1.3 Analysis of lyssaviral glycoproteins

For genotypes 2 through 6, the same monoclonal antibody mapping panel was basically applied to each lyssavirus glycoprotein antigen in an indirect ELISA to determine if the same epitopes were present. These results are shown in Table 3.2. All antigenic sites are represented in all genotypes except Site IIa, which is missing from the Sri Lanka dog isolate of genotype 1, and from genotypes 2 and 4.

Also shown in Table 3.2 are the binding affinities of each monoclonal antibody for each lyssavirus antigen recognized, determined by ammonium thiocyanate elution. Affinities are expressed as affinity index values based on the molar amount of ammonium thiocyanate required to reduce the binding of the monoclonal antibody to the antigen by 50 %. For comparative purposes, affinity indexes below 0.5 will be considered to be low affinity.

3.1.4 Immunological analysis of lyssavirus glycoprotein epitopes

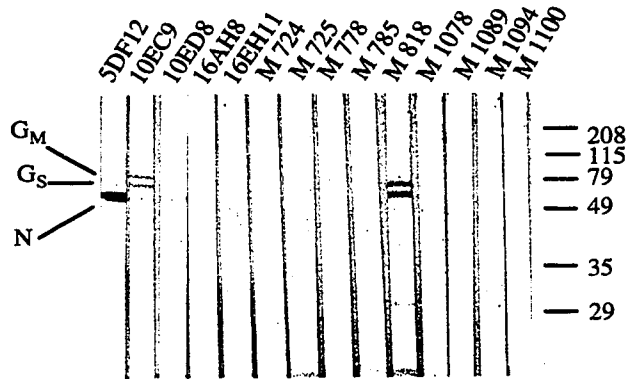
The lyssavirus epitopes defined by the glycoprotein mapping panel were subjected to western blot analysis to determine if any epitopes were linear. Figure 3.1

Table 3.2 Glycoprotein epitopes represented in all lyssavirus genotypes

		Lyssavirus Genotype							
		1	1	1	2	3	4	5	6
Antigenic Site	Epitope	ERA	Lima	Sri Lanka	Lagos Bat	Mokola	Duvenhage	EBL-1	EBL-2
I	10EC9*	2.38	1.25	0.92	0.37	0.37	0.33	0.58	0.47
	M 725	2.43					0.09		
	M 818*	4.37	3.32	2.96	3.60	3.43	3.41	2.96	3.05
Ia	16EH11	0.46	1.98				0.32		2.84
	M 1089	3.71	1.93	1.68	1.17	2.34	1.48	2.15	2.98
II	M 778	3.18	1.82				1.25		
	M 1094	1.08	2.32	1.84	2.07	2.32	1.19	2.32	2.32
III	10ED8	1.32	1.78	1.52	2.20	2.62	1.48	2.20	2.62
	M 724	2.17			0.41		1.05		
	M 1100	3.16	2.19	1.82	1.31	2.05	1.20	2.32	1.99
IIIa	16AH8	0.42	2.02			4.00		2.40	4.00
	M 785	2.42	2.31			2.50		2.50	2.68
IV	M 1078	2.77	1.68	1.68	1.86	2.97	1.30	1.57	2.60

Values in the table are the relative binding affinities of the monoclonal antibodies of the glycoprotein mapping panel estimated from graphs of ammonium thiocyanate elution experiments (see page 38 for details of calculations).

* These epitopes of antigenic site I are linear (see page 47). All other epitopes are conformational.


5DF12
10EC9
10ED8
16AH8
16EH11
M 724
M 725
M 778
M 785
M 818
M 1078
M 1089
M 1094
M 1100
G_M
G_S
N
208
115
79
49
35
29

shows that two epitopes (10EC9 and M 818) of antigenic site I are linear. Both the soluble (G_s) and membrane bound (G_m) forms of the glycoprotein are discernable in these blots.

Dr. A.I. Wandeler provided the results of virus neutralization tests using the anti-glycoprotein monoclonal antibodies (refer to Appendix I). These showed that the epitopes of antigenic sites I, Ia, and IIIa are neutralizable, while those of sites II, III, and IV are non-neutralizable.

3.2 Nucleoprotein

3.2.1 Selection of monoclonal antibodies for mapping lyssaviral nucleoproteins

Monoclonal antibodies for mapping lyssaviral nucleoproteins were chosen from a panel of 193 different antibodies generated against viruses representing all six lyssavirus genotypes. The composition of this panel is shown in Table 3.3. Based on their binding to acetone fixed antigen in lyssavirus infected cells, as visualized by immuno-fluorescence, similar monoclonal antibodies were grouped together. Dr. A.I. Wandeler provided these immunofluorescent staining results which were obtained for all lyssavirus genotypes. These results were initially used to limit the number of antibodies that would be used for mapping. A representative was chosen from each group showing similar staining patterns for further analysis by competitive ELISA.

Homologous competitive ELISAs were performed on antigen prepared from each genotype using all the genotype specific antibodies that were chosen for that genotype based on immunofluorescent staining patterns. Whenever possible, competitions were

Table 3.3 Composition of the panel of anti-nucleoprotein monoclonal antibodies

a) Mabs available for mapping,

Lyssavirus Genotype		Number of Mabs Available
1: Rabies	(vaccine strains)	13
	(Lima dog isolate)	10
	(Sri Lanka dog isolate)	29
2: Lagos Bat		36
3: Mokola		40
4: Duvenhage		50
5: EBL-1		14
6: EBL-2		1
TOTAL		193

b) Mabs used for mapping

Lyssavirus Genotype		Number of Mabs Used
1: Rabies	(vaccine strains)	8
	(Lima dog isolate)	3
	(Sri Lanka dog isolate)	7
2: Lagos Bat		4
3: Mokola		4
4: Duvenhage		6
5: EBL-1		2
6: EBL-2		1
TOTAL		35

done reciprocally. For some antibodies, ascites was not available for purification, therefore labeling with biotin could not be performed. For these, one way competitions were performed. Analysis of the inhibition patterns allowed the identification of monoclonal antibodies that recognized the same epitope. When this occurred, one monoclonal antibody was chosen to represent the epitope in the final mapping panel. Following similar experiments with all lyssavirus antigens tested, a mapping panel of 35 monoclonal antibodies was selected as shown in Table 3.3b (refer to Appendix 2 for details).

The number of epitopes represented by homologous monoclonal antibodies on antigens from each genotype ranged from 1-18, depending on how many homologous monoclonal antibodies were available for each genotype. Because most of the available monoclonal antibodies were generated against genotype 1 viruses, 18 different epitopes were found for this genotype. In contrast, only one homologous monoclonal antibody was available for EBL-2 (genotype 6) and therefore only one epitope was defined for this genotype using homologous antibodies. Two homologous epitopes were represented for EBL-1 (genotype 5), however all other genotypes had at least 4 homologous epitopes represented.

3.2.2 Competitive ELISAs using the nucleoprotein mapping panel on each lyssavirus genotype nucleoprotein

To determine which epitopes were present on the nucleoproteins of each genotype, titrations of the same panel of 35 anti-nucleoprotein monoclonal antibodies were performed on each lyssavirus antigen by indirect ELISA. Each monoclonal

antibody was titrated using doubling dilutions beginning from a 1:50 dilution. If a monoclonal antibody did not give an A_{414} of 1.0 after 10 minutes at a minimum dilution of 1:50 on any antigen, then the represented epitope was considered to be not present on that antigen. This allowed the establishment of individual mapping panels for each genotype, which were unique subsets of the original mapping panel of 35 monoclonal antibodies. Tables 3.4-3.11 show only the monoclonal antibodies that represent epitopes that are present on each individual antigen and not the entire mapping panel of 35 monoclonal antibodies. These mapping panels were used in two way competitive ELISAs to elucidate the epitope map of each nucleoprotein. The mapping panels and inhibition results for each lyssavirus genotype are given in Tables 3.4 to 3.11.

Examination of the competitive inhibition results revealed that two monoclonal antibodies represent the same epitope. These are 20CB11 (genotype 1) and 38HF2 (genotype 6). All other monoclonal antibodies used represent unique lyssavirus nucleoprotein epitopes, therefore the original mapping panel used in these experiments represents 34 unique lyssavirus nucleoprotein epitopes.

All 34 nucleoprotein epitopes represented by the nucleoprotein mapping panel are not present in each genotype or even on all antigenic variants within a genotype. Intra- and inter-genotypic variation is evident from Tables 3.4 to 3.11. Each lyssavirus nucleoprotein has in its own antigenic repertoire some epitopes that are unique and some that are shared with other genotypes. The data indicate that there are five major antigenic sites in genotype 1 with one containing three sub-sites within it (Site IV). All five major sites are present in all genotypes except for genotype 5 (EBL-1) which is missing Site V. Also, genotype 4 (Duvenhage) has one additional site (VI) that the other genotypes do

Table 3.4 Competitive ELISA results using the nucleoprotein mapping panel on the rabies virus (genotype 1) vaccine strain ERA

Biotin labeled Mab

Unlabeled Mab	5DF12	11DG10	26FE4	39EE5	11CD4	20AC3	7DH2	20CB11	32HH2	32KA6	M 993	M 853	26BG8	7AG8	32FE10	24FF11	M 862	M 840	11DD1	20CH4	20GF4	11DB9		Antigenic Site
5DF12	92	80	52	0	36	0	72	10	0	7	0	44	29	44	3	19	17	23	26	48	29	53	**5DF12**	
11DG10	87	86	88	0	43	0	71	29	43	21	28	49	15	35	1	7	15	23	13	46	17	52	**11DG10**	I
26FE4	39	80	89	50	44	34	80	81	81	51	69	67	39	52	24	8	22	41	22	50	40	60	**26FE4**	
39EE5	0	22	59	96	3	0	22	22	12	19	9	5	23	22	5	10	6	12	16	5	20	15	**39EE5**	Ia
11CD4	0	40	22	0	55	0	62	14	0	7	6	28	27	37	4	16	11	18	22	34	27	45	**11CD4**	II
20AC3	0	52	43	0	43	95	81	16	4	11	13	36	40	49	9	23	10	21	26	40	34	59	**20AC3**	III
7DH2	0	51	40	0	44	17	83	24	23	27	19	47	45	43	17	25	18	27	20	45	25	36	**7DH2**	
20CB11	13	54	65	5	36	11	77	98	97	97	96	83	57	75	7	15	38	52	24	62	26	60	**20CB11**	
32HH2	10	56	73	7	29	8	74	97	96	95	95	78	76	66	53	51	68	60	12	48	33	57	**32HH2**	
32KA6	9	50	46	0	33	15	73	94	89	95	93	85	89	87	61	52	78	75	61	47	33	54	**32KA6**	IVa
M 993	4	25	13	0	9	0	35	86	87	91	89	67	0	40	26	11	40	43	12	22	18	27	**M 993**	
M 853	0	53	56	0	37	0	73	56	50	73	48	81	85	65	77	44	65	58	52	48	23	58	**M 853**	
26BG8	0	44	20	0	34	5	71	14	9	10	5	28	82	48	4	16	11	14	24	32	30	52	**26BG8**	
7AG8	2	44	38	0	37	6	65	57	40	94	59	82	92	80	94	93	91	86	78	74	25	54	**7AG8**	
32FE10	0	43	0	0	27	0	66	18	10	43	5	81	86	71	90	59	81	79	68	76	42	47	**32FE10**	IVb
24FF11	3	22	0	0	10	0	23	29	43	61	15	68	35	56	86	67	79	75	63	71	35	14	**24FF11**	
M 862	0	54	23	0	25	5	73	38	36	79	34	80	91	78	91	74	86	81	66	66	32	56	**M 862**	
M 840	0	52	35	0	22	0	74	25	20	68	14	77	90	73	90	80	88	81	64	60	31	56	**M 840**	
11DD1	1	39	25	0	36	0	64	23	12	83	16	84	92	87	96	96	94	91	90	88	44	49	**11DD1**	
20CH4	0	36	9	0	32	0	71	1	6	5	0	57	53	51	48	25	23	28	31	68	29	46	**20CH4**	IVc
20GF4	0	40	19	0	26	0	67	0	0	17	18	67	5	46	75	76	28	35	39	81	93	49	**20GF4**	
11DB9	1	40	24	0	32	3	50	19	0	7	9	27	26	40	6	16	7	15	21	32	25	80	**11DB9**	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.5 Competitive ELISA results using the nucleoprotein mapping panel on the rabies virus (genotype 1) isolate Lima dog

Biotin labeled Mab

Unlabeled Mab	5DF12	11DG10	26FE4	39EE5	11CD4	20AC3	7DH2	20CB11	32HH2	32KA6	M 993	M 853	7AG8	26BG8	M 862	M 840	24FF11	32FE10	M 855	20CH4	20GF4	11DB9		Antigenic Site
5DF12	98	91	64	13	26	0	61	4	5	17	14	29	32	9	19	16	17	12	59	46	39	0	5DF12	I
11DG10	95	97	93	20	23	0	59	24	36	43	52	34	46	0	20	22	11	0	44	39	23	1	11DG10	
26FE4	43	89	90	66	34	26	61	88	80	73	83	67	63	23	24	40	24	3	55	51	44	16	26FE4	Ia
39EE5	0	20	76	98	0	0	8	27	24	32	19	13	28	20	19	0	33	17	11	0	40	0	39EE5	
11CD4	0	16	24	10	52	0	40	14	7	16	6	3	30	8	1	1	14	10	44	33	28	0	11CD4	II
20AC3	0	21	42	14	21	99	60	9	16	24	17	15	41	8	11	1	22	16	49	33	39	5	20AC3	III
7DH2	0	26	37	17	27	0	80	11	18	20	20	7	29	7	14	4	19	14	46	26	23	0	7DH2	
20CB11	0	26	46	22	17	0	57	99	98	99	99	94	83	20	37	58	18	6	51	54	35	10	20CB11	
32HH2	0	27	67	21	9	0	60	98	97	98	99	85	80	41	85	69	73	34	58	50	41	9	32HH2	
32KA6	0	16	46	15	17	0	49	94	91	98	98	96	88	71	90	87	77	58	62	46	43	0	32KA6	IVa
M 993	0	19	15	14	0	0	9	89	91	95	96	83	46	16	42	62	16	19	16	27	39	0	M 993	
M 853	0	22	45	2	9	0	46	65	27	88	64	95	85	62	91	90	77	69	57	39	39	0	M 853	
7AG8	0	10	16	2	13	0	31	16	17	61	32	74	66	27	78	82	50	38	34	49	11	0	7AG8	
26BG8	0	10	15	16	13	0	45	39	22	92	51	97	87	97	99	99	99	98	53	61	38	0	26BG8	
M 862	0	23	5	9	3	0	46	39	44	90	40	96	87	85	98	99	94	92	50	47	44	0	M 862	IVb
M 840	0	16	30	5	3	0	47	26	13	72	22	83	83	67	94	95	88	70	61	36	42	0	M 840	
24FF11	0	9	0	14	3	0	11	38	46	81	30	94	68	84	97	98	93	90	8	89	64	0	24FF11	
32FE10	0	4	0	1	1	0	31	19	20	74	7	95	84	96	99	97	98	99	44	90	66	0	32FE10	
M 855	0	28	28	10	12	9	43	36	44	57	27	39	60	32	26	20	37	23	92	6	75	0	M 855	IVc
20CH4	0	0	6	2	3	0	40	11	0	23	20	34	52	12	24	19	28	15	42	76	36	0	20CH4	
20GF4	0	3	11	0	4	0	35	4	0	28	29	63	55	19	28	17	94	58	88	77	99	0	20GF4	
11DB9	11	41	50	26	27	14	0	16	23	27	25	16	36	13	16	3	23	19	47	41	30	95	11DB9	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.6 Competitive ELISA results using the nucleoprotein mapping panel on the rabies virus (genotype 1) isolate Sri Lanka dog

