CHAPTER II

LITERATURE REVIEWS

Rabies virus (RV) is a neurotropic RNA virus that is responsible for encephalomyelitis in mammals classified in the *Rhabdoviridae* family of the *Mononegavirale* order of viruses. It is further divided into the *Lyssavirus* genus (from the Greek *rhabdos*, meaning "rod"), rabies virus and rabies-related viruses belong to a separate genus.(13) The family *Rhabdoviridae* together with the families *Paramyxoviridae, Filoviridae* and *Bornaviridae* constitute the "superfamily" taxon, order *Mononegaviridae*, because all members of the order are RNA viruses that contain nonsegmented, negative-sense, single-strand RNA genomes.(14) The genus *Lyssavirus* presently contains seven virus genotypes.(15,16,17,18) One of these defines rabies virus (RV) (genotype1, serotype1), whereas the other six present specific rabies-related lyssaviruses, reflecting the genetic diversity of viruses that share with rabies virus, the primary etiologic agent of rabies, the unique capability to produce a rabies-like encephalomyelitis. The rabies-related lyssaviruses include Lagos bat virus (LBV) (genotype 2, serotype 2), Mokola virus (MOKV) (genotype 3, serotype 3), Duvenhage virus (DUVV) (genotype 4, serotype 4), European bat lyssavirus type 1 (EBL-1) (genotype 5), European bat lyssavirus type 2 (EBL-2) (genotype 6) and Australian bat lyssavirus (ABLV) (genotype 7). Three other viruses included in the genus *Lyssavirus,* Kotonkon (KOTV), Obodhiang (OBOV) and Rochambeau (RBUV), which have a serologic (antigenic) relationship distant from all other genus members and have not been involved in rabies-like infections of mammals.(11)

2.1 Molecular composition and morphology of rabies virus particles.

All lyssaviruses share many biologic and physico chemical features as well as amino acid sequence characteristic that classify them with other rhabdoviruses. These

include the bullet-shaped morphology, helical nucleocapsid (NC) or ribonucleoprotein (RNP) core and structural proteins of the virus. The five structural proteins of the virion contain the genomic RNA tightly encapsidated by the viral nucleoprotein (N) and RNA polymerase complex, consisting of the large protein (L) and its co-factor, the phosphoprotein (P). Both L and P proteins are involved in transcription and replication. One matrix protein (M) interacts with both the nucleocapsid and the plasma membrane and probably plays an important role in the virion maturation process. The fifth is a type I transmembrane glycoprotein (G). Three of the viral proteins are located in the RNP core. They are the N, the noncatalytic polymerase-associated P and the catalytic L (RNA polymerase). All three proteins are involved in the RNA polymerase activity of the virion. Both the N and P are phosphorylated in rabies virus, unlike in other rhabdoviruses, in which only the P is phosphorylated.(20,21,22,23) The P in rabies virus is phosphorylated by a unique cellular protein kinase and specific isomers of protein kinase C.(24) The number of molecules of each protein per virion has been estimated in different laboratories with somewhat variable results.(25) It can be argued reasonably that these differences in estimates of protein molecules per virion simply may be a reflection of variables associated with the way the studies were conducted in the different laboratories.

Nevertheless, one stoichiometric relationship that emerges from these estimates with respect to the RNP composition that appears to be valid is the 2:1 ratio of the N and P molecules per virion. During nascent protein synthesis and replication of viral progeny RNA, these two proteins interact in the same 2:1 ratio to bind to the newly synthesized progeny RNA. Also, the L is typically the protein produced in least amount. The remaining two structural proteins of the rabies virion, the G and M, are associated with the lipid-bilayer envelope that surrounds the RNP core. The M lines the viral envelope, forming an inner leaflet between the envelope and RNP core, whereas the G produces the spikelike projections or peplomers on the surface of the viral envelope.(26) Rabies virions also have been found to contain such cellular

proteins as actin and heat shock proteins of the hsp70 type, similar to other negative-strand RNA viruses.(27,28,29)

