CHAPTER II

LITERATURE REVIEW

RABIES VIRUS

1. Background

Rabies in man has been called "hydrophobia" due to the prominent clinical dread of water. The disease appears to have been first recognized in dogs, and that the responsible etiologic agent was known as "Rabies virus" by Pasteur in 1880. Rabies virus is a rhabdovirus of the Lyssavirus group. Its typical distinct bullet-shaped morphology is shared with other rhabdoviruses which infect vertebrates, invertebrates and plants although without any apparent genetic interrelationship. The core of the rabies virus is a helical structure of negative stranded RNA and a closely associated layer of protein ribonucleocapsid that forms a cylinder consisting of 30 to 35 coils, approximately 50 nm wide and 165 nm long (22). The surface envelope, derived from host cell membrane, is covered by regularly spaced knoblike spikes, 6 to 7 nm long, except at the flat end of the particle where the membrane is frequently depressed.

The replication of rabies virus is similar to that of other negative-stranded RNA viruses, which occurs in the

cytoplasm of the infected cells. The first event is to initiate transcription into mRNA by RNA-dependent RNA polymerase (virus transcriptase) and mRNA is then translated to form 40S positive stranded RNAs, which serve as template for subsequent production of negative stranded progeny RNA (23).

2. Viral Antigens

Kaplan (3) defined five proteins of rabies virus as nucleoprotein, nonstructural protein and polymerase designated N,NS and L respectively, are linked to the viral RNA to form helical structure. The glycoprotein (G) and the matrix protein (M) are associated with membrane. The G protein is responsible for eliciting and reacting with the neutralizing antibody as well as for the induction of the cell-mediated immune response and for conferring immunity against a lethal challenge in animals (24,25,26). The M protein and lipid in the viral envelope appears to be associated with hemagglutinating properties. The L protein is associated with transcriptase activity necessary for the replication of rabies virus. The major antigens of viral nucleocapsid are phosphorylated N and NS proteins. There are three antigenic sites on N and two on NS protein (27). Two major antigenic sites of N and one of NS protein have already been located by chemical and enzymatic cleavage and these sites can be recognized by both T and B cells (28). Although antibodies to nucleocapsid can be detected in the serum of vaccinees who received inactivated vaccines (29), it is still uncertain whether these nucleocapsid proteins are targets in host defense as the

glycoprotein.

The panel of monoclonal antibodies against nucleocapsid and glycoprotein can demonstrate antigenic variation among isolates of rabies virus from different animal species in different geographical areas, and has also provided a means to differentiate between rabies and rabies related viruses that are indistinguishable using fluorescent nucleocapsid antibody tests.

At present, the standard vaccine strains appear to give adequate protection throughout the world but extension of the antigen variations might lead to some revision of the recommendations for vaccine and antiserum production in relation to local requirements.

3. Negri Body

Negri body, the most helpful diagnostic finding in rabies, was described by Negri in 1903 (30). These intracytoplasmic bodies are dense masses of rabies viral protein and virions (31). By light microscopy, they appear as round-to-ovoid dark-blue structures in toluidine blue-stained sections and purple to red in Giemsa-stained smears. In hematoxylin and eosin stain, they appear as acidophilic irregular structures of various sizes (average of 1-27 μm) and shapes. Three different morphologic types of rabies virus-associated Negri bodies have been described by Miyamoto and Matsumoto (32). By electron microscopy, the structure of type 1 particle is round-to-elongated with an average width of 120 to 130 mμ. They have

slightly electron-dense homogeneous core surrounded by a single or double agranular osmiophilic membrane. Other particles (type 2) are round-to-slighty elongated, more electron-dense core surrounded by a clear halo bounded by a single agranular dense membrane. The last type is bullet-shaped, 80-90 mμ wide, surrounded by a double membrane, and seen both within and outside the Negri bodies. Negri bodies are usually found in Purkinje cells of the cerebellum, neurons of hippocampus, brainstem, spinal cord as well as cerebral cortex, pyramidal-ganglion cells of Ammon's horn, axons and dendrites encounter within the neuropil and glial cells.