Biotin labeled Mab

Unlabeled Mab	5DF12	11DG10	26FE4	39EE5	11CD4	20AC3	7DH2	20CB11	32HH2	32KA6	M 993	M 853	7AG8	26BG8	M 862	M 840	24FF11	32FE10	20CH4	20GF4	11DB9		Antigenic Site
5DF12	**98**	**84**	**50**	11	18	0	38	0	0	0	3	11	13	0	11	9	0	0	13	9	0	**5DF12**	
11DG10	**96**	**96**	**92**	25	11	0	39	11	36	18	51	24	22	0	13	3	0	0	7	0	0	**11DG10**	I
26FE4	48	**85**	**89**	71	17	0	48	**86**	**83**	59	**89**	61	54	0	40	37	7	0	44	27	7	**26FE4**	Ia
39EE5	0	0	46	**98**	0	0	1	15	13	0	32	12	25	6	3	0	29	0	7	39	0	**39EE5**	
11CD4	3	0	17	12	**47**	0	8	0	7	17	9	3	15	0	2	0	10	14	9	11	0	**11CD4**	II
20AC3	0	5	29	14	30	**98**	41	0	13	18	18	12	21	2	13	19	18	14	16	23	0	**20AC3**	III
7DH2	8	8	38	16	23	0	**73**	7	22	23	24	7	20	1	12	19	15	26	10	16	0	**7DH2**	
20CB11	16	18	53	23	8	0	41	**99**	**98**	**99**	**99**	**95**	79	4	65	69	15	0	35	16	13	**20CB11**	
32HH2	8	10	68	26	4	0	52	**98**	**95**	**99**	**99**	**90**	76	10	91	70	53	22	41	28	6	**32HH2**	
32KA6	4	0	33	13	6	0	21	**94**	**90**	**98**	**99**	**98**	87	54	97	93	53	44	35	32	0	**32KA6**	
M 993	19	6	11	20	0	0	10	**89**	**94**	**98**	**99**	**91**	53	2	67	67	7	0	21	35	7	**M 993**	IVa
M 853	5	4	42	8	0	0	22	66	60	**90**	76	**97**	**85**	58	**97**	**93**	67	67	27	28	0	**M 853**	
7AG8	0	0	5	1	9	0	9	3	12	56	34	**80**	**56**	13	**91**	**87**	36	10	31	0	0	**7AG8**	
26BG8	0	0	0	13	5	0	15	12	7	**94**	60	**98**	**84**	**95**	**99**	**99**	**98**	**98**	66	29	0	**26BG8**	
M 862	0	0	0	13	0	0	17	27	35	**81**	47	**96**	**85**	71	**97**	**97**	**85**	73	49	34	0	**M 862**	IVb
M 840	0	0	1	9	0	0	25	10	1	**61**	30	**86**	**79**	58	**95**	**95**	**79**	**61**	41	38	0	**M 840**	
24FF11	4	2	0	22	1	0	9	22	39	**79**	31	**97**	**77**	78	**99**	**98**	**90**	**84**	**88**	50	0	**24FF11**	
32FE10	0	0	0	2	0	0	13	5	11	**60**	2	**98**	**81**	**96**	**99**	**99**	**97**	**99**	**89**	59	0	**32FE10**	
20CH4	0	0	0	0	0	0	0	0	0	0	0	39	44	0	45	33	27	4	70	25	0	**20CH4**	IVc
20GF4	0	0	0	4	2	0	3	0	0	0	23	66	38	0	51	36	**85**	11	70	**97**	0	**20GF4**	
11DB9	26	24	43	29	25	0	0	11	25	26	32	16	23	3	18	21	17	29	22	19	**95**	**11DB9**	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.7 Competitive ELISA results using the nucleoprotein mapping panel on Lagos Bat virus (genotype 2)

Biotin labeled Mab

Unlabeled Mab	39EE5	11CD4	20AC3	20CB11	32KA6	M 613	M 1016	M 617	M 594	M 1004	M 600	M 855	11DB9		Antigenic Site
39EE5	**98**	0	0	1	0	18	0	8	0	25	16	0	0	**39EE5**	Ia
11CD4	28	**45**	0	8	5	5	0	0	3	31	18	16	0	**11CD4**	II
20AC3	31	0	**98**	9	4	9	0	1	7	29	23	18	0	**20AC3**	III
20CB11	39	0	0	**99**	**98**	**99**	**99**	**99**	57	**84**	67	46	0	**20CB11**	
32KA6	30	0	0	**68**	**98**	**99**	**46**	**99**	49	78	60	27	0	**32KA6**	
M 613	31	0	0	**98**	**99**	**99**	**99**	**83**	19	78	60	37	18	**M 613**	IVa
M 1016	26	0	1	**78**	**85**	**99**	**99**	43	8	58	48	22	12	**M 1016**	
M 617	3	0	0	**92**	**99**	79	0	**99**	**99**	**96**	**89**	29	4	**M 617**	
M 594	21	0	11	13	**99**	31	0	**99**	**98**	**87**	56	49	0	**M 594**	IVb
M 1004	8	0	0	0	55	11	0	**87**	24	**95**	**96**	**88**	5	**M 1004**	
M 600	12	0	0	0	65	23	0	**79**	0	**97**	**99**	**93**	0	**M 600**	IVc
M 855	16	0	0	20	34	39	0	**40**	12	**94**	**73**	**93**	12	**M 855**	
11DB9	42	2	0	12	12	12	0	4	6	25	17	21	**92**	**11DB9**	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.8 Competitive ELISA results using the nucleoprotein mapping panel on Mokola virus (genotype 3)

Biotin labeled Mab

Unlabeled Mab	39EE5	11CD4	20AC3	20CB11	32HH2	M 993	M 1016	M 1030	M 1004	11DB9		Antigenic Site
39EE5	**98**	0	18	52	55	64	44	56	57	23	**39EE5**	Ia
11CD4	41	**54**	0	27	10	37	3	5	10	12	**11CD4**	II
20AC3	51	23	**94**	58	26	52	30	4	30	42	**20AC3**	III
20CB11	44	12	10	**96**	**96**	**91**	**95**	33	6	14	**20CB11**	IVa
32HH2	47	7	3	**93**	**96**	**88**	**91**	65	48	3	**32HH2**	
M 993	54	0	14	**86**	**89**	**80**	**80**	43	31	8	**M 993**	
M 1016	60	8	23	**86**	**90**	**81**	**95**	37	45	15	**M 1016**	
M 1030	59	3	23	77	74	53	51	**99**	21	10	**M 1030**	IVb
M 1004	53	3	14	51	52	43	51	42	**98**	9	**M 1004**	
11DB9	65	27	35	54	8	39	10	14	16	**97**	**11DB9**	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.9 Competitive ELISA using the nucleoprotein mapping panel on Duvenhage virus (genotype 4)

Biotin labeled Mab

Unlabeled Mab	5DF12	39EE5	11CD4	20AC3	M 853	M 862	M 840	20CB11	M 859	M 613	M 600	M 855	11DB9	M 878		Antigenic Site
5DF12	**94**	0	21	0	0	11	0	0	13	75	7	0	21	10	**5DF12**	I
39EE5	0	**98**	0	0	2	2	0	18	15	30	36	0	3	16	**39EE5**	Ia
11CD4	0	0	**61**	0	4	4	0	0	1	8	0	0	0	0	**11CD4**	II
20AC3	0	0	15	**96**	2	7	0	0	0	0	3	2	32	4	**20AC3**	III
M 853	3	1	11	0	**99**	**99**	**99**	**98**	**99**	**98**	**90**	12	4	21	**M 853**	
M 862	4	7	2	0	**79**	**97**	**99**	**70**	**85**	**90**	**92**	40	0	21	**M 862**	
M 840	0	6	4	0	**41**	**95**	**98**	**37**	**84**	**87**	**86**	24	0	11	**M 840**	
20CB11	9	4	6	0	**51**	**69**	**73**	**99**	**99**	**99**	**90**	28	25	10	**20CB11**	IVa
M 859	5	12	4	0	**83**	**65**	**70**	**97**	**99**	**99**	**89**	35	11	17	**M 859**	
M 613	0	2	4	0	10	16	34	**99**	**99**	**99**	**87**	18	0	14	**M 613**	
M 600	0	0	0	0	0	0	0	0	0	54	**96**	**52**	0	10	**M 600**	IVb
M 855	0	9	6	0	17	14	6	24	23	53	**91**	**99**	1	21	**M 855**	IVc
11DB9	0	3	12	0	3	6	0	0	0	6	7	0	**75**	0	**11DB9**	V
M 878	0	13	10	0	15	8	2	20	19	21	45	0	4	**97**	**M 878**	VI

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.10 Competitive ELISA results using the nucleoprotein mapping panel on EBL-1 (genotype 5) virus

Biotin labeled Mab

Unlabeled Mab	5DF12	11DG10	39EE5	11CD4	20AC3	20CB11	M 840		Antigenic Site
5DF12	**91**	**89**	0	23	0	5	12	**5DF12**	I
11DG10	**90**	**97**	0	23	0	10	21	**11DG10**	
39EE5	2	1	**95**	2	0	9	1	**39EE5**	Ia
11CD4	0	15	0	**57**	0	16	9	**11CD4**	II
20AC3	0	18	0	23	**95**	17	9	**20AC3**	III
20CB11	7	33	0	15	0	**99**	**74**	**20CB11**	IVa
M 840	0	15	0	18	0	0	**64**	**M 840**	IVb

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

Table 3.11 Competitive ELISA results using the nucleoprotein mapping panel on EBL-2 virus (genotype 6)

Biotin labeled Mab

Unlabeled Mab	5DF12	11DG10	39EE5	11CD4	20AC3	7DH2	20CB11	M 853	M 840	M 862	17EE12	20CH4	24FF11	M 1004	11DB9		Antigenic Site
5DF12	**99**	**97**	21	32	0	52	18	14	11	11	1	8	13	9	0	**5DF12**	I
11DG10	**97**	**98**	26	28	0	42	20	20	14	20	3	9	10	4	0	**11DG10**	
39EE5	11	26	**99**	18	0	20	63	23	38	41	13	39	33	44	21	**39EE5**	Ia
11CD4	1	11	21	**62**	0	14	10	12	6	11	10	1	10	6	0	**11CD4**	II
20AC3	0	24	20	29	**99**	**53**	19	16	13	18	14	11	16	13	6	**20AC3**	III
7DH2	0	15	21	26	0	**78**	20	13	0	9	10	7	9	11	0	**7DH2**	
20CB11	0	0	3	10	0	13	**99**	**90**	56	30	35	46	32	0	0	**20CB11**	
M 853	0	7	11	18	0	31	**88**	**90**	61	43	44	47	56	5	0	**M 853**	IVa
M 840	0	4	6	15	0	30	**75**	**81**	**98**	**89**	72	56	**96**	35	0	**M 840**	
M 862	0	9	12	17	0	31	**83**	**95**	**99**	**99**	**97**	68	**99**	25	5	**M 862**	
17EE12	0	0	12	0	0	17	**87**	**93**	**98**	**97**	**98**	**83**	**97**	43	0	**17EE12**	
20CH4	0	0	0	2	0	0	57	**73**	**46**	31	31	**92**	**77**	21	0	**20CH4**	IVb
24FF11	0	0	11	9	0	5	53	**88**	**87**	50	44	**73**	**87**	47	0	**24FF11**	
M 1004	0	0	10	8	0	19	65	**75**	**81**	16	12	**71**	**99**	**99**	0	**M 1004**	
11DB9	28	41	34	41	1	0	18	12	10	21	16	15	16	13	**96**	**11DB9**	V

Each value represents per cent inhibition of the binding of the biotin labeled Mab by the unlabeled Mab (see Methods page 37 for details). Shaded areas represent antigenic sites while boxed, unshaded areas represent areas of interaction between antigenic sites. Numbers in bold represent Mabs directed against the same, or spatially close, epitopes.

not share. These results are summarized in Table 3.12. Site IV has been divided into 3 sub-sites to accommodate the complexity of this site. Large numbers of epitopes in this region of the nucleoprotein interact with each other and must be spatially close to each other due to protein folding, or are overlapping epitopes. Despite the large amount of epitope overlap and interaction, definite clustering of epitopes is seen in Site IV and for this reason three sub-sites were established.

3.2.3 Monoclonal antibody affinities to lyssaviral nucleoproteins

The binding affinities of the anti-nucleoprotein monoclonal antibodies to N protein were estimated following the same method as for the anti-glycoprotein monoclonal antibodies. Affinities are expressed as an affinity index based on the molar amount of ammonium thiocyanate required to reduce the binding of a monoclonal antibody to its antigen by 50%. Table 3.13 summarizes these results. Again, for comparative purposes, an affinity index of less than 0.5 is considered low.

3.2.4 Western blot analysis of lyssavirus nucleoprotein epitopes

The lyssavirus epitopes defined by the nucleoprotein mapping panel were subjected to western blot analysis to determine which nucleoprotein epitopes were linear. Monoclonal antibodies were tested against the genotype antigen against which they were generated. Figure 3.2 shows that eight monoclonal antibodies generated against genotype 1 epitopes define linear epitopes; all other epitopes are conformational. Linear epitopes were recognized in all major antigenic sites, but are missing from Site VI (present on Duvenhage only) and sub-sites IVb and IVc.

Table 3.12 Epitope composition of antigenic sites and genotype specificity of epitopes on lyssavirus nucleoproteins.