Although the explanation for cellular proteins in mature virions is not entirely clear, it is possible that the molecular chaperones, such as the heat shock protein calnexin, that associate with the viral proteins during synthesis are incorporated into virions after binding to and assisting in G folding.(30) Also, a small fraction of calnexin and possibly other chaperone proteins may escape from the endoplasmic reticulum (ER), where they function to ensure proper protein folding before being expressed on the cell surface where virus budding occurs.(31) In a similar manner, cytoskeleton proteins normally expressed on the cell surface may be incorporated into virions as a consequence of their proximal location and function in virus budding.(29,32) Other cytoskeletal proteins may ensure intracellular transport of viral RNP.(33,34) Cellular kinases that activate the transcriptional function of P in rabies virus also may be packaged into rabies virions.(24) At the center of the bullet-shaped virus particle is a core of the helical RNA (the viral genome) and protein, the RNP core that extends along its longitudinal axis and is surrounded by a lipid-protein envelope. The RNA is single-stranded, nonsegmented and has negative-sense or minus-strand polarity.(35) This implies that isolated (naked) minus-strand genome RNA is not infectious and it cannot be translated directly into protein.(11) The RNP that becomes the tightly coiled core of all virions is produced from a flexible right-handed helix structure that has a periodicity of approximately 7.5 nm per turn. The RNP core in standard size infectious virions measures approximately 165 x 50 nm but measures between 4.2 and 4.6 mm in length when relaxed and fully extended, like a tread, outside the virion.(36,37)

During virus assembly, the RNP core is surrounded by M, one of the two membrane proteins of the virus, to form the "skeleton" structure of the virus.(38) As virus particles mature and bud through the cellular membrane, the skeleton structure acquires the lipid bilayer envelope that is 7.5 to 10 nm thick surrounding the mature

virion. Located on the external surface of the viral envelope are the surface projections that measure 8.3-10 nm in length, each projection or spike containing three molecules (a trimer) of the viral G.(39) These have been described when viewed in the EM as the "short spikes extending outward with the appearance of hollow knobs at their distal ends".(36) It is estimated that the height of the "hollow knobs" or "heads" of the spike is about 4.8 nm, the rest of the spike is made up of the thin "stalk" on which the head rests.(39)

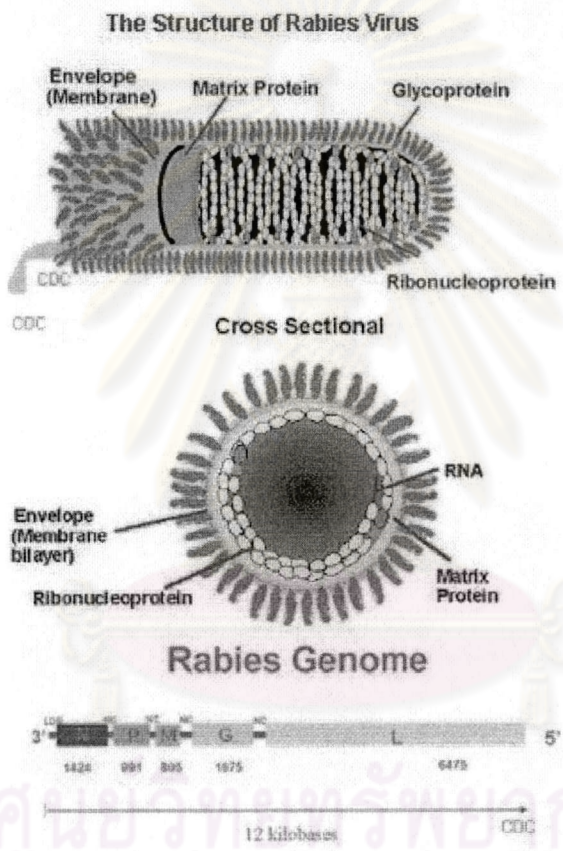

Figure 1 : structure of rabies virus

2.2 Genome organization

The RNA is single-stranded, nonsegmented and has negative-sense or minus-strand polarity. This implies that isolated (naked) minus-strand genome RNA is not