Histopathologic changes of the central nervous system also depend on the strain of rabies virus. In experimental rabies infection with "fixed" rabies strain, massive damage of neurons with widespread inflammation are frequently found and Negri bodies are usually absent. This is in contrast to that found in infection with "street" rabies strain. Neuronal structure is usually presented in the latter group and Negri body is evident (33).

CLINICAL FEATURES OF RABIES

In human, rabies presents in one of two forms; encephalitic or paralytic which are analogous to furious and dumb rabies in the dog respectively. A wound or abrasion of the skin, usually inflicted by a rabid animal, is the major portal entry of virus which is excreted in saliva. However, a small number of cases have been reported after nonbite exposures. These included

contact with aerosolized rabies virus in caves inhabited by rabid bats (34) and in laboratory accidents with infected aerosolized tissues (35). Exposure of saliva of rabid animals to conjunctiva, mucous membranes of mouth, genitalia and on abrasions can also cause rabies (36). Rabies has occurred in recipients who received corneal transplants from donors who died of undiagnosed rabies (11). The transplacental route has never been reported in human, but well documented in cattle (37).

The risk of developing clinical rabies depends on the amount of virus content in saliva, severity of bite, and location of wound. Virus multiplies in muscle and connective tissue but remains localized for periods, then progresses along the axoplasm of peripheral nerves to ganglia and eventually to the central nervous system (38).

Once infection occurs, the clinical course can be divided into 5 stages (36) : incubation period, prodrome, acute neurological phase, coma, and dead or recovery.

1. Incubation Period

The interval between exposure and the appearance of illness attributable to rabies is the incubation period. This stage is extremely variable, usually 3 to 8 weeks but ranging from 1 day to 5 years. It depends upon the size of the viral inoculum, the severity of the wound, and the length of the neural path from the wound to the brain. The short incubation period usually follows bites on the face, head and neck.

2. Prodrome

The initial symptoms of clinical rabies occur at the time of viral invasion into the central nervous system. These symptoms are usually either variable or nonspecific, and may be described as tingling, itching, burning, coldness, numbness or as simply as pain at the site of bite. Local symptoms, starting at the site of bite, gradually spread to involve the whole limb and occasionally involve the whole body. About one-third to two-thirds of all patients with rabies can be expected to notice some sort of abnormal sensation in the region of the bite at the start of illness. Other symptoms include fever, easy fatigability, gastrointestinal symptoms, musculoskeletal pain which may resemble influenza or an upper respiratory tract infection. These symptoms are non-diagnostic and also occur in most patients who have been bitten but never develop the disease.

3. Acute Neurological Phase

Encephalitic rabies : This form of rabies occurs in more than 80% of human cases. The earliest neurological symptom is hyperactivity that resembles intense anxiety reaction or nervousness. Fever is usually the only objective finding. With hours to days, the patient exhibits alternating intervals of confusion and calm. During confusional state, signs of autonomic disturbances are occasionally observed. As the disease progresses, confusion becomes extreme, and may evolve to wild agitation and aggressiveness. This period of irritability is gradually replaced by depression of consciousness and coma.

Most encephalitic rabies patients have aero- and hydrophobia. Some victims have these phobic signs as an initial symptom prior to any hyperactivity. Aerophobia and hydrophobia may not persist throughout the whole course. The clinical pattern of encephalitic rabies is shown in Figure 1.

Paralytic Rabies : One-fifth of human cases may present as paralytic rabies. It is less readily diagnosed because of its atypical presentation. Certain animal vectors, different virus strains, a bite site on the lower limbs, and unsuccessful postexposure immunization may predispose to the clinical presentation . For example, human paralytic rabies has been associated with vampire bat bites (39) and with squirrel bites (40).