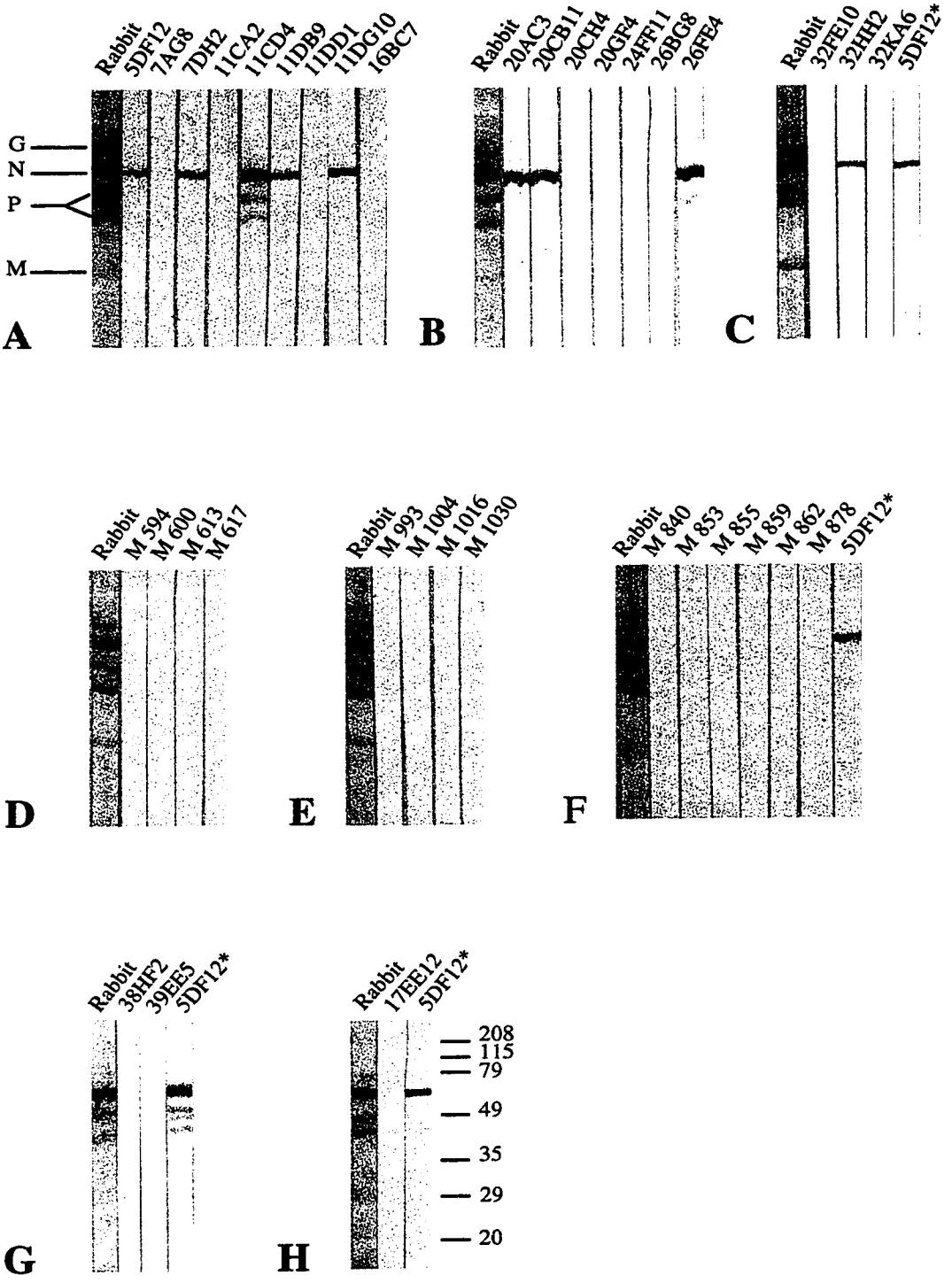
Antigenic Site	Linear Epitope	Epitopes present, as represented by monoclonal antibodies, in lyssavirus genotype: 1[a]	1[b]	1[c]	2[d]	3	4	5	6
I	Yes	5DF12	5DF12	5DF12			5DF12	5DF12	5DF12
	Yes	11DG10	11DG10	11DG10				11DG10	11DG10
Ia	Yes	**26FE4**[e]	**26FE4**	**26FE4**					
		39EE5[f]	39EE5	39EE5	39EE5	39EE5	39EE5	39EE5	39EE5
II	Yes	11CD4	11CD4	11CD4	11CD4	11CD4	11CD4	11CD4	11CD4
III	Yes	20AC3	20AC3	20AC3	20AC3	20AC3	20AC3	20AC3	20AC3
	Yes	7DH2	7DH2	7DH2					7DH2
IVa	Yes	20CB11	20CB11	20CB11	20CB11	20CB11	20CB11	20CB11	20CB11
	Yes	32HH2	32HH2	32HH2		32HH2	M 613		M 853
		32KA6	32KA6	32KA6	32KA6		**M 859**		M 840
		M 993	M 993	M 993	M 613	M 993	M 853		M 862
		M 853	M 853	M 853	M 1016	M 1016	M 862		**17EE12**
			26BG8	**26BG8**	**M 617**		M 840		
		7AG8	**7AG8**	**7AG8**	**M 594**				
IVb		**32FE10**	**32FE10**	**32FE10**	M 1004	M 1004			M 1004
		24FF11	24FF11	24FF11	M 600	**M 1030**	M 600		24FF11
		M 862	M 862	M 862					20CH4
		M 840	M 840	M 840				M 840	
		11DD1							
IVc		20CH4	20CH4	20CH4					
		20GF4	**20GF4**	**20GF4**					
			M 855		M 855		M 855		
V	Yes	11DB9	11DB9	11DB9	11DB9	11DB9	11DB9		11DB9
VI							**M 878**		

[a] ERA vaccine strain of rabies virus, genotype 1
[b] Lima dog rabies virus isolate, genotype 1
[c] Sri Lanka dog rabies virus isloate, genotype 1
[d] Genotypes 2-6 = Lagos bat, Mokola, Duvenhage, EBL-1, and EBL-2, respectively
[e] Boxed Mabs = genotype specific epitopes (refer to Discussion)
[f] Shaded Mabs = lyssavirus specific epitopes

Table 3.13 Anti-nucleoprotein monoclonal antibody panel binding affinities for native lyssavirus N protein epitopes.

	ERA	LIMA	SRI LANKA	LAGOS	MOKOLA	DUVENHAGE	EBL-1	EBL-2	
5DF12	0.41	0.32	0.34			0.33	0.40	0.28	5DF12
7AG8	1.21	1.14	1.24						7AG8
7DH2	2.26	1.30	1.43					0.82	7DH2
11CD4	0.91	0.91	0.85	0.69	0.60	0.60	0.65	0.73	11CD4
11DB9	2.50	2.29	2.68	0.76	0.48	0.48		2.11	11DB9
11DD1	1.08								11DD1
11DG10	0.95	0.62	0.43				0.62	0.43	11DG10
16BC7	0.90	0.66	0.61						16BC7
20AC3	0.52	0.40	0.40	0.79	0.82	0.40	0.40	0.47	20AC3
20CB11	2.58	2.35	2.58	2.52	1.70	2.06	2.45	2.20	20CB11
20CH4	1.10	0.79	0.68					1.32	20CH4
20GF4	0.54	0.48	0.48						20GF4
24FF11	1.58	1.06	1.02					1.47	24FF11
26BG8		0.92	1.09						26BG8
26FE4	0.79	0.75	0.68						26FE4
32FE10	1.17	0.73	0.62						32FE10
32HH2	2.23	2.03	2.11		1.82				32HH2
32KA6	0.92	0.62	0.81	0.55					32KA6
M 594				1.03					M 594
M 600				0.81		0.23			M 600
M 613				0.93		0.58			M 613
M 617				0.29					M 617
M 993	1.03	0.94	1.47		0.37				M 993
M 1004				0.27	0.43			0.85	M 1004
M 1016				0.53	0.78				M 1016
M 1030					0.72				M 1030
M 840	0.68	0.53	0.49			0.57	0.28	0.68	M 840
M 853	0.85	0.85	0.85			1.04		0.85	M 853
M 855		0.89		0.70		0.63			M 855
M 859						1.28			M 859
M 862	0.87	0.78	0.57			1.07		1.03	M 862
M 878						1.23			M 878
38HF2	2.17	2.86	1.82	2.43	1.73	2.30	2.74	2.37	38HF2
39EE5	2.76	2.43	2.28	1.24	0.59	2.23	2.33	2.03	39EE5
17EE12								0.56	17EE12

Blank spaces in the table indicate no binding of the monoclonal antibody to that particular antigen.


Rabbit
5DF12
7AG8
7DH2
11CA2
11CD4
11DB9
11DD1
11DG10
16BC7
G
N
P
M
A
Rabbit
20AC3
20CB11
20CH4
20GF4
24FF11
26BG8
26FE4
B
Rabbit
32FE10
32HH2
32KA6
5DF12*
C
Rabbit
M 594
M 600
M 613
M 617
D
Rabbit
M 993
M 1004
M 1016
M 1030
E
Rabbit
M 840
M 853
M 855
M 859
M 862
M 878
5DF12*
F
Rabbit
38HF2
39EE5
5DF12*
G
Rabbit
17EE12
5DF12*
208
115
79
49
35
29
20
H

4.0 Discussion

4.1 Glycoprotein

Topographical epitope mapping of viral proteins has been useful in the past for gaining knowledge on the antigenic and structural nature of the proteins of several viruses. These include Venezuelan equine encephalomyelitis virus (Roehrig et al. 1982), Japanese encephalitis virus (Kimura-Kuroda and Yasiu 1983), bovine corona virus (Deregt and Babiuk 1987), bovine viral diarrhea virus (Paton et al. 1992) and dengue 2 virus (Roehrig et al. 1998) to name a few. The rabies virus glycoprotein has also been mapped topographically using a limited number of monoclonal antibodies (Lafon et al. 1983, 1984; Bunschoten et al. 1989; Benmansour et al. 1991).

Previous studies on the rabies virus glycoprotein give only limited insight into the topology of the glycoprotein overall. In all reported studies, the results are only for vaccine strains of genotype 1 (rabies virus). ERA and CVS (Challenge Virus Standard) strains of rabies virus were used in most cases, and the Pitman-Moore strain was used in one study. Also, all mapping has been done using neutralizing monoclonal antibodies and cross neutralization tests. Epitopes were mapped by generating monoclonal antibody escape mutants (Wiktor and Koprowski 1980) followed by gene sequencing. Escape mutants from non-neutralizable monoclonal antibodies cannot be detected, therefore very little attention has been paid to non-neutralizable epitopes. Although non-neutralizable epitopes can be mapped by ELISA using peptides or polypeptides as the coating antigen, this work has not been done and might only detect sequential epitopes. Information gained by this type of physical mapping is sequence data only that does not describe

secondary protein structure at all. Maps obtained from cross neutralization tests were confirmed by very limited competitive binding assays. Lafon et al. (1983, 1984) used only five monoclonal antibodies in competitive binding assays to partially confirm their cross neutralization results. The monoclonal antibodies chosen for this assay however did not represent all the antigenic sites that were identified by this group.

Benmansour et al. (1991) have tried to standardize the nomenclature used for describing antigenic sites and epitopes in rabies viruses. These authors have suggested that the term antigenic site (both major and minor) be reserved to define regions of the glycoprotein that are defined by several monoclonal antibodies arising from different fusions. Regions of the protein that are defined by a single monoclonal antibody should be simply referred to as epitopes. In reality, the antigenic nature may not be that simple, therefore in this study, all unique areas of the glycoprotein will be referred to as antigenic sites. According to Benmansour's system of nomenclature, previous studies of the rabies virus glycoprotein describe two major antigenic sites, one minor site, and several epitopes. These correspond to former antigenic sites I, IV, V, and VI defined by Lafon et al. (1993, 1994). Minor sites are defined by other groups of overlapping epitopes that can be recognized by several monoclonal antibodies isolated from separate fusions.

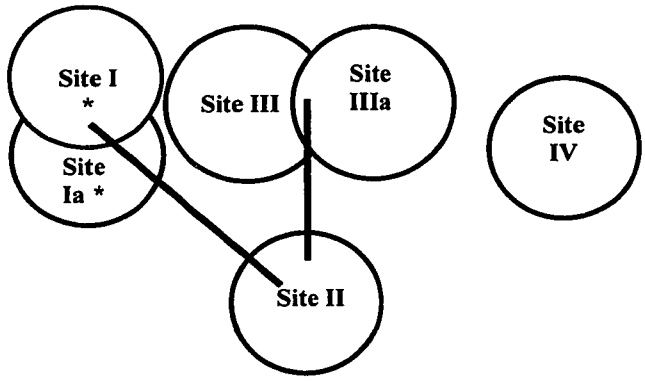
In a study of epitopes using competitive binding assays, the results obtained are only as good as the monoclonal antibodies used. This study shares the same disadvantage as others in that all the monoclonal antibodies used for epitope mapping of the glycoprotein were generated exclusively against genotype 1 viruses. In particular, vaccine strains were used which are all attenuated viruses that have been adapted to cell culture and so are highly diverged from wild isolates (Sacramento et al. 1992;

Benmansour et al. 1992; Wandeler et al. 1994). A distinction this study has over others is that four of the monoclonal antibodies used for mapping were generated against a wild carnivore rabies virus isolated from a striped skunk in Ontario. It is therefore likely that in this study, epitopes previously unrecognized have been identified with these four monoclonal antibodies. In fact, this is most likely the case with the monoclonal antibody M 1078, which defines our antigenic site IV (refer to Table 3.1 and Figure 4.1).

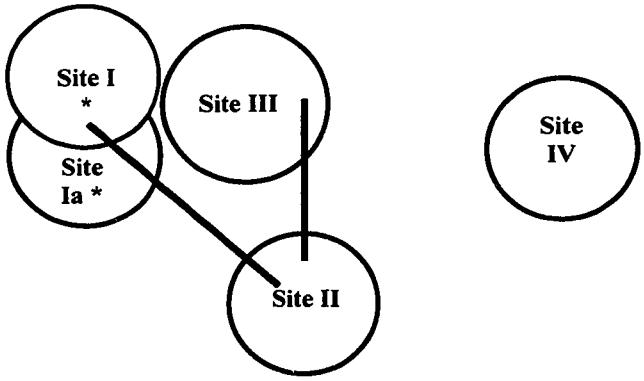
The monoclonal antibodies used in this study for mapping the glycoprotein of the ERA stain of rabies virus were all used in two-way competitive binding assays. Thirteen antibodies were used in the mapping experiments. Examination of the inhibition patterns obtained from two-way competitive binding assays allowed for the elucidation of four major antigenic sites and two sub-sites (Figure 4.1).

In all likelihood, the antigenic sites described by Lafon et al. (1983, 1984) as sites II and III correspond to our sites I and III, including their minor sites. This is likely because 97 % of the monoclonal antibodies used in previous studies belonged to these sites and 76 % of the monoclonal antibodies used here belong to our sites I and III. For ease of description in this work, antigenic sites have been defined as non-overlapping regions of the glycoprotein. Where overlap occurs, these regions have been called minor sites and have been given the suffix "a". Although sites III and IV are only described by two and one monoclonal antibodies respectively, they will continue to be referred to as antigenic sites. In Table 3.1, many epitopes are shown as interacting with epitopes outside of their antigenic sites. This must be due to stearic interactions between sites.

Antigenic site I is characterized by three distinct epitopes and one epitope overlapping antigenic site Ia. The epitopes represented by Mabs M 725 and 10EC9 (both



a



b

Figure 4.1 Schematic glycoprotein epitope maps for all lyssavirus genotypes based on reciprocal blocking in competitive binding assays. Large circles represent antigenic sites and heavy lines show possible linkages between sites. Genotypes 1 (ERA and Lima dog isolates), 3, 5, and 6 are represented in panel "a". Panel "b" shows genotypes 1 (Sri Lanka dog), 2, and 4. * indicates antigenic sites with neutralizable epitopes.

site I) must be physically close to each other because they almost completely inhibit each other in two-way competitive binding assays. The same is probably true for 10EC9 (site I) and 16EH11 (site Ia), even though 16EH11 is only able to inhibit 10EC9 by 36 %. The affinity of 10EC9 is five times greater than 16EH11, which may explain this apparently one-sided competition. 16EH11 is also probably on the "edge" of the epitope cluster because there is virtually no competition between it and the Mabs that define epitopes M 725 and M 818 (both also site I). This lack of interaction is not due to differences in affinities because no one-way inhibition is observed. M 818 did not fully inhibit itself in self-competition. This may be due to the Mab being poorly labeled with biotin, or may be due to some intrinsic characteristic of this Mab or epitope. The same may be true of M 1089 in site Ia. This Mab has a high affinity yet is unable to highly inhibit any other Mab. It is grouped with 16EH11 because there is a one-way competition between these Mabs. 16EH11 inhibits M 1089 nearly as much as M 1089 does itself. The reason for this may be that M 1089 represents an epitope that lays buried underneath 16EH11. If this were the case, M 1089 would never be able to inhibit 16EH11. It may be that 16EH11, which is the overlap between sites I and Ia, may belong fully to site I and simply overlap, or bury, the isolated epitope defined by Mab M 1089. An epitope from antigenic site IIIa, defined by M 785, also inhibits M 1089 (site Ia) fairly strongly (64 %) in a one-way competition, which supports the view that M 1089 represents a buried epitope. How this buried epitope became available for B cell recognition by the donor mouse is open to speculation. The epitope may have been exposed during processing of the antigen by the infected mouse cells for MHC presentation to T lymphocytes. The

epitope was also probably made available for detection in the ELISAs due to partial denaturation of the protein by the high pH of the antigen coating buffer.

Antigenic sites III and IIIa represent an interesting set of epitopes. Mabs M 724 and 10ED8 (both site III) are able to completely inhibit M 1100 (site III) from binding but have a nil inhibitory effect on each other. Also, these two Mabs inhibit M 1100 in a one-way competition only. This indicates that the epitope represented by M 1100 must be sandwiched between M 724 and 10ED8. These two epitopes must also be positioned so that they are on the exterior of the glycoprotein. M 1100 is the overlapping epitope between sites III and IIIa. The two epitopes of site IIIa, M 785 and 16AH8, completely inhibit M 1100 in one-way competition. Because these Mabs have no effect on the binding of other site III Mabs, it appears that they must also bury the M 1100 epitope, but from a different angle than the site III epitopes. Also, because 16AH8 does not inhibit M 785 (both site IIIa), it must also be buried slightly by M 785. A possible three-dimensional model of sites III and IIIa could place M 1100 at the XY origin and going out along the X axis would be first 16AH8 followed by M 785. Along the Y axis, 10ED8 would be on the positive side and M 724 on the negative side. This possible model is illustrated in Figure 4.2.