infectious and it cannot be translated directly into protein.(11) The first event in infection, therefore, is transcription of the genome RNA to produce complementary (positive strand) monocistronic messenger RNA (mRNA) molecules from each of the viral genes or cistrons in the genome. The viral proteins are synthesized from themonocistronic mRNAs. The organization and general features of the rabies virus genome RNA are similar to other negative-strand RNA viruses within the *Mononegavirales* order and, in particular, to other rhabdoviruses.(40) At the 3'end (first 58 nucleotides) of the 11,932 nucleotides genome RNA of rabies virus (PV strain) is a noncoding (extragenic) leader (Le) sequence. Immediately downstream of the Le sequence, in sequential order, are the five structure genes (N, P, M, G, and L) followed by noncoding trailer (Tr) sequence (last 70 nucleotides) at the 5' end.(41,42) The genes are separated by relatively short (dinucleotide o pentanucleotide) sequences that represent the intergenic regions (stretch of nucleotides from the 5' end of one gene to the 3' start of the next gene) of the genome. These short intergenic regions are located between the N and P genes (2 nucleotides) and between the P and M genes and the M and G genes (each 5 nucleotides long). The remaining intergenic region between the G and L genes contains a long stretch of 423 nucleotides in the rabies virus genome, 504 nucleotides in the Mokola virus genome,(43) 475 nucleotides in the Australian bat lyssavirus genome.(44) The G-L intergenic region is sufficiently long to represent a potential gene but lacks an ORF for a detectable protein. It has been given the designation of remnent gene or pseudogene (Ψ), recognizing that it once represented an ORF of sufficient size to code for a recoverable protein.(42) Interestingly, in this long intergenic region, two sequences stand out that appear to give credence to its former function. One is a sequence motif that resembles the rabies consensus mRNA start signal (UUAU), which is located 10 nucleotides downstream (or UUGU in MOKV, 20 nucleotides downstream) from the stop signal for the mRNA of the G gene in the rabies virus genome. The other is a stretch of 25 nucleotides located upstream from the L gene, which resembles a polyadenylation (poly A) signal

located at the end of mRNA molecules. These signals suggest that the virus may have inherited and since lost a protein ORF in its evolution, analogous to the nonviral protein of the infectious hematopoietic necrosis virus, a fish rhabdovirus.(42) Strangely, the Ψ region represents the most divergent area of the genome.(45)

The Le sequence at the 3'end of the genome RNA serves a multifunctional purpose in rabies viruses. Within the 3' terminal Le sequence, a specific *cis*-acting signal (a specific nucleotide sequence "acting within" the genome RNA) functions as a signal (or promoter) for template recognition by the viral RNA transcriptase (L-alone) or RNA polymerase complex (L and P). This particular signal initiates genome RNA transcription.(46,47,48,49) Within the first 10 to 20 nucleotides at the 3' and 5' ends of the rabies virus RNA genome there is a high level of sequence complementary, including an exact base complementarity between the first and the last 11 nucleotides at the 3' and 5' ends of the genome RNA, respectively. This is compelling evidence that the promoter sequences, which are shared in the Le and TrC (3' end of the antigenome RNA that is complementary to the 5' end of the genome) regions, provide a common function in transcription and replication.(11)

During transcription, a positive stranded leader RNA and five mRNAs are synthesized. The replication process yields nucleocapsids containing full length antisense genomic RNA, which in turn serves as a template for the synthesis of positive sense genomic RNA. The only external viral protein that governs the viral tropism.(50,51)

Human rabies is almost always attributing to a bite exposure, bitten by rabid animals generally inoculate virus-laden saliva through the skin into muscle and subcutaneous tissue. Other routes of infection are rare. A bite to the areas with abundant nerve supply alone is not the sole determinant for rabies risk. It is also the severity of the wound (transdermal bite with bleeding), particularly if deep enough to reach the muscles, at areas where muscle surfaces contain high density of nicotinic acetylcholine receptor (AchR) that determines the likelihood of developing rabies.

During the incubation period the virus can replicate locally in muscle cells or attach directly to nerve endings. Having gained access to peripheral nerves, it travels in a retrograde direction within the axoplasm. When the reaches the central nervous system, there is massive replication on membranes within neurons.