The major signs in encephalitic rabies appear late in paralytic cases. The clinical differences are the relative sparing of consciousness in patients with paralytic rabies. Weakness usually starts in the bitten extremity and progressively involves all limbs. During the early phase, the obvious signs are myoedema at percussion sites (41) and piloerection. This myoedema is noted the start of neurological phase, even during the prodrome, and persists until the preterminal stage. Only half of the paralytic patients experience aero-and hydrophobia. The course of the disease is less fulminant and the survival is longer than that in encephalitic rabies. The clinical pattern of paralytic rabies is shown in Figure 2.

4. Coma

When coma supervenes, both groups are indistinguishable. It is extremely difficult to diagnose rabies in a comatose patient. Meticulous history taking and diagnostic laboratory tests must be performed to exclude from other causes of central nervous system infection especially in rabies endemic areas.

5. Recovery

The fate of victims has not changed despite intensive care plus treatment with adenine-arabinoside and interferon. Three well documented cases of recovery have been reported (42,43,44). Concentrations of rabies antibodies in blood and cerebrospinal fluid performed serially were considered too high to have resulted from vaccine.

Clinical presentations of dog rabies are similar to that observed in human. Incubation period in dog after exposure, however, is shorter than that in human and usually between three and twelve weeks but may vary from few days to more than six months (12).

Furious Rabies : One-fourth of rabid dogs may present as furious, or aggressive mad-dog form. Extreme excitation, exaggerated response to stimuli, and aggressive behavior usually lasts 1 to 7 days (12,45). Abnormal behavior includes attempt to eat wood, soil, stones or other foreign objects which when found in the stomach at necropsy have long been considered pathognomonic of

rabies. Dilation of the pupils and a nonfocusing stare may give the eyes a squinting, frightening appearance. Salivation may be stimulated and the head characteristically held at a downward angle, causing drooling or frothing of tenacious saliva. Characteristics of dog with furious rabies are frothy-mouth, the holding of the tail tightly between the rear legs. Attacking people or other animals without warning, and biting their cages are also main characters. Between paroxysms of fury, it lies quietly but stimuli may precipitate it once more into paroxysmal rage.

Dumb Rabies : The whole clinical course usually lasts 1 to 4 days (46). Paralysis commonly begins at the head and neck, then one or both hind legs, causing the dogs to sway or fall. The jaws fall open and muscles of deglutition become paralyzed, causing drooling of saliva and inability to swallow either food or water, but this is unrelated to the symptom of hydrophobia which is seen only in human cases. Increasing paralysis of the hindquarters extends progressively forwards, although not always uniformly bilateral. The rabid dog becomes comatose and death ensues, usually from respiratory failure.

Rabies in dog is either paralytic type from the outset, or rapidly becomes so, after initially presented as furious rabies.

Difference between dog and human rabies is that clinical forms in dog cannot be separated into two distinct entities as those in human. Dumb rabies in dog, instead of

having longer survival period, usually has a rapid course, thus indicating that dog with dumb features actually is furious rabid dog that have a very short, unrecognized aggressive stage.

PATHOGENESIS

Rabies infection almost always follows the bite of a rabid animal harboring the virus in saliva, although can be acquired via nasal routes, mucosal and aerosol exposures. In order to complete its cycle with production of clinical signs and further transmission of the infection, the virus has to be transported centripetally from the site of the bite to the central nervous system where multiplication takes place and then carried centrifugally to neural and extraneural sites such as salivary glands. This cycle is modified and even interrupted by the influence of genetic susceptibility factors in the host, immunologic pressure and finally by the strain and virulence of the virus. These complex mechanisms, undoubtedly, play a role in several puzzling aspects of rabies infection which include the long incubation period, two uniquely different clinical manifestations, abortive infections as well as recovery, and rapidly progressive fatal outcome once clinical signs appear. Pathogenesis studies have been carried out for over a century and shed important new light on these aspects.