Antigenic site II is distinct from other sites on the glycoprotein in that there are no strongly overlapping epitopes that completely inhibit binding of site II monoclonal antibodies. There is however some inhibition of site III Mabs by Mabs defining all other antigenic sites but site IV. This could mean that site III is brought in close proximity of the other sites by secondary structure. In Figure 4.1, the lines joining site II to sites I and III may represent disulfide bridges between these areas. Two disulfide bridges are

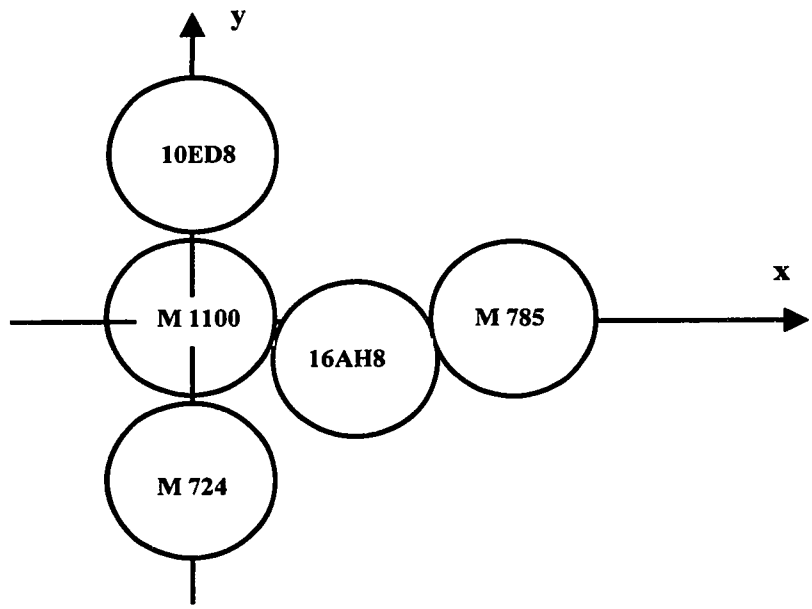


Figure 4.2 Possible model for the spatial arrangement of epitopes of antigenic sites III and IIIa of the ERA strain of rabies virus glycoprotein as determined by reciprocal competitive binding assays.

present in the rabies virus glycoprotein (Dietzschold et al. 1982) so this model is possible. It is also possible that folding of the protein brings these areas together without the need for disulfide bridges.

No other monoclonal antibodies were able to compete fully with M 1078 (site IV) in competitive binding assays. Mabs from site I (M 818) and site IIa (M 785) were able to partially inhibit M 1078 up to the 50 % level, which is almost certainly due to folding properties of the glycoprotein. This may be an epitope that is lost from cell culture adapted viruses such as the vaccine strains used to create most monoclonal antibodies.

The amount of epitope conservation between rabies virus and the other lyssavirus members is quite significant. Seven out of the thirteen epitopes defined by the monoclonal antibodies used in this study are conserved throughout all six lyssavirus genotypes (refer to Table 3.2). All the antigenic sites are also present except for site IIIa in the Sri Lanka dog isolate of genotype 1, and genotypes 2 and 4. If all lyssaviruses use the same or similar cell surface moieties for binding by their glycoproteins, such high levels of conservation would be expected. This type of knowledge of the antigenic make up of lyssaviruses may be valuable in designing new lyssavirus vaccines.

The glycoproteins of three different genotype 1 viruses were analyzed in this study. It is interesting that the two viruses that show the most epitopic similarity are rabies virus ERA strain and the Lima dog isolate. These are both viruses isolated in the Americas whereas the other genotype 1 isolate was obtained from Sri Lanka, in the old world. The North American isolates share 11 out of 13 epitopes whereas the isolate from Sri Lanka has only 7 epitopes in common with either of them. This is probably a good example of evolutionary divergence of three rabies viruses from a common ancestor.

Duvenhage virus (genotype 4), EBL-1 virus (genotype 5) and EBL-2 virus (genotype 6) are also all very similar. Before phylogenetic analysis established that these three virus types belong in separate genotypes (Bourhy et al. 1993a), they were all considered to be serotype 4 (Schneider and Cox 1994). The epitope distribution seen in these experiments provides support for the fact that these viruses are very closely related and appear to agree with earlier monoclonal antibody studies that placed these genotypes together. These data also show that monoclonal antibody analysis alone is not enough to establish the true relationships between viruses. When the binding affinities of the monoclonal antibodies to these three genotypes are examined and compared by their affinities to rabies virus, differences become apparent. In most cases, the affinities of the monoclonal antibodies are much higher for rabies virus since they were generated against rabies virus. The much lower affinities for genotypes 4, 5, and 6 indicate that changes at the amino acid level have taken place in these viruses, which in turn indicates genetic divergence. Conversely, some of the monoclonal antibodies bind with much higher affinities to these genotypes than to rabies virus (ERA strain). Cell culture adaptation of ERA strain may have altered some wild-type epitopes shared by other lyssaviruses. Possibly the wild-type epitope fits the monoclonal antibody better than the altered vaccine epitopes. Nevertheless, genotypes 4, 5, and 6 are all bat viruses that show close similarities to rabies virus. These data support the idea that all terrestrial rabies may have evolved from an adaptation of ancestral bat rabies to terrestrial carnivores (Wandeler 1991).

The two other African genotypes, Lagos bat and Mokola, are very similar except for the fact that Lagos bat virus lacks antigenic site IIIa as defined by the monoclonal

antibodies used for these experiments. These two genotypes share only eight and nine epitopes respectively with genotype 1 (ERA strain). These genotype groupings based on antigenic similarities when compared to the phylogenetic trees obtained for the various lyssaviruses (Tordo et al. 1998) show complete agreement. Lagos bat virus and Mokola virus are clustered together at the furthest point from rabies virus. Similarly, Duvenhage virus, EBL-1 virus and EBL-2 virus are clustered nearer to rabies virus.

All the epitopes defined by the monoclonal antibodies generated against the wild rabies virus isolate (fox rabies virus isolate) are common to all isolates tested from all lyssavirus genotypes. These are clearly immunodominant epitopes that may have been altered in cell culture adapted viruses. The gene for the fox rabies virus glycoprotein was cloned from mouse brain material (Wandeler, personal communication) and so was never adapted to cell culture. This may have allowed these major epitopes of the glycoprotein to be preserved for presentation to mouse lymphocytes used for hybridoma production. The lyssaviruses used for preparing ELISA antigen were very low passage number (up to passage 3 or 4) in cell culture. Presumably, some of the wild-type, immunodominant epitopes must have been preserved without significant amino acid changes. Wild-type epitopes must be more conserved than cell culture adapted epitopes. These data indicate that it may be important to include epitopes that represent wild lyssavirus isolates when developing new vaccines. For complete protection, a vaccine should present wild-type epitopes whenever possible. Epitopes responsible for virulence would necessarily still need to be attenuated.

Most of the topographical epitopes identified on the glycoprotein are conformational, which is in agreement with previously published results about rabies

virus glycoprotein epitopes. Two out of the thirteen topographical epitopes identified are linear and may be useful as peptide vaccine candidates. These two epitopes are present in all six genotypes. The epitope represented by Mab 10EC9 (site I) is more variable across the genotypes than the epitope represented by Mab 818 (site I) as evidenced by the affinity results. 10EC9 has high affinities only for the ERA lab strain and Lima dog isolate of rabies viruses. Its affinity is decreased in all other genotypes. Conversely, the epitope represented by M 818 has very high affinity across all lyssavirus genotypes, indicating that this is a very conserved epitope, probably crucial in some aspect of viral pathogenesis.

4.2 Nucleoprotein

Very little is known about the antigenic structure of lyssavirus nucleoproteins. Since the nucleoprotein is not a neutralizable protein, it has not received the attention that the glycoprotein has in the past. Lafon and Wiktor (1985) identified three antigenic sites on the ERA rabies virus glycoprotein. Two of these sites are linear and one conformational (Dietzschold et al. 1987c, 1988). Since these studies, no other experiments have been done to further understand the antigenic nature of the rabies virus nucleoprotein. The utility of studying the nucleoprotein at the nucleotide level has been exploited by several groups (Kissi et al. 1995; Smith et al. 1992; Nadin-Davis et al. 1993, 1994) for epidemiological and phylogenetic analyses. At the nucleotide level, vaccine strains of rabies viruses that have been sequenced show an extremely high level (99 %) of homology (Wunner et al. 1988). This appears also to be true for the epitope mapping results obtained in this study.

Competitive Mab binding assays using the six lyssavirus genotype nucleoproteins have not been reported in the literature. This study used an extensive panel of monoclonal antibodies generated against representatives of all lyssavirus genotypes. The statement made previously about the results of competitive binding assays being only as good as the monoclonal antibodies used is equally true for the nucleoprotein as it was for the glycoprotein. The initial selection of anti-nucleoprotein monoclonal antibodies available for this epitope mapping study represented all genotypes well except for genotype 6. Only one monoclonal antibody, 17EE12, was available for this genotype. Unlike the monoclonal antibodies available for the glycoprotein, only 7 % of the nucleoprotein Mabs were generated against vaccine strains of rabies virus. This may have proved beneficial in identifying wild-type epitopes that might have been missed by using only vaccine derived Mabs. Genotypes 2, 3, and 4 were initially represented by more than 30 anti-nucleoprotein monoclonal antibodies each. Genotype 5 was represented by fourteen monoclonal antibodies. The number of useful monoclonal antibodies that would represent each genotype was reduced after examining the homologous inhibition results between all genotype specific Mabs and their immunizing antigens in competitive binding assays. Mabs that gave identical inhibition patterns were deemed to be against the same epitope and were grouped together. A representative of each group was selected for use in the final mapping panel based on Mab isotype and ease of labeling with biotin. The final nucleoprotein mapping panel consisted of eighteen genotype 1 Mabs, four genotype 2 Mabs, four genotype 3 Mabs, six genotype 4 Mabs, two genotype 5 Mabs, and 1 genotype 6 Mab. In contrast to the glycoprotein mapping panel, only 25 % of the Mabs were vaccine derived and Mabs from all genotypes were

used. The mapping panel could be improved by the addition of more monoclonal antibodies generated against genotypes 2-6 if they were available.

Analysis of competitive binding results showed there to be five major antigenic sites on lyssavirus nucleoproteins and four sub-sites. The deduced topology of these sites is illustrated in Figure 4.3. Antigenic sites I-III are all characterized by a relatively small number of the mapping panel Mabs. Site I is characterized by two or three Mabs. One Mab, 26FE4 (site I) overlaps with site Ia in genotype 1 only. 26FE4 is not present on any other genotype, but because of this overlap of a single epitope, the epitope defined by Mab 39EE5 (site Ia) was made a sub-site of site I. Sites II and III are described by one and two Mabs, respectively. Antigenic site IV is a very large antigenic region that is identified by up to fifteen epitopes. This region has been subdivided into three distinct but overlapping regions (sub-sites). Site V is defined by a single Mab, as is site VI which is present in genotype 4 only.

The similarity of the antigenic sites between all lyssaviruses is very great. As expected, even greater similarity is seen between the three genotype 1 isolates studied. Out of 35 epitopes recognized by the mapping panel of monoclonal antibodies used, 32 are present in all three isolates. This represents a 91 % homology between these isolates at the epitope level. The ERA rabies virus strain is a vaccine strain and so has been well adapted to cell culture. It has one epitope, represented by Mab 11DD1, that is unique to ERA and may represent a cell culture adaptation of the virus. The ERA strain also lacks a canine specific epitope, defined by Mab 26BG8 (site IVa), that is present on both the Lima and Sri Lanka dog isolates. This epitope is also missing from all other lyssaviruses examined and so may represent a host specific epitope involved in maintaining rabies

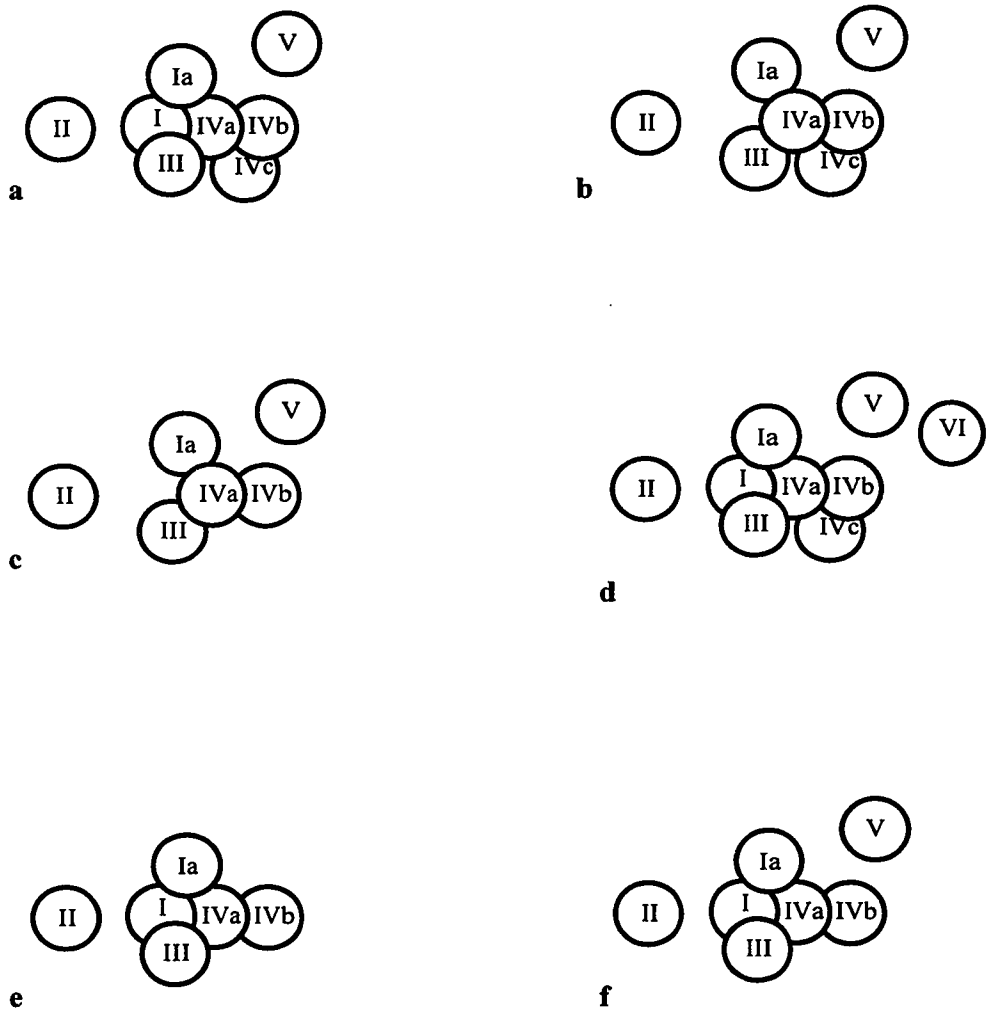


Figure 4.3 Epitope maps of lyssavirus nucleoproteins as deduced from competitive binding assays. Antigenic sites are represented by circles and designated by roman numerals. Each lyssavirus genotype is represented as follows: a) genotype 1, including ERA, Lima dog, and Sri Lanka dog isolates of rabies virus; b) genotype 2, Lagos bat virus; c) genotype 3, Mokola virus; d) genotype 4, Duvenhage virus; e) genotype 5, European bat lyassavirus type 1 (EBL-1); f) genotype 6, EBL-2

viruses in canine hosts. Only one other difference in the epitope profiles of the rabies viruses is the epitope represented by Mab M 855 (site IVc). This is a genotype 4 Mab that is found also on the Lima dog isolate (genotype 1) and genotype 2. The significance of this epitope is unknown. Also of note with the genotype 1 viruses is that none of the genotype 2 (Lagos bat) and only one of the genotype 3 (Mokola) epitopes are present. Up to 50 % of the genotype 4 (Duvenhage) epitopes are present in genotype 1 viruses as well as both the genotype 6 epitopes. Though many of the non-rabies epitopes are missing from genotype 1, they clearly fall into the same antigenic sites established for genotype 1. This is clearly further evidence of phylogenetic divergence from a common ancestor. When one looks at the phylogenetic trees available for the lyssaviruses, these epitope relationships agree completely. Mokola and Lagos bat viruses are the furthest diverged from rabies virus while Duvenhage virus and EBL-2 virus are more closely related phylogenetically to the rabies viruses. These data certainly add strength to the phylogenetic data previously reported (Bourhy et al. 1993a).