2.3 Transport of rabies virus to the CNS

Rabies virus migrates along peripheral nerves towards the central nervous system at about 12-100 mm per day via the fast axonal transport system. Because this movement is strictly retrograde, It is used experimentally to track neural pathways. Infection is thought to be via sensory as well as motor nerves, because antigen was detected in sensory nerve endings and dorsal root ganglia soon after peripheral inoculation in several studies.(52) Rabies virus has been used as a neuroanatomical tracer in order to define circuits of synaptically linked neurons in rodents and primates, and *in vivo* studies have provided evidence that axonal transport of rabies virus occurs exclusively in the retrograde direction. Recently reports have provided evidence that the rabies virus phosphoprotein, particularly involving amino acid residues at position 143 and 147 interacts strongly with the 10-kDa cytotoplasmic dynein light chain (LC8).(53) LC8 is a component of both cytoplasmic dynein and myosin V and is important in both microtubule-directed organelle transport and in actin-based vesicle transport in axons. However, the role of the interaction of the rabies virus phosphoprotein and dynein for axonal transport of the ribonucleocapsid complex has not yet been demonstrated. Mutants with a deletion in amino acid residues of the phosphoprotein encompassing a conserved LC8-interacting motif and simultaneous substitution of the arginine at position 333 of the glycoprotein showed neuroattenuation in mice. Interestingly, mutants with deletions in the LC8 binding region of the phosphoprotein remained as pathogenic as their parent virus after transmuscular inoculation of suckling mice, indicating that LC8 is actually dispensable

in young mice for the spread of pathogenic rabies virus from a peripheral site to the CNS.(54)

Recently study demonstrated that the glycoprotein (G) is important for the transsynaptic spread of rabies virus between neurons by bind with neuronal receptor. Yan *et al* examined the role of rabies virus glycoprotein in determining the topographic distribution of rabies virus infection 7 days after stereotaxic inoculation of virus into the hippocampus of rats using a variety of rabies virus strains and recombinant viruses, including a rabies virus recombinant constructed using the vascular stomatitis virus glycoprotein. With all of the recombinant virus, the viral distribution was similar to that of parental viruses from which the glycoprotein was derived.(55,56) Hence, further evidence is provided that the rabies virus glycoprotein exerts a very important influence on the distribution of rabies virus infection in the nervous system. Mazarakis and team have also recently demonstrated that rabies virus glycoprotein-pseudotyped lentivirus (equine infectious anemia virus)-based vectors enhance gene transfer to neurons by facilitating retrograde axonal transport. Hence, a variety of studies emphasize the importance of the rabies virus glycoprotein in the uptake, transport, transsynaptic spread and topographic distribution of the infection in the nervous system.(57,58)

2.4 Spread of virus within the CNS

Viral replication is intraneuronal, but the mechanism of interneuronal spread is unknown. Once rabies virus reaches the CNS, rapid amplification occurs in neuronal perikarya and virus disseminates via plasma membrane budding and direct cell to cell transmission or by transsynaptic propagation. Following stereotaxic inoculation into the striatum, rabies virus has been shown to travel by retrograde fast axonal transport (200-400 mm/day). It is not known exactly which tract is preferentially involved. *In vivo*, rabies virus also can spread in the cell, particularly cells of peripheral nerves and neuronal cells of the CNS, through intraaxonal transport in a microtubule network-

dependent process, dynein light chain (LC8) by bind with phosphoprotein of rabies virus. Neurons are the CNS cells selectively involved, although infection of astrocytes and glail cells in animals and humans has been reported.(10,59,60,61) The fact that budding of virus is very rarely seen at synapses by electron microscopy suggests that infectious naked nucleocapsids are transferred across synapses.

However, interneuronal infection is dependent on the presence of viral glycoprotein, which suggests that intact virus can cross the synapse. The mechanism of uptake is unknown, but one possibility is through the synaptic vesicle recycling system. Direct transmission of virus occurs from cell to cell across synaptic junctions. At the onset of illness when evidence of neuronal dysfunction appears, there is little or no apparent histopathological change. Centrifugal spread of virus from the central nervous system in somatic and autonomic nerves deposits virus in many tissues, including skeletal and cardiac muscle adrenal glands, kidney, retina, cornea, pancreases and nerves around hair follicles. Productive viral replication with budding from plasma membranes takes place predominantly in the salivary glands, excreting virus that is transmissible to other mammals.(52)

2.5 Neuronal dysfunction and death

In human beings, the symptoms of encephalitis and even death can occur with only minor histopathological changes. Natural rabies is normally characterized by severe neurologic signs and fatal outcome with relatively mild neuropathologic changes in the CNS, supporting the idea that neuronal dysfunction, rather than neuronal cell death, must play an important role in producing the disease.

Clinical and laboratory evidence suggests that functional changes and clinical manifestations (including mood and behavior, motor weakness, etc.) be due to a differential response of the various CNS regions. Cerebrum symptoms dominate the clinical picture in furious rabies and spinal cord and/or peripheral nerve symptoms in dumb rabies. Similar regional CNS rabies antigen distribution can be found in both

forms. There is also no correlation between the site of bite and virus localization. Brainstem, thalamus, basal ganglia and spinal cord are preferential sites of rabies viral infection in both forms of rabies. Moreover minimal or no rabies viral antigen was found in the hippocampus and neocortex. During the early phase of clinical rabies (within 72 hrs.) serum cortisol levels were significantly elevated as compared to those with viral encephalitis other than rabies and others with immune-mediated encephalitis. This indicates early stimulation of limbic structures, including the hypothalamus, which may relate to behavioral changes.

The presence and amount of virus in the CNS does not appear to determine the clinical and functional severity of the disease. Access of the virus to the CNS does not necessarily lead to rapid development of symptoms and death. High titers of virus in the brain and spinal cord can be found in animals long before clinical signs appear.(10)

2.5.1 Abnormality of neurotransmitter functions

Abnormality of neurotransmitter functions affecting serotonin, opioid, GABA and muscarinic acetylcholine transmission have been found experimentally, in some cases in specific brain areas, and not always associated with the presence of virus. Results show no clear explanation of the limbic-system dysfunction suggested by the classic clinical features. Changes in neurotransmitter functions could lead to failure of brain networking and regulation of responses.(52) Moreover, rabies virus nucleocapsid inhibits the actin-bundling effect induced by dephosphosynapsin I, a neuron-specific protein which is known to exert a control on the actin-based cytoskeleton.(62)

2.5.2 Ion channels changed

Dysfunction of ion channels has been shown in rabies virus infected cultured mouse neuroblastoma NA cells with the whole cell patch clamp technique.(63) The infection reduced the functional expression of voltage-dependent sodium channels and inward rectifier potassium channels and there was a decreased resting membrane

potential reflecting membrane depolarization. There was no change in the expression of delayed rectifier potassium channels, indicating that nonselective dysfunction of ion channels had not occurred. The reduction in sodium channels and inward rectifier potassium channels could prevent infected neurons from firing action potentials and generating synaptic potentials, resulting in functional impairment.

2.5.3 Nitric oxide neurotoxicity

The role of nitric oxide toxicity in neuronal dysfunction in rabies is not clear,(64) but it could be related to the LC8 inhibition of neuronal nitric oxide synthase, through interaction with the viral phosphoprotein.(65) Koprowski and coworkers have hypothesized that nitric oxide neurotoxicity may mediate neuronal dysfunction in rabies. Induction of inducible nitric oxide synthase mRNA and increased brain levels of nitric oxide have been demonstrated in rabies virus infected rodents, but the significance of these findings is uncertain.(64,66) The role of nitric oxide in rabies pathogenesis needs further study.

2.5.4 Neuronal cell death

Neurotropic viruses may cause cell death by either apoptosis or necrosis. Each of these forms of cell death is associated with typical morphologic features. Morphologically, apoptosis is characterized by nuclear and cytoplasmic condensation of single parenchyma cells followed by fragmentation of the nuclear chromatin and subsequent formation of multiple fragments of condensed nuclear material and cytoplasm.(67) Phagocytosis of this material occurs, although an inflammatory reaction normally is absent. In contrast, cellular death due to necrosis is characterized by preservation of cell outlines, and there is variable swelling of the cell and of organelles. Cellular fragmentation occurs as a late event in necrosis. There are derangements in energy and substrate metabolism in necrosis that result in breaks in the plasma membrane and organellar membranes. Apoptosis is associated with

endonuclease-mediated cleavage of the DNA of nuclear chromatin, resulting in DNA fragments with sizes in multiples of a single nucleosome length (180 bp). The internucleosomal cleavage of the DNA results in a ladder appearance on agarose gel electrophoresis, whereas in necrosis there is less specific degradation of DNA into a smear containing fragments of different sizes.