Though binding patterns of anti-nucleoprotein monoclonal antibodies have been studied in the past with the rabies-like viruses (genotypes 2-6) for epidemiological purposes, the epitopes on these proteins have never been functionally or physically mapped. This is therefore the first description of the antigenic structure of non-rabies lyssavirus nucleoproteins. As stated earlier, there is a remarkable similarity between the nucleoprotein epitope maps generated for all six genotypes. The only difference between the epitope nucleoprotein maps of genotypes 1 and 2 is that genotype 2 is missing site I as defined by the assembled panel of monoclonal antibodies. Perhaps if more monoclonal antibodies were available against Lagos bat virus, some would be able to

identify site I. Antigenic site I may also represent a region of the nucleoprotein that is more highly diverged than other regions because it is not critical to nucleoprotein function and lyssavirus survival. To emphasize the degree of divergence of both Lagos bat and Mokola viruses from rabies viruses, only six of the eighteen rabies virus specific nucleoprotein epitopes from all antigenic sites are present on these viruses.

Like Lagos bat virus, Mokola virus (genotype 3) also lacks antigenic site 1 as defined by this mapping panel. As well, antigenic site IVc is also missing. Phylogenetically, Mokola virus is the most diverged of all the lyssaviruses from rabies virus. With two antigenic sites missing, the Mokola virus nucleoprotein epitope map is also one of the most diverged epitopically of all the lyssavirus nucleoproteins from rabies virus. All Mokola virus antigenic sites, other than site IV, are defined by single epitopes. Again, it must be assumed that a more extensive panel of monoclonal antibodies may have resulted in identifying more mono-specific and cross-reactive epitopes in these antigenic sites.

Using the monoclonal antibodies available, Duvenhage virus (genotype 4) is the most antigenically complex of all the lyssaviruses. All five antigenic sites identified on the rabies virus (genotype 1) nucleoprotein are present on Duvenhage virus nucleoprotein and one additional site not found on any other lyssavirus has been identified. Site VI, defined by Mab M 878 on genotype 4 only, is separate from all other sites on the nucleoprotein since no inhibition is seen with any other monoclonal antibody in competitive binding assays. The binding affinity of M 878 (site VI) is moderate and certainly not high enough to block all competitors from binding. Like the canine epitope defined in wild rabies viruses by Mab 26BG8 (site IVa) , M 878 may also define a host

specific epitope necessary for maintenance of epizootics in the primary host of Duvenhage virus.

EBL-1 virus (genotype 5), next to Mokola virus, has the second most differences in its antigenic make up when compared to the other lyssaviruses. Only 8 of the 35 monoclonal antibodies that make up the nucleoprotein mapping panel bind to EBL-1 virus nucleoprotein. EBL-1 virus is missing antigenic sites IVc and V. If stringency was increased in determining whether an epitope was present or not in a genotype, site IVb might also be considered to be missing. M 840, the only site IVb Mab that binds to EBL-1 virus, does so with very low affinity, indicating that this epitope does not recognize the monoclonal antibody very well. Unfortunately, this Mab has weak affinities for all the lyssavirus genotypes, therefore an idea of the amount of divergence of this epitope from the authentic epitope can not be made. From the competitive binding assay results it is clear that M 840 (site IVb) is present on EBL-1 virus, indicated by its strong inhibition of the site IVa monoclonal antibodies.

EBL-2 virus (genotype 6) is very close to both genotypes 1 and 4 in its epitope topography. Only site IVc is missing from EBL-2 virus, however many of the epitopes that define the whole of site IV are missing. Also, with this genotype, the division between sub-sites IVa and IVb are not as clear cut as in other genotypes. There are low to moderate levels of inhibition all over the site IV area. Perhaps some differences in folding, or the shape of the nucleoprotein of this genotype bring all site IV epitopes into closer proximity to each other.

The type of the nucleoprotein epitopes is indicated by the immunoblotting results with all the monoclonal antibodies against their homologous antigens. Of the 34 unique

epitopes defined by the mapping panel, nine, or approximately 26 %, are linear. This is higher than the 15 % linear epitopes found on the glycoprotein in this study, and much higher than the few linear glycoprotein epitopes described in the literature. What makes these results very intriguing is that all the linear epitopes were found only with monoclonal antibodies generated against genotype 1 viruses. Linear epitopes were found on the nucleoproteins of the other lyssavirus genotypes, but only using genotype 1 Mabs. Why none of the monoclonal antibodies generated against the other lyssavirus genotypes recognized linear epitopes is unclear. Certainly more monoclonal antibodies were generated against rabies viruses than most of the other genotypes and this may account for the greater number of linear epitopes detected. These genotype 1 monoclonal antibodies came from eight different fusions using four different rabies viruses, which most likely adds diversity to the epitopes detected. An almost equal number of monoclonal antibodies was generated against genotype 4 (50 Mabs) as generated against genotype 1 (53 Mabs), yet no linear epitopes were defined by any genotype 4 specific Mabs. The difference however is the genotype 4 Mabs, as well as all other non-rabies genotypes, were generated from one fusion, each using only one virus representative. Perhaps to be able to identify linear epitopes in the rabies-like viruses (genotypes 2-6), more fusions will be required to allow for individual differences in antigen recognition in mice to be realized. Different viral epitopes may be immunodominant in different mice and this difference may be necessary to achieve more complete epitope pictures of these viruses.

The vast majority of the nucleoprotein epitopes are conformational as would be expected. Secondary structure is important in the normal functioning of the nucleo-

protein and this complexity is evident in the epitope structures. Three times more epitopes are conformational than linear. Another reason for the majority of epitopes being conformational is that all mice were immunized with the nucleoprotein in its native form as part of the ribonucleoprotein complex purified from virus. In this form, the nucleoprotein would have been bound to both the RNA genome as well as the phosphoprotein, making its secondary structure critical. For purposes of generating monoclonal antibodies against strictly linear epitopes the nucleoprotein would probably have to be dissociated from the RNP complex and be denatured using a reducing agent. Linear epitopes may be useful for generating peptide vaccines but their usefulness would probably be limited. Secondary and tertiary structures are vital to the proper functioning of the nucleoprotein so monoclonal antibodies generated against denatured epitopes may have little practical use.

Along with obtaining topographical mapping information about the lyssavirus nucleoproteins, the collected data have also revealed both lyssavirus and genotype specific epitopes. Four nucleoprotein epitopes are lyssavirus specific in that they are detected on all lyssaviruses examined in this study (refer to Table 3.12). One of the anti-nucleoprotein monoclonal antibodies that define these epitopes was generated against genotype 5 (EBL-1) (39EE5) and three against genotype 1; two against the Sri Lanka dog isolate (20AC3 and 20CB11), one against ERA strain (11CD4). Also, the three genotype 1 epitopes are linear (refer to Figure 3.2). These epitopes must be heavily constrained due to some essential role they play and are therefore resistant to evolutionary pressures.

Several epitopes are genotype 1 specific (refer to Table 3.12). One of these, 26BG8, appears to be canine rabies specific while another, 11DD1, is ERA virus vaccine

and *Lasiurus sp* specific (data not shown). 11DD1 reacts with all *Lasiurus sp.* isolates tested (Wandeler, personal communication), but not with any other terrestrial rabies isolates. *Lasiurus* is a bat genus that maintains its own rabies virus variants, all of which have the epitope defined by 11DD1. That several bat viruses share an epitope found only on an attenuated vaccine strain isolated from a dog is most intriguing. This may be a clue as to the evolution of terrestrial rabies arising from bat rabies. It may be that this is an ancient epitope that has been lost in terrestrial rabies virus isolates but either has been retained by ERA strain or has mutated back in to ERA strain. Alternatively, perhaps the reason ERA strain is attenuated is because it evolved from a modern day bat virus that in evolutionary terms recently became adapted to a dog. The specific natures of these epitopes may change when these monoclonal antibodies are applied to other viruses not used in this study, but to date, the epitope defined by 11DD1 has never been found on a terrestrial carnivore rabies isolate.

Among the other genotypes, at least one genotype specific epitope was found for each except for genotype 5 (EBL-1). The epitopes defined by Mab M 594 and M 617 are only present on genotype 2, Lagos bat virus. The Mab that is specific for genotype 3, Mokola virus, is M 1030. Mabs M 859 and M 878 define unique epitopes on genotype 4, Duvenhage virus, and 17EE12 defines a unique epitope on genotype 6, EBL-2. These monoclonal antibodies that are genotype specific may be very useful in the diagnosis of known lyssavirus infections and in identifying possible new lyssavirus genotypes.

4.3 Evidence for a common ancestral rhabdovirus to lyssaviruses being a bat virus

The very large amount of epitope and antigenic site conservation between the six lyssavirus genotypes can only mean that lyssaviruses have evolved from a common rhabdovirus ancestor. The method of choice for reconstructing phylogenies is by analyzing genome sequences, however the epitopes recognized in this study are the phenotypic expressions on which natural selection acts. They also present hints that rabies in bats might have preceded rabies in terrestrial mammals. The first clue is found with the epitope represented by Mab 11DD1 (site IVb). This epitope is found on all *Lasiurus sp.* bat viruses so far identified in North America but is only found on the ERA strain of rabies virus in carnivores. Since the ERA strain was first isolated from a dog, this may be a case of transfer of host specificity from one host (bat) to another (dog).

Another piece of evidence to support the ancestral bat rhabdovirus theory is that four out of the six lyssavirus genotypes are bat viruses. The vast number of distinct bat lyssavirus isolates that currently circulate in bat populations is in complete contrast to the carnivore isolates of genotype 1. While only one rabies virus strain currently circulates in the fox and skunk populations of Ontario, there are more than 10 distinct bat virus isolates that circulate in the Big Brown bat (*Eptesicus fuscus*) in the same area. The large diversity of bat viruses may indicate that lyssaviruses have been in bat species considerably longer than in carnivores.

5.0 CONCLUSIONS

In this study, the complex antigenic nature of two structural proteins (glycoprotein and nucleoprotein) of lyssaviruses has been explored. In agreement with previous studies of the rabies virus glycoprotein, several major antigenic sites have been defined. The major difference between this study and others is that two-way competitive binding assays were performed for the mapping of all epitopes defined. Monoclonal antibodies used for the mapping of the nucleoprotein were generated against all six lyssavirus genotypes , whereas, as in previous studies, only anti-rabies virus monoclonal antibodies were used for mapping of antigenic sites on the glycoprotein. Two-way competitions allow for confidence in epitope placement that cannot be achieved with one-way competitions.

The data from these experiments show there are four major antigenic sites and two sub-sites that are for the most part conserved in glycoproteins in all lyssavirus genotypes. Thirteen epitopes define the glycoprotein antigenic sites and seven of these are conserved in all six lyssavirus genotypes. Only one glycoprotein antigenic sub-site is missing from two of the viruses tested. Most of the epitopes identified on the glycoprotein are conformational, a finding which is in agreement with all previously published results. Two out of thirteen epitopes identified are linear.

For the nucleoprotein, five major antigenic sites were defined by 34 monoclonal antibodies representing unique epitopes. Antigenic site IV is defined by several overlapping epitopes that fall into three overlapping antigenic sub-sites. The topographical epitope maps of the nucleoproteins of the six genotypes are very similar

with at least three antigenic sites conserved in all six genotypes. Nine of the thirty-four epitopes identified are linear. As with the glycoprotein, the vast majority of the nucleoprotein epitopes are conformational, indicating that the nucleoprotein retains a considerable amount of secondary structure which may be important in the normal function of the nucleoprotein.

Results from both the glycoprotein and nucleoprotein experiments support the proposal for the existence of six lyssavirus genotypes as defined by genomic analyses. In fact, these data support theories that claim that all lyssaviruses have evolved from an ancestral rhabdovirus. The clear conservation of antigenic sites among all lyssaviruses tested can only mean divergent evolution from a common ancestor, probably by a process that is known as adaptive radiation in evolutionary biology. Essential epitopes necessary for virus propagation may be represented by these conserved epitopes present in all genotypes. Perhaps less essential epitopes have diverged more from the ancestral virus and are represented by the genotype specific epitopes.

The main goal of this study was to identify and map epitopes that define antigenic sites of two lyssavirus structural proteins, the glycoprotein and nucleoprotein. This has been achieved and now leads to many possible continuing avenues of pursuit. An interesting addition to this work will be its extension to cover the newly isolated Australian pteropid bat lyssavirus (genotype 7?) as well as the very diverse group of North American genotype 1 bat rabies viruses. We know that bat rabies viruses are very different from terrestrial rabies viruses. These differences should be examined in light of the fact that a large proportion of all rabies virus deaths in the industrialized western world is now caused by bat transmitted viruses.

A comparison of the anti-glycoprotein monoclonal antibodies used in these experiments with the published monoclonal antibodies against the rabies virus glycoprotein should be made. This will help to physically map the revealed epitopes on the lyssavirus glycoprotein. Similarly, the epitopes of the nucleoprotein should also be physically mapped on the core protein.

Though the information presented here is by no means a complete picture of lyssavirus antigenicity, much useful information has been gained. First, information that supports theories of rabies virus epidemiology and evolution has been obtained. Second, the complex nature of lyssavirus structural proteins is somewhat better understood as a result of these experiments. Still, many questions remain, for example, what this study has not uncovered is the nature of lyssavirus persistence in nature. The question of virus host specificity has not been answered, even though genotype specific epitopes have been identified. Why is fox rabies virus adapted to foxes and not able to maintain itself in other carnivores? The adaptation of a virus variant to a particular host cannot be simply explained by specific adaptation to cell surface receptors. Adaptation to a specific host must include the induction of clinical disease patterns that fit the host's natural life history and population biology. These questions and others about the complex relationship between lyssaviruses and their hosts are still waiting to be answered. Perhaps more answers will be obtained when the other lyssavirus proteins are more closely examined. However, this study of two structural proteins has provided important hints as to which direction to search to find answers to the questions of specific adaptation.

6.0 REFERENCES

1. **Abelseth, M. K.** 1964. Propagation of rabies virus in pig kidney cell culture. Can. Vet. J. **5**:84-87.

2. **Anilionis, A., W. H. Wunner, and P. J. Curtis.** 1981. Structure of the glycoprotein gene in rabies virus. Nature **294**:275-78.

3. **Astoul, E., M. Lafage, and M. Lafon.** 1996. Rabies superantigen as a Vbeta T-dependent adjuvant. J. Exp. Med.**183**:1623-31.

4. **Bacon, P. J.** 1985. A systems analysis of wildlife rabies epizootics, p. 109-130. *In* P. J. Bacon (ed.), Population dynamics of rabies in wildlife. Academic Press, London.

5. **Baer, G. M., J. H. Shaddock, R. Quirion, T. V. Dam, and T. L. Lentz.** 1990. Rabies susceptibility and acetylcholine receptor [letter]. Lancet **335:** 664-665.