During viral infection, the destruction of infected cells by apoptosis can be considered as an innate cellular response to limit viral propagation. Apoptosis can occur either through an intrinsic reaction within the infected cells or by the action of cytotoxic T cells, which induce the apoptosis of virus infected cells by triggering the secretion of cytokines such as TNF-alpha the release of perforin and granzymes and by activating Fas in the target cells. In contrast, viruses can facilitate their dissemination by developing anti-apoptotic strategies or by evading cytotoxic T lymphocyte (CTL) and natural killer (NK) cell attack. Viruses can inhibit the activation of the caspases induced by death receptors, encode analogues of the anti-apoptotic protein Bcl-2, inactivate p53 or secrete neurotropic factor. In addition, viruses use numerous mechanisms to prevent presentation by MHC molecules or to evade CTL or NK killing.(68)

The challenge virus strain (CVS) of fixed rabies virus has been observed to induce apoptotic cell death in rat prostatic adenocarcinoma cells(65), mouse neuroblastoma cells(69) and in mouse embryonic hippocampal neurons.(70) *In vitro* and *in vivo* experiments using cultured rat prostatic adenocarcinoma (AT3) and mouse neuroblastoma cells and suckling and adult ICR mice infected with CVS strain of fixed rabies virus showed characteristic morphological features of apoptosis, evidence of oligonucleosomal DNA fragmentation and of Bax protein. The apoptosis is a caspase dependent programme and *Bcl-2* transfected AT3 cells were resistant to apoptosis.(65,71) Apoptosis was first observed and most marked in pyramidal neurons of the hippocampus, where there was a little infection, and in neurons scattered in the neocortex in CVS-infected adult mice. More apoptosis was evident in the brain of

suckling mice than in those of adult mice. Further the presence of rabies antigen does not correlate with the apperance of apoptosis.(65,72) Purkinje cells expressed viral antigen but did not show apoptosis, whereas neurons in the external granular layer of the cerebellum did not express viral antigen, but demonstrated greater apoptotic changes. These imply that rabies virus induces apoptosis *in vivo* by both direct and indirect mechanisms. Apoptosis can also be induced by the cytokine-nitric oxide process via initiation of a breakdown of sphingomyelin and ceramide production and glutamate exitoxicity etc.(10)

Further more apoptosis appears to be one of the most important defense mechanisms against rabies virus infection. It leads to depolymerization of actin filaments, which would prevent transport of viral nucleocapsid protein and neuronal spread of virus. The extent of apoptosis correlates with the amount of expression of rabies virus G protein in infected neurons.(73,74,75) Down regulation of G protein expression in neuronal cells contributes to pathogenesis by preventing apoptosis.(76) However, the process of cell death is also modulated by the types of the neurons infected and the quality of immune response to infecting virus, as well as its virulence.(77) There is a delay of apoptosis in spinal cord motoneurons after rabies virus infection compared with hippocampal cells.(78,79)

Guigoni and coulon observed that primary cultures of CVS-infected purified rat spinal motoneurons did not show major evidence of apoptosis over a period of 7 days, wheras infected neurons did not survive more than 2 days in crude primary spinal cord cultures.(79) This survival was not dependent on the presence of factors in the culture medium. In contrast, cultures of purified hippocampus neurons showed apoptosis in over 90% of neurons within 3 days. These results suggest that different neuronal cell types respond differently to rabies virus infection, and that the presence of glial cells and/or neurons other than motoneurons are essential for apoptosis of spinal motoneurons. Physical contact with glia or synaptic contact with other spinal cord neorons may be necessary for induction of apoptosis in motoneurons, but not for

apoptosis of hipppocampal neurons. However, apoptosis in infected cultured cells, including embryonic cells, does not closely correspond to what is observed in infected animals. Peripherally inoculated animals with CVS strains do not show the prominent apoptosis that is observed in neurons after intracerebral inoculation.(80) Conflicting results have been reported by different investigators with respect to the occurrence of neuronal apoptosis after intracerebral inoculation of different street (wild type) rabies virus variants in mice.(81,82) Hence, in rabies virus infection, there are complex mechanisms involved in cell death or survival of neurons both in vitro and in animal models using different viral strains and routes of inoculation.