6. **Baer, G. M.** 1991. Vampire bat and bovine paralytic rabies, p. 389-403. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

7. **Baer, G. M., and J. S. Smith.** 1991. Rabies in nonhematophagous bats, p. 341-346. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

8. **Barge, A., Y. Gaudin, P. Coulon, and R. W. Ruigrok.** 1993. Vesicular stomatitis virus M protein may be inside the ribonucleocapsid coil. Virology **67**:7246-7253.

9. **Barik, S., and A. K. Banerjee.** 1992. Sequential phosphorylation of the phosphoprotein of vesicular stomatitis virus by cellular and protein kinases is essential for transcription activation. J. Virol. **66**:1109-1118.

10. **Benmansour, A., M. Brahimi, C. Tuffereau, P. Coulon, F. Lafay, and A. Flamand.** 1992. Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogeneous nature of the viral population. Virology **187**:33-45.

11. **Benmansour, A., H. Leblois, P. Coulon, C. Tuffereau, Y. Gaudin, A. Flamand, and F. Lafay.** 1991. Antigenicity of rabies virus glycoprotein. J. Virol. **65**:4198-4203.

12. **Bingham, J., and C. M. Foggin.** 1993. Jackal rabies in Zimbabwe. Onderstepoort J. Vet. Res. **60**:365-366.

13. **Bjorklund, H. V., K. H. Higman, and G. Kurath.** 1996. The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame virus: analysis of relationships with other rhabdoviruses. Virus Res. **42:**65-80.

14. **Black, B. L., R. B. Rhodes, M. McKenzie, and D. S. Lyles.** 1993. The role of vesicular stomatitis virus matrix protien in inhibition of host-directed gene expression is genetically separable from its function in virus assembly. J. Virol. **67:**4814-4821.

15. **Blancou, J., M. F. A. Aubert, and M. Artois.** 1991. Fox rabies, p. 257-290. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

16. **Boulger, L. R., and J. S. Porterfield.** 1958. Isolation of a virus from Nigerian fruit bats. Trans. Royal Soc. Trop. Med. Hyg. **52:**421-424.

17. **Bourhy, H., B. Kissi, M. Lafon, D. Sacramento, and N. Tordo.** 1992. Antigenic and molecular characterization of bat rabies virus in Europe. J. Clin. Microbiol. **30:**2419-2426.

18. **Bourhy, H., B. Kissi, and N. Tordo.** 1993a. Molecular diversity of the Lyssavirus genus. Virology **194:**70-81.

19. **Bourhy, H., B. Kissi, and N. Tordo.** 1993b. Taxonomy and evolutionary studies on lyssaviruses with special reference to Africa. Onderstepoort J. Vet. Res. **60:**277-282.

20. **Bourhy, H., P. Sureau, and N. Tordo.** 1990. From rabies to rabies-related viruses. [Review]. Vet. Microbiol. **23:**115-128.

21. **Bracci, L., S. K. Ballas, A. Spreafico, and P. Neri.** 1997. Molecular mimicry between the rabies virus glycoprotein and human immunodeficiency virus-1 GP120: cross-reacting antibodies induced by rabies vaccination. Blood **90:**3623-3628.

22. **Broughan, J. H., and W. H. Wunner.** 1995. Characterization of protein involvement in rabies virus binding to BHK-21 cells. Arch. Virol. **140:**75-93.

23. **Bunschoten H., M. Gore, I. J. T. M. Classen, F. G. C. M. Uytdehaag , B. Dietzschold, W. H. Wunner, and A. D. M. E. Osterhaus.** 1989. Characterization of a new virus-neutralizing epitope that denotes a sequential determinant on the rabies virus glycoprotein. J. Gen. Virol. **70:**291-298.

24. **Burger, S. R., A. T. Remaley, J. M. Danley, J. Moore, R. J. Muschel, W. H. Wunner, and S. L. Spitalnik.** 1991. Stable expression of rabies virus glycoprotein in Chinese hamster ovary cells. J. Gen. Virol. **72:**359-367.

25. **Celis, E., D. Ou, B. Dietzschold, L. Otvos, and H. Koprowski.** 1989. Rabies virus-specific T cell hybridomas: identification of class II MHC-restricted T cell epitopes using synthetic peptides. Hybridoma **8:**263-75.

26. **Charlton, K. M., W. A. Webster, and G. A. Casey.** 1991. Skunk rabies, p. 307-324. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

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

28. **Chenik, M., K. Chebli, Y. Gaudin, and D. Blondel.** 1994. In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein. J. Gen. Virol. **75:**2889-2896.

29. **Chenik, M., M. Schnell, K. K. Conzelmann, and D. Blondel.** 1998. Mapping the interacting domains between the rabies virus polymerase and phosphoprotein. J. Virol. **72:**1925-1930.

30. **Cherkassky, B. L.** 1989. Ecological classification of lyssaviruses, p. 85-87. *In* O. Thraenhart, H. Koprowski, K. Bögel, and P. Sureau (eds.), Progress in rabies control. Wells Medical, Kent.

31. **Clark, K. A., S. U. Neill, J. S. Smith, P. J. Wilson, V. W. Whadford, and G. W. McKirahan.** 1994. Epizootic canine rabies transmitted by coyotes in south Texas [see comments]. JAVMA **204:**536-540.

32. **Clinton, G. M., S. P. Little, F. S. Hagen, and A. S. Huang.** 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. Cell **15:**1455-1462.

33. **Conti, C., B. Hauttecoeuer, M. J. Morelec, B. Bizzini, N. Orsi, and H. Tsiang.** 1988. Inhibition of rabies virus infection by a soluble membrane fraction from the rat central nervous system. Arch. Virol. **98:**73-86.

34. **Conzelmann, K. K., J. H. Cox, L. G. Schneider, and H. J. Thiel.** 1990. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology **175:**485-499.

35. **Conzelmann, K. K., and M. Schnell.** 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J. Virol. **68:**713-719.

36. **Coulon, P., P. Rollin, M. Aubert, and A. Flamand.** 1982. Molecular basis of

rabies virus virulence. I. Selection of avirulent mutants of the CVS strain with anti-G monoclonal antibodies. J. Gen. Virol. **61:**97-100.

37. **Coulon, P., P. Rollin, and A. Flamand.** 1983. Molecular basis of rabies virus virulence. II. Identification of a site on the CVS glycoprotein associated with virulence. J. Gen. Virol. **68:**693-696.

38. **Cox, J. H., F. Weiland, B. Dietzschold, and L. G. Schneider.** 1981. Re-evaluation of the structural proteins M1 and M2 of rabies virus, p. 721-725. *In* D. H. L. Bishop, and R. W. Compans (eds.), Replication of negative strand viruses. Elsevier, New York.

39. **Crandall, R. A.** 1991. Arctic fox rabies, p. 291-306. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

40. **De, B. P., G. B. Thornton, D. Luk, and A. K. Banerjee.** 1982. Purified matrix protein of vesicular stomatitis virus blocks viral transcription in vitro. Proc. Natl. Acad. Sci. USA **79:**7137-7141.

41. **Delagneau, J. F., P. Perrin, and P. Atanasiu.** 1981. Structure of the rabies virus: spatial relationships of the proteins G, M1, M2 and N. Ann. Virol. Inst. Pasteur Paris **132E:**473-493.

42. **Deregt, D., and L. A. Babiuk.** 1987. Monoclonal antibodies to bovine coronavirus: characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology **161:**410-420.

43. **Dietzschold, B., and H. C. Ertl.** 1991. New developments in the pre- and post-exposure treatment of rabies. [Review]. Crit. Rev. Immunol. **10:**427-439.

44. **Dietzschold, B., M. Gore, H. Ertl, E. Celis, L. Otvos, and H. Koprowski.** 1989. Analysis of protective immune mechanisms induced by rabies nucleoprotein, p. 295-305. *In* D. Kolakofski, and B. Mahy (eds.), Genetics and pathogenicity of negative strand viruses. Elsevier, New York.

45. **Dietzschold, B., M. Gore, D. Marchadier, H. S. Niu, H. M. Bunschoten, L. Otvos Jr, W. H. Wunner, H. C. Ertl, A. D. Osterhaus, and H. Koprowski.** 1990. Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. J. Virol. **64:**3804-3809.

46. **Dietzschold B., C. E. Rupprecht, M. Tollis, M. Lafon, J. Mattei, T. J. Wiktor, and H. Koprowski.** 1988. Antigenic diversity of the glycoprotein and nucleocapsid of rabies and rabies related viruses: implications for epidemiology and control of rabies. Rev. Infect. Dis. **10 Suppl. 4:**S785-S798.

47. **Dietzschold B., M. Tollis, C. E. Rupprecht, E. Celis, and H. Koprowski.**

1987a. Antigenic variation in rabies and rabies-related viruses: cross-protection independent of glycoprotein mediated virus-neutralizing antibody. J. Infect. Dis. **156:**815-822.

48. **Dietzschold, B., H. Wang, C. E. Rupprecht, E. Celis, M. Tollis, H. Ertl, E. Herber-Katz, and H. Koprowski.** 1987b. Induction of protective immunity against rabies by immunization with rabies virus nucleoprotein. Proc. Natl. Acad. Sci. USA **84:**9165-969.

49. **Dietzschold, B., T. J. Wiktor, R. MacFarlan, and A. Varrichio.** 1982. Antigenic structure of rabies virus glycoprotein: ordering and immunological characterization of the large CNBr cleavage fragments. J. Virol. **44:**595-602.

50. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80:**70-74.

51. **Dietzschold, B., M. Lafon, H. Wang, L. Otvos Jr., E. Celis, W. H. Wunner, and H. Koprowski.** 1987c. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. Virus Res. **8:**103-125.

52. **Domingo, E.** 1992. Genetic variation and quasi-species. [Review]. Curr. Opinion Genet. Dev. **2:**61-63.

53. **Domingo, E., and J. J. Holland.** 1988. High error rates, population equilibrium, and evolution of RNA replicating systems, p. 3-36. *In* E. Domingo, J. J. Holland, and P. Ahlquist (eds.), RNA Genetics Vol. III. Variability of RNA genomes. CRC Press, Boca Raton.

54. **Domingo, E., E. Martinez-Salas, F. Sobrino, J. Carlos de la Torre, A. Portela, J. Ortin, C. Lopez-Galindez, P. Pérez-Brena, N. Villanueva, R. Najera, S. VandePol, D. Steinhauer, N. DePolo, and J. Holland.** 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: Biological relevance - A review. Gene **40:**1-8.

55. **Donnelly-Roberts, D. L., and T. L. Lentz.** 1991. Structural and conformational similarity between synthetic peptides of curaremimetic neurotoxins and rabies virus glycoprotein. Brain Res. Mol. Brain Res. **11:**107-113.

56. **Durrer, P., Y. Gaudin, R. W. Ruigrok, R. Graf, and J. Brunner.** 1995. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. J. Biol. Chem. **270:**17575-17581.

57. **Eigen, M., and C. K. Biebricher.** 1988. Sequence space and quasispecies

distribution, p. 211-245. *In E.* Domingo, J. J. Holland, and P. Ahlquist (eds.), RNA genetics. Vol. III, Variability of RNA genomes. CRC Press, Boca Raton.

58. **Eigen, M., and P. Schuster.** 1977. The hypercycle: a principle of natural self-organization. Naturwiss **64:**541-565.

59. **Elmgren, L. D., and A. I. Wandeler.** 1996. Competitive ELISA for the detection of rabies virus neutralizing antibodies, p. 200-208. *In* F.-X. Meslin, M. M. Kaplan, and H. Koprowski (eds.), Laboratory techniques in rabies, 4th ed. World Health Organization, Geneva.

60. **Emerson, S. U., and M. Schubert.** 1987. Localization of the binding domains for the RNA polymerase L and the ribonucleocapsid template within different halves of the NS phosphoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA **84:**5655-5659.

61. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. **10:**1348-1356.

62. **Ertl, H. C. J., B. Dietzschold, M. Gore, L. Otvos Jr., J. K. Larson, and W. H. Wunner.** 1989. Induction of rabies virus-specific T helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol. **63:**2885-2892.

63. **Everard, C. O. R., and J. D. Everard.** 1985. Mongoose rabies in Grenada, p. 43-69. *In* P. J. Bacon (ed.), Population dynamics of rabies in wildlife. Academic Press, London.

64. **Flamand, A., T. J. Wiktor, and H. Koprowski.** 1980a. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. I. The nucleocapsid protein. J. Gen. Virol. **48:**97-104.

65. **Flamand, A., T. J. Wiktor, and H. Koprowski.** 1980b. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. II. The glycoprotein. J. Gen. Virol. **48:**105-109.

66. **Foggin, C. M.** 1983. Mokola virus infection in cats and a dog in Zimbabwe. Vet. Rec. **113:**115.

67. **Fu, Z. F., B. Dietzschold, C. L. Schumacher, W. H. Wunner, H. C. Ertl, and H. Koprowski.** 1991. Rabies virus nucleoprotein expressed in and purified from insect cells is efficacious as a vaccine. Proc. Natl. Acad. Sci. USA **88:**2001-2005.

68. **Fu, Z. F., W. H. Wunner, and B. Dietzschold.** 1994a. Immunoprotection by rabies virus nucleoprotein. [Review]. Curr. Topics Microbiol. Immunol.**187:**161-172.

69. **Fu, Z. F., Y. Zheng, W. H. Wunner, H. Koprowski, and B. Dietzschold.** 1994b. Both the N- and the C-terminal domains of the nominal phosphoprotein of rabies virus are involved in binding to the nucleoprotein. Virology **200:**590-597.

70. **Gao, Y., and J. Lenard.** 1995. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. EMBO J. **14:**1240-1247.

71. **Gastka, M., J. Horvath, and T. L. Lentz.** 1996. Rabies virus binding to the nicotinic acetylcholine receptor alpha subunit demonstrated by virus overlay protein binding assay. J. Gen. Virol. **77:**2437-2440.

72. **Gaudin, Y., H. Raux, A. Flamand, and R. W. Ruigrok.** 1996. Identification of amino acids controlling the low-pH-induced conformational change of rabies virus glycoprotein. J. Virol. **70:**7371-7378.

73. **Gaudin, Y., R. W. Ruigrok, M. Knossow, and A. Flamand.** 1993. Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J. Virol. **67:**1365-1372.

74. **Gaudin, Y., R. W. Ruigrok, C. Tuffereau, M. Knossow, and A. Flamand.** 1992. Rabies virus glycoprotein is a trimer. Virology **187:** 627-632.

75. **Gaudin, Y., C. Tuffereau, A. Benmansour, and A. Flamand.** 1991a. Fatty acylation of rabies virus proteins. Virology **184:**441-444.

76. **Gaudin, Y., C. Tuffereau, P. Durrer, A. Flamand, and R. W. Ruigrok.** 1995. Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation. J. Virol. **69:**5528-5534.

77. **Gaudin, Y., C. Tuffereau, D. Segretain, M. Knossow, and A. Flamand.** 1991b. Reversible conformational changes and fusion activity of rabies virus glycoprotein. J. Virol. **65:**4853-4859.

78. **Goding, J. W.** 1986. Monoclonal antibodies: principles and practice, 2nd Edition, p. 241-280. Academic Press, London.

79. **Hiramatsu, K., K. Mifune, K. Mannen, A. Nishizono, H. Kawano, Y. Ito, and A. Kawai.** 1992. Mapping of the antigenic determinants recognized by monoclonal antibodies against the M2 protein of rabies virus. Virology **187:**472-479.

80. **Holland, J. J., J. C. de la Torre, D. K. Clarke, and E. Duarte.** 1991. Quantitaion of relative fitness and great adaptability of clonal populations of RNA viruses. J. Virol. **65:**2960-2967.

81. **Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. Vandepol.** 1982. Rapid evolution of RNA genomes. Science **215:**1577-1585.

82. **Hooper, D. C., I. Pierard, A. Modelska, L. Otvos Jr., Z. F. Fu, H. Koprowski, and B. Dietzschold.** 1994. Rabies ribonucleocapsid as an oral immunogen and immunological enhancer. Proc. Natl. Acad. Sci. USA **91:**10908-10912.

83. **Hummeler, K., H. Koprowski, and T. J. Wiktor.** 1967. Structure and development or rabies virus in tissue culture. J. Virol. **1:**152-170.

84. **Kawai, A., and K. Morimoto.** 1994. Functional aspects of lyssavirus proteins. [Review]. Curr. Topics Microbiol. Immunol. **187:**27-42.

85. **Kemp, G. E., O. R. Causey, D. L. Moore, A. Odelola, and A. Fabiyi.** 1972. Mokola virus. Further studies on IbAn 27377, a new etiological agent of zoonosis in Nigeria. Am. J. Trop. Med. Hyg. **21:**356-359.

86. **Kimura-Kuroda, J., and K. Yasiu.** 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of japanese encephalitis virus, using monoclonal antibodies. J. Virol. **45:**124-132.

87. **King, A.** 1989. The rabies-related viruses of Africa and Europe, p. 153-158. *In* O.Thrsenhart, H. Koprowski, K. Bögel, and P. Sureau (eds.), Progress in rabies control. Wells Medical, Kent.

88. **King, A. A.** 1993. Monoclonal antibody studies on rabies-related viruses. Onderstepoort J. Vet. Res. **60:**283-287.

89. **King, A. A., C. D. Meredith, and G. R. Thomson.** 1994. The biology of southern African lyssavirus variants. [Review]. Curr. Topics Microbiol. Immunol. **187:**267-295.

90. **King A., and J. Crick.** 1988. Rabies-related viruses, p. 177-199. *In* J. B. Campbell and K. M. Charlton (eds.), Rabies. Kluwer, Boston.

91. **Kissi, B., N. Tordo, and H. Bourhy.** 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology **209:**526-537.

92. **Koprowski, H., T. J. Wiktor, and M. K. Abelseth.** 1985. Cross reactivity and cross protection: Rabies and rabies related viruses, p. 30-39. *In* E. Kuwert, C. Mérieux, H. Koprowski, and K. Bögel (eds.), Rabies in the tropics. Springer-Verlag, New York.

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

94. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.

95. **Lafay, F., A. Benmansour, K. Chebli, and A. Flamand.** 1996. Immunodominant epitopes defined by a yeast-expressed library of random fragments of the rabies virus glycoprotein map outside major antigenic sites. J. Gen. Virol. **77:**339-346.

96. **Lafon, M.** 1993. Rabies virus superantigen. [Review]. Res. Immunol. **144:**209-213.

97. **Lafon, M., H. Bourhy, and P. Sureau.** 1988. Immunity against the European bat rabies (Duvenhage) virus induced by rabies vaccines: An experimental study in mice. Vaccine **6:**362-368.

98. **Lafon, M., and A. Galelli.** 1996. Superantigen related to rabies. [Review]. Sem. Immunopathol. **17:**307-318.

99. **Lafon, M., J. Ideler, and W. H. Wunner.** 1984. Investigation of the antigenic structure of rabies virus glycoprotein by monoclonal antibodies. Dev. Biol. Stand. **57:**219-225.

100. **Lafon, M., and M. Lafage.** 1987. Antiviral activity of monoclonal antibodies specific for the internal proteins N and NS of rabies virus. J. Gen. Virol. **68:**3113-3123.

101. **Lafon, M., M. Lafage, A. Martinez-Arends, R. Ramirez, F. Vuillier, D. Charron, V. Lotteau, and D. Scott-Algara.** 1992. Evidence for a viral superantigen in humans. Nature **358:**507-510.

102. **Lafon, M., and T. J. Wiktor.** 1985. Antigenic sites on the ERA rabies virus nucleoprotein and non-structural protein. J. Gen. Virol. **66:**2125-2133.

103. **Lafon, M., T. J. Wiktor, and R. I. MacFarlan.** 1983. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. J. Gen. Virol. **64:**843-851.

104. **Lentz, T. L.** 1990. Rabies virus binding to an acetylcholine receptor alpha-subunit peptide. J. Mol. Recognition **3:**82-88.

105. **Lentz, T. L., T. G. Burrage, A. L. Smith, J. Crick, and G. Tignor.** 1982. Is the acetlycholine receptor a rabies virus receptor? Science **215:**182-184.

106. **Lentz, T. L., E. Hawrot, and P. T. Wilson.** 1987. Synthetic peptides

corresponding to sequences of snake venom neurotoxins and rabies virus glycoprotein bind to the nicotinic acetlycholine receptor. Proteins: Struc. Func. Genet. **2:**298-307.

107. **Lentz, T. L., P. T. Wilson, E. Hawrot, and D. W. Speicher.** 1984. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science **226:**847-848.

108. **Lodmell, D. L., J. W. Sumner, J. J. Esposito, W. J. Bellini, and L. C. Ewalt.** 1991. Raccoon poxvirus recombinants expressing the rabies virus nucleoprotein protect mice against lethal rabies virus infection. J. Virol. **65:**3400-3405.

109. **Lumio, J. H. M., R. Roine, L. Kefonen, M. Haltia, M. Valle, E. Neuvonen, and J. Lahdevirta.** 1986. Human rabies of bat origin in Europe. Lancet **378:**8477

110. **Luo, T. R., N. Minamoto, H. Ito, H. Goto, S. Hiraga, N. Ito, M. Sugiyama, and T. Kinjo.** 1997. A virus-neutralizing epitope on the glycoprotein of rabies virus that contains Trp251 is a linear epitope. Virus Res. **51:**35-41.

111. **Lyles, D. S., M. McKenzie, and J. W. Parce.** 1992. Subunit interactions of vesicular stomatitis virus envelope glycoprotein stabilized by binding to the viral matrix protein. J. Virol. **66:**349-358.

112. **Macdonald, R. A., C. S. Hosking, and C. L. Jones.** 1988. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J. Immunol. Methods **106:**191-194.

113. **Marrack, P., and J. Kappler.** 1990. The staphylococcal enterotoxins and their relatives. Science **248:**705-711.

114. **Masters, P. S., and A. K. Banerjee.** 1988. Resolution of multiple complexes of phosphoprotein NS with nucleocapsid protein N of vesicular stomatitis virus. J. Virol. **62:**2651-2657.

115. **Mebatsion, T., J. H. Cox, and J. W. Frost.** 1992. Isolation and characterization of 115 street rabies virus isolates from Ethiopia by using monoclonal antibodies: identification of 2 isolates as Mokola and Lagos bat viruses. J. Infect. Dis. **166:**972-977.

116. **Melki, R., Y. Gaudin, and D. Blondel.** 1994. Interaction between tubulin and the matrix protein of vesicular stomatitis virus: possible implications in the viral cytopathic effect. Virology **202:**339-347.

117. **Meredith, C. D., A. P. Rossouw, and H. Van Praag Koch.** 1971. An unusual case of human rabies thought to be of Chiropteran origin. S. African Med. J. **45:**767-769.

118. **Mifune, K., M. Ohuchi, and K. Mannen.** 1982. Hemolysis and cell fusion by rhabdoviruses. FEBS Lett. **137:**293-297.

119. **Morimoto, K., D. C. Hooper, H. Carbaugh, Z. F. Fu, H. Koprowski, and B. Dietzschold.** 1998. Rabies virus quasispecies: implications for pathogenesis. Proc. Natl. Acad. Sci. USA **95:**3152-3156.

120. **Morimoto, K., A. Ohkubo, and A. Kawai.** 1989. Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of rabies virus. Virology **173:**465-477.

121. **Morimoto, K., M. Patel, S. Corisdeo, D. C. Hooper, Z. F. Fu, C. E. Rupprecht, H. Koprowski, and B. Dietzschold.** 1996. Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. Proc. Natl. Acad. Sci. USA **93:**5653-5658.

122. **Morzunov, S. P., J. R. Winton, and S. T. Nichol.** 1995. The complete genome structure and phylogenetic relationships of infectious haematopoietic virus. Virology **173:**175-192.

123. **Nadin-Davis, S. A., G. A. Casey, and A. Wandeler.** 1993. Identification of regional variants of the rabies virus within the Canadian province of Ontario. J. Gen. Virol. **74:**829-837.

124. **Nadin-Davis, S. A., G. A. Casey, and A. I. Wandeler.** 1994. A molecular epidemiological study of rabies virus in central Ontario and western Quebec. J. Gen. Virol. **75:**2575-2583.

125. **Nadin-Davis, S. A., W. Huang, and A. I. Wandeler.** 1997. Polymorphism of rabies viruses within the phosphoprotein and matrix protein genes. Arch. Virol. **142:**979-992.

126. **Nel, L. H., G. R. Thomson, and B. F. Von Teichman.** 1993. Molecular epidemiology of rabies virus in South Africa. Onderstepoort J. Vet. Res. **60:**301-306.

127. **Newcomb, W. W., G. J. Tobin, J. J. McGowan, and J. C. Brown.** 1982. In vitro reassembly of vesicular stomatitis virus skeletons. J. Virol. **41:**1055-1062.

128. **Ni, Y., Y. Tominaga, Y. Honda, K. Morimoto, S. Sakamoto, and A. Kawai.** 1995. Mapping and characterization of a sequential epitope on the rabies virus glycoprotein which is recognized by a neutralizing monoclonal antibody, RG719. Microbiol. Immunol. **39:**693-702.

129. **Novella, I. S., E. A. Duarte, S. F. Elena, A. Moya, E. Domingo, and J. J. Holland.** 1995. Exponential increases of RNA virus fitness during large population transmissions. Proc. Natl. Acad. Sci. USA **92:**5841-5844.

130. **Paton, D. J., J. P. Lowings, and A. D. T. Barrett.** 1992. Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. Virology **190:**763-772.

131. **Poch, O., B. M. Blumberg, L. Bougueleret, and N. Tordo.** 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J. Gen. Virol. **71:** 1153-1162.

132. **Poch, O., N. Tordo, and G. Kieth.** 1988. Sequence of the 3386 3'-nucleotides of the genome of the AvO1 strain rabies virus: structural similarities of the protein regions involved in transcription. Biochimie **70:**1019-1029.

133. **Prehaud, C., P. Coulon, F. Lafay, C. Thiers, and A. Flamand.** 1988. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J. Virol. **62:**1-7.

134. **Raux, H., P. Coulon, F. Lafay, and A. Flamand.** 1995. Monoclonal antibodies which recognize the acidic configuration of the rabies glycoprotein at the surface of the virion can be neutralizing. Virology **210:**400-408.

135. **Rayssiguier, C., L. Cioe, E. Withers, W. H. Wunner, and P. J. Curtis.** 1986. Cloning of rabies virus matrix protein mRNA and determination of its amino acid sequence. Virus Res. **5:**177-190.

136. **Reagan, K. J., and W. H. Wunner. 1985.** Rabies virus interaction with various cell lines is independent of the acetylcholine receptor. Arch. Virol. **84:**277-283.

137. **Roehrig, J. T., R. A. Bolin, and R. G. Kelly.** 1998. Monoclonal antibody mapping of the envelope glycoprotein of the Dengue 2 virus, Jamaica. Virology **246:**317-328.

138. **Roehrig, J. T., J. W. Day, and R. M. Kinney.** 1982. Antigenic analysis of the surface glycoproteins of a Venezuelan equine encephalomyelitis virus (TC-83) using monoclonal antibodies. Virology **118:**269-278.

139. **Rohde, R. E., S. U. Neill, K. A. Clark, and J. S. Smith.** 1997. Molecular epidemiology of rabies epizootics in Texas. Clin. Diag. Virol. **8:**209-217.

140. **Rose, J. K., R. F. Doolittle, A. Anilionis, P. J. Curtis, and W. H. Wunner.** 1982. Homology between the glycoproteins of vesicular stomatitis virus and rabies virus. J. Virol. **43:**361-364.

141. **Rupprecht C. E., B. Dietzschold, W. H. Wunner, H. Koprowski.** 1991. Antigenic relationships of lyssaviruses, p. 69-100. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

142. **Rupprecht, C. E., J. S. Smith, M. Fekadu, and J. E. Childs.** 1995. The ascension of wildlife rabies: a cause for public health concern or intervention? Emerg. Infect. Dis.**1:**107-114.

143. **Sacramento, D., H. Badrane, H. Bourhy, and N. Tordo.** 1992. Molecular epidemiology of rabies virus in France: comparison with vaccine strains. J. Gen. Virol. **73:**1149-1158.

144. **Sacramento, D., H. Bourhy, and N. Tordo.** 1991. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol. Cell. Probes **5:**229-240.

145. **Schneider, L. G.** 1982. Antigenic variants of rabies virus. Comp. Immunol. Microbiol. Infect. Dis. **5:**101-107.

146. **Schneider, L. G., and J. H. Cox.** 1994. Bat lyssaviruses in Europe. [Review]. Curr. Topics Microbiol. Immunol. **187:**207-218.

147. **Schneider, L. G., B. Dietzschold, R. E. Dierks, W. Matthaaeus, P. J. Enzmann, and K. Strohmaier.** 1973. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. J. Virol. **11:**748-755.

148. **Schneider, L. G., B. J. H. Barnard, and H. P. Schneider.** 1985. Application of monoclonal antibodies for epidemiological investigations and oral vaccination studies: African viruses, p. 47-59. *In* E. Kuwert, C. Merieux, H. Koprowski, and K. Bögel (eds.), Rabies in the tropics. Springer, Berlin.

149. **Schnell, M. J., and K. K. Conzelmann.** 1995. Polymerase activity of in vitro mutated rabies virus L protein. Virology **214:**522-530.

150. **Seif, G., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoproteins. J. Virol. **53:**926-934.

151. **Shakin-Eshleman, S. H., A. T. Remaley, J. R. Eshleman, W. H. Wunner, and S. L. Spitalnik.** 1992. N-linked glycosylation of rabies virus glycoprotein. Individual sequons differ in their glycosylation efficiencies and influence on cell surface expression. J. Biol. Chem. **267:**10690-10698.

152. **Shope, R. E., F. A. Murphy, A. K. Harrison, O. R. Causey, G. E. Kemp, D. I. H. Simpson, and D. L. Moore.** 1970. Two African viruses serologically and morphologically related to rabies virus. J. Virol. **6:**690-692.

153. **Smith, J. S., L. A. Orciari, P. A. Yager, H. D. Seidel, and C. K. Warner.** 1992. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. J. Infect. Dis. **166:**296-307.

154. **Smith, J. S., P. A. Yager, W. J. Bigler, and E. C. Hartwig Jr.** 1990. Surveillance and epidemiologic mapping of monoclonal antibody-defined rabies variants in Florida. J. Wildlife Dis. **26:**473-485.

155. **Smith, J. S., P. A. Yager, and L. A. Orciari.** 1993. Rabies in wild and domestic carnivores of Africa: epidemiological and historical associations determined by limited sequence analysis. Onderstepoort J. Vet. Res. **60:**307-314.

156. **Smith, J. S..** 1988. Monoclonal antibody studies of rabies in insectivorous bats of the United States. Rev. Infect. Dis. **10 Suppl. 4**:S637-S643.

157. **Smith, J. S., and G. M. Baer.** 1988. Epizootiology of rabies: the Americas, p. 267-299. *In* J. B. Campbell, and K. M. Charleton *(eds.),* Rabies. Kluwer Academic Publishers, Boston.

158. **Smith, J. S., and A. A. King.** 1996. Monoclonal antibodies for the identification of rabies and non-rabies lyssaviruses, p. 145-156. *In* F.-X. Meslin, M. M. Kapaln, and H. Koprowski (eds.), Laboratory techniques in rabies, 4th ed. World Health Organization, Geneva.

159. **Smith, J. S., J. W. Sumber, L. F. Roumillat, G. M. Baer, and W. G. Winkler.** 1984. Antigenic characteristics of isolates associated with a new epizootic of raccoon rabies in the United States. J. Infect. Dis. **149:**769-774.

160. **Sokol, F.** 1975. Chemical composition and structure of rabies virus, p. 79-102. *In* G. M. Baer (ed.), The natural history of rabies, 1st ed. Academic Press, Orlando.

161. **Sokol, F., and H. F. Clark.** 1973. Phosphoproteins, structural components of rhabdoviruses. Virology **52:**246-263.

162. **Sokol, F., H. F. Clark, T. J. Wiktor, M. L. McFalls, D. H. L. Bishop, and J. F. Obijeski.** 1974. Structural phosphoproteins associated with ten rhabdoviruses. J. Gen. Virol. **24:**433-445.

163. **Sokol, F., H. D. Schlumberger, T. J. Wiktor, H. Koprowski, and K. Hummeler.** 1969. Biochemical and biophysical studies on the nucleocapsid and on the RNA of rabies virus. Virology **38:**651-655.

164. **Sokol, F., D. Stancek, and H. Koprowski.** 1971. Structural proteins of rabies virus. J. Virol. **7:**241-249.

165. **Steinhauer, D., and J. J. Holland.** 1987. Rapid evolution of RNA viruses. Ann. Rev. Microbiol. **41:**409-433.

166. **Superti, F., B. Hauttecoeur, M.-J. Morlelec, P. Goldoni, B. Bizzini, and H. Tsiang.** 1986. Involvement of gangliosides in rabies virus infection. J.

Gen. Virol. **67**:47-56.

167. **Sureau, P., P. Rollin, and T. J. Wiktor.** 1983. Epidemiologic analysis of antigenic variations of street rabies virus: detection by monoclonal antibodies. Am. J. Epidemiol. **117**:605-609.

168. **Takacs, A. M., T. Das, and A. K. Banerjee.** 1993. Mapping of interacting domains between the nucleocapsid protein and the phosphoprotein of vesicular stomatitis virus by using a two-hybrid system. Proc. Natl. Acad. Sci. USA **90**:10375-10379.

169. **Takacs, A., and A. Banerjee.** 1995. Efficient interaction of vesicular stomatitis virus P protein with the L protein or the N protein in cells expressing the recombinant proteins. Virology **208**:821-826.

170. **Tignor, G. H., F. A. Murphy, H. F. Clark, R. E. Shope, P. Madore, S. P. Bauer, S. M. Buckley, and C. D. Meredith.** 1977. Duvenhage virus: morphological, biochemical, histopathological and antigenic relationships to the rabies serogroup. J. Gen. Virol. **37**:595-611.

171. **Tignor, G. H., and A. L. Smith.** 1972. Vaccination and challenge of mice with viruses of the rabies group. J. Infect. Dis. **125**:322-324.

172. **Tollis, M., B. Dietzschold, C. B. Volia, and H. Koprowski.** 1991. Immunization of monkeys with rabies ribonucleoprotein (RNP) confers protective immunity against rabies. Vaccine **9**:134-136.

173. **Tordo, N.** 1996. Characteristics and molecular biology of the virus, p. 28-51. *In* F.-X. Meslin, M. M. Kaplan, and H. Koprowski (eds.), Laboratory techniques in rabies, 4th ed. World Health Organization, Geneva

174. **Tordo, N., H. Badrane, H. Bourhy, and D. Sacramento.** 1993. Molecular epidemiology of lyssaviruses: focus on the glycoprotein and pseudogenes. [Review]. Onderstepoort J. Vet. Res. **60**:315-323.

175. **Tordo, N., K. Charlton, and A. Wandeler.** 1998. Rhabdoviruses: rabies, p. 665-692. *In* L. Collier, A. Balows, and M. Sussman (eds.), Topley & Wilson's Microbiology and Microbial Infections, 9th ed. Vol. 1: Virology. Arnold, London.

176. **Tordo, N., and A. Kouknetzoff.** 1993. The rabies virus genome: an overview. [Review]. Onderstepoort J. Vet. Res. **60**:263-269.

177. **Tordo N., and O. Poch.** 1988. Structure of rabies virus, p. 25-45. *In* J. B. Campbell and K. M. Charlton (eds.), Rabies. Kluwer, Boston.

178. **Tordo, N., O. Poch, A. Ermine, G. Keith, and F. Rougeon.** 1988. Completion of the rabies virus genome sequence determination: highly conserved

domains along the L (polymerase) proteins of unsegmented negative-strand RNA viruses. Virology **165:**165-176.

179. **Tordo, N., O. Poch, G. Keith, and F. Rougeon.** 1986. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. USA **83:**3914-3918.

180. **Tordo, N., P. De Hann, R. Goldbach, and O. Poch.** 1992. Evolution of negative-stranded RNA genomes. Sem. Virol. **3:**341-357.

181. **Tuffereau, C., S. Fischer, and A. Flamand.** 1985. Phosphorylation of the N and M1 proteins of rabies virus. J. Gen. Virol. **66:**2285-2289.

182. **van der Heijden, R. W., J. P. Langedijk, J. Groen, F. G. UytdeHaag, R. H. Meloen, and A. D. Osterhaus.** 1993. Structural and functional studies on a unique linear neutralizing antigenic site (G5) of the rabies virus glycoprotein. J. Gen. Virol. **74:**1539-1545.

183. **Vernon, S. K., A. R. Neurath, and B. A. Rubin.** 1972. Electron microscopic studies on the structure of rabies virus. J. Ultrastruc. Res. **41:**29-40.

184. **Wagner, R. R.** 1990. Rhabdoviridae and their replication, p. 867-881. *In* B. N. Fields, D. M. Knipe (eds.), Fields Virology, 2nd ed. Raven Press, New York.

185. **Wandeler, A. I., S. A. Nadin-Davis, R. R. Tinline, and C. E. Rupprecht.** 1994. Rabies epidemiology: some ecological and evolutionary perspectives. [Review]. Curr. Topics Microbiol. **187:**297-324.

186. **Wandeler, A. I.** 1991. Carnivore rabies: ecological and evolutionary aspects. Hystrix **3:**121-135.

187. **Whitt, M. A., L. Buonocore, C. Prehaud, and J. K. Rose.** 1991. Membrane fusion activity, oligomerization, and assembly of the rabies virus glycoprotein. Virology **185:**681-688.

188. **WHO.** 1994. WHO workshop on genetic and antigenic molecular epidemiology of lyssaviruses. World Health Organization, Geneva.

189. **Wiktor, T. J., and H. Koprowski.** 1978. Monoclonal antibodies against rabies virus produced by somatic cell hybridization: detection of antigenic variants. Proc. Natl. Acad. Sci. USA **75:**3938-3942.

190. **Wiktor, T. J., and H. Koprowski.** 1980. Antigenic variants of rabies virus. J. Exp. Med. **12:**99-112.

191. **Winkler, W. G., and S. R. Jenkins.** 1991. Raccoon rabies, p. 325-340. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

192. **Wojczyk, B., S. H. Shakin-Eshleman, R. W. Doms, Z. Q. Xiang, H. C. Ertl, W. H. Wunner, and S. L. Spitalnik.** 1995. Stable secretion of a soluble, oligomeric form of rabies virus glycoprotein: influence of N-glycan processing on secretion. Biochemistry **34:**2599-2609.

193. **Wunner, W. H., B. Dietzschold, R. I. Macfarlan, C. L. Smith, E. Golub, and T. J. Wiktor.** 1985a. Localization of immunogenic domains on the rabies virus glycoprotein. Ann. Virol. Inst. Pasteur Paris **136E:**353-362.

194. **Wunner, W. H., B. Dietzschold, C. L. Smith, and M. Lafon.** 1985b. Antigenic variants of CVS rabies virus with altered glycosylation sites. Virology **140:**1-12.

195. **Wunner W. H.** 1991. The chemical composition and molecular structure of rabies, p. 31-67. *In* G.M. Baer (ed.), The natural history of rabies. 2nd ed. CRC Press, Boca Raton.

196. **Wunner W. H., J. K. Larson, B. Dietzschold, and C. L. Smith.** 1988. The molecular biology of rabies viruses. Rev. Infect. Dis. **10, Suppl. 4:**S771-S784.

197. **Wunner, W. H., K. J. Reagan, and H. Koprowski.** 1984. Characterization of saturable binding sites for rabies virus. J. Virol. **50:**691-697.

198. **Wunner, W. H., C. H. Calisher, R. G. Dietzgen, A. O. Jackson, E. W. Kitajima, M. Lafon, J. C. Leong, S. Nichol, D. Peters, J. S. Smith, and P. J. Walker.** 1995. Rhabdoviridae, p. 275-288. In Murphy, F. A., C. M. Fauguet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers (eds.), Virus taxonomy. Sixth report of the international committee on taxonomy of viruses. Springer-Verlag, Wien, New York.

199. **Yang, J., D. C. Hooper, W. H. Wunner, H. Koprowski, B. Dietzschold, and Z. F. Fu.** 1998. The specificity of rabies virus RNA encapsidation by nucleoprotein. Virology **242:**107-117.

Appendix 1: Monoclonal Antibodies Used For Epitope Mapping of Lyssavirus Glycoproteins

Mab Designation	Antigen Source	Lyssavirus Genotype	Ig Isotype	Neutralizable Epitope	U of Bern or ADRI
10AA9	SAD[1] (whole cell)	1: Rabies	G1		U of Bern
10DB12	SAD (whole cell)	1: Rabies	G1	YES	U of Bern
10EC9	SAD (whole cell)	1: Rabies	G1	YES	U of Bern
10ED8	SAD (whole cell)	1: Rabies	G2b		U of Bern
10HG7	SAD (whole cell)	1: Rabies	G2b		U of Bern
12AA1	SAD (whole cell)	1: Rabies	G2b		U of Bern
16AB1	HEP[2] (whole cell)	1: Rabies	G2b		U of Bern
16AH8	HEP (whole cell)	1: Rabies	G2a	YES	U of Bern
16DB4	HEP (whole cell)	1: Rabies	G2a	YES	U of Bern
16EH11	HEP (whole cell)	1: Rabies	G2a	YES	U of Bern
M 722	ERA[3] (ARG virus)[4]	1: Rabies	G1	YES	ADRI
M 723	ERA (ARG virus)	1: Rabies	G2b		ADRI
M 724	ERA (ARG virus)	1: Rabies	G2a		ADRI
M 725	ERA (ARG virus)	1: Rabies	G3	YES	ADRI
M726	ERA (ARG virus)	1: Rabies	G1		ADRI
M 727	ERA (ARG virus)	1: Rabies	G2a	YES	ADRI
M 730	ERA (ARG virus)	1: Rabies	G1		ADRI
M 732	ERA (ARG virus)	1: Rabies	G2b		ADRI
M 777	ERA (ARG virus)	1: Rabies	G1	YES	ADRI
M 778	ERA (ARG virus)	1: Rabies	G1		ADRI
M 784	ERA (ARG virus)	1: Rabies	G1		ADRI
M 785	ERA (ARG virus)	1: Rabies	G2a	YES	ADRI
M 786	ERA (ARG virus)	1: Rabies	G2a	YES	ADRI
M 799	ERA (ARG virus)	1: Rabies	G1	YES	ADRI
M 800	ERA (ARG virus)	1: Rabies	G1	YES	ADRI
M 804	ERA (ARG virus)	1: Rabies	G1	YES	ADRI
M 805	ERA (ARG virus)	1: Rabies	G1		ADRI
M 809	ERA (ARG virus)	1: Rabies	G2b		ADRI
M 813	ERA (ARG virus)	1: Rabies	G1	YES	ADRI
M 814	ERA (ARG virus)	1: Rabies	G2a		ADRI
M 815	ERA (ARG virus)	1: Rabies	G1		ADRI
M 818	ERA (ARG virus)	1: Rabies	G2a		ADRI
M 819	ERA (ARG virus)	1: Rabies	G2a		ADRI
M 820	ERA (ARG virus)	1: Rabies	G2a	YES	ADRI
M 1078	FOX[5] (ARG virus)	1: Rabies	G2b		ADRI
M 1089	FOX (ARG virus)	1: Rabies	G2a	YES	ADRI
M 1094	FOX (ARG virus)	1: Rabies	G2a		ADRI
M 1100	FOX (ARG virus)	1: Rabies	G2a		ADRI

[1] Street Alabama Dufferin rabies vaccine strain
[2] High Egg Passage rabies vaccine strain
[3] Evelyn Rokitniki Abelseth rabies vaccine strain
[4] Adenovirus Rabies Glycoprotein recombinant
[5] Fox rabies isolate from southern Ontario

Appendix 2: Monoclonal Antibodies Used For Epitope Mapping of Lyssavirus Nucleoproteins

Mab Designation	Antigen Source	Lyssavirus Genotype	Immunoglobulin Isotype	U of Bern or ADRI
5DF12	SAD[1] RNP[2]	1: Rabies	G1	U of Bern
7AG8	SAD RNP	1: Rabies	G1	U of Bern
7DH2	SAD RNP	1: Rabies	G1	U of Bern
11CD4	SAD RNP	1: Rabies	G1	U of Bern
11DB9	SAD RNP	1: Rabies	G1	U of Bern
11DD1	SAD RNP	1: Rabies	G1	U of Bern
11DG10	SAD RNP	1: Rabies	G1	U of Bern
20AC3	Sri Lanka dog RNP	1: Rabies	G1	U of Bern
20CB11	Sri Lanka dog RNP	1: Rabies	G2a	U of Bern
20CH4	Sri Lanka dog RNP	1: Rabies	G1	U of Bern
20GF4	Sri Lanka dog RNP	1: Rabies	G1	U of Bern
24FF11	Sri Lanka dog RNP	1: Rabies	G2a	U of Bern
26BG8	Sri Lanka dog RNP	1: Rabies	G1	U of Bern
26FE4	Sri Lanka dog RNP	1: Rabies	G2a	U of Bern
32FE10	Lima dog RNP	1: Rabies	G2b	U of Bern
32HH2	Lima dog RNP	1: Rabies	G1	U of Bern
32KA6	Lima dog RNP	1: Rabies	G1	U of Bern
M 594	Lagos Bat	2: Lagos Bat	G1	ADRI
M 600	Lagos Bat	2: Lagos Bat	G1	ADRI
M 613	Lagos Bat	2: Lagos Bat	G2a	ADRI
M 617	Lagos Bat	2: Lagos Bat	G1	ADRI
M 993	Mokola	3: Mokola	G2a	ADRI
M 1004	Mokola	3: Mokola	G2a	ADRI
M 1016	Mokola	3: Mokola	G2a	ADRI
M 1030	Mokola	3: Mokola	G2a	ADRI
M 840	Duvenhage	4: Duvenhage	G1	ADRI
M 853	Duvenhage	4: Duvenhage	G1	ADRI
M 855	Duvenhage	4: Duvenhage	G2a	ADRI
M 859	Duvenhage	4: Duvenhage	G2a	ADRI
M 862	Duvenhage	4: Duvenhage	G2a	ADRI
M 878	Duvenhage	4: Duvenhage	G1	ADRI
38HF2	EBL-1	5: EBL-1	G2a	U of Bern
39EE5	EBL-1	5: EBL-1	G1	U of Bern
17EE12	EBL-2	6: EBL-2	G2a	U of Bern

[1] Street Alabama Dufferin
[2] Ribonucleoprotein

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- D. Deregt, A. A. F. de Vries, M. J .B. Raamsman, **L. D. Elmgren**, and P. J. M. Rottier. 1994. Monoclonal antibodies to equine arteritis virus proteins identify the G_L protein as a target for virus neutralization. J. Gen. Virol. **75**:2439-2444.

- **L. D. Elmgren** and A. I. Wandeler.1996. Competitive ELISA for rabies serology. In: Meslin, F. X., ed. Laboratory Techniques in Rabies. World Health Organization, Geneva.

- **L. D. Elmgren** and A. I. Wandeler. In preparation. Epitope mapping of lyssavirus structural proteins I: Glycoprotein

- **L. D. Elmgren** and A. I. Wandeler. In preparation. Epitope mapping of lyssavirus structural proteins II: Nucleoprotein.