Transit of rabies virus to the central nervous system following viral inoculation has been studied largely in rodents using routine histology, fluorescent antibody staining and electron microscopy. Following intramuscular inoculation in

newborn hamsters, closely mimicking exposure from a deep animal bite, an earliest evidence of viral replication was found in muscle cells at the peripheral inoculation site (19,47,48,49). The role of the neuromuscular and neurotendinal spindles as well as nicotinic receptor to acetylcholine has been suggested (50,51,52) By electron microscopy, viral particles were found budding in moderate numbers from the plasma membranes of infected but undamaged muscle cells into extracellular spaces. The viral genome was then taken up into the unmyelinated nerve endings of the peripheral nerves of both sensory and motor pathways at these sensory stretch proprioceptors and neuromuscular junctions respectively. The role of acetylcholine receptor, as rabies-binding site or productive receptor (53) has been confirmed by successful competitive inhibition experiment with alpha bungarotoxin and d-tubocurarine (51). Monoclonal antibodies to alpha subunit of the acetylcholine receptor also specifically block viral binding in vitro (54). Sequence homology of amino acid between rabies viral glycoprotein and snake venom curaremimetic neurotoxins has also been demonstrated (55). However, these findings do not imply that binding of rabies virus must occur solely at acetylcholine receptor since many cells such as Hela cell that can be infected do not contain this receptor.

Rabies virus may remain at the inoculation site for a considerable period, since amputating the foot of the mice seven weeks after footpad inoculation markedly reduced mortality rate (56). Immunosuppression increased mortality and shortened the time at which the virus stayed at the inoculation site (57). It

is possible that some immune mechanisms are involved in eventual release of virus from myocytes, thus influencing the duration of this eclipse period. Rabies virus reaches the central nervous system by passive transport through retrograde axoplasmic flow (58,59). Inhibition of axoplasmic flow of the sciatic nerve by colchicine and vinblastine prevented rabies in experimentally infected mice. The same can be achieved by nerve section (60). Sequential studies demonstrated viral accumulation at dorsal root ganglia and at soma of motor neurons where there were ribosomes necessary for viral replication. In the spinal cord, there is uncertainty about the relative involvement of sensory and motor pathways. Schneider (61) found the dorsal and lateral horns predominantly affected.

Once rabies virus has reached the central nervous system, rapid dissemination occurs. Budding from the plasma membranes of neurons and glia cells with presence of virus in the intercellular spaces and evidence of direct cell-to-cell transmission or direct transneuronal transfer from perikarya and dendrites to adjacent axon terminals have been documented (62,63,64,65). Recently, Gillet et al (66) has provided evidence that rabies virus travels by retrograde fast axonal transport (200-400 mm/day) following stereotaxic inoculation into the striatum. Based on studies by Johnson (67,68), there is selective vulnerability of neurons to rabies infection in the limbic system with relative sparing of neocortex in the early stage of invasion. These findings readily explain behavioral abnormalities, loss of natural timidity, abnormal sexual

behavior, and aggressiveness in clinical rabies. Eventually, widespread infection of neurons leads to terminal coma and death. Neurons are the central nervous system cells selectively involved in rabies infection although infection of astrocytes and glial cells has been reported in animals and humans (21,63,69,70). Glial cells are significantly less susceptible than neurons to rabies virus infection in vitro (71). The presence of highly sialylated gangliosides of GT1b, GQ1b, play a role in the binding of rabies virus to the membrane of chick embryo related cells. This might explain neuronotropism since larger amounts of these gangliosides are found in neurons than in glial cells (72,73).

The clinical manifestations of rabies have been considered to be due to direct viral invasion of the nervous system. The ascending wave of rabies infection in central nervous system precedes shortly the sign of paralysis in animals (19). The relative scarcity of inflammation in the brain tissue with absence of cell destruction point to derangement in functions as the main cause of lethality. Preliminary studies in mouse neuroblastoma-rat glioma hybrid cells (74,75) and in rabies infected rat brain (76,77) showed that there were modifications in opiate and muscarinic acetylcholine receptor affinity. Functional alterations of neurons have been well demonstrated during the evolution of experimental fixed rabies infection (78). Three distinct phases of brain activities and sleep pattern changes were characterized and are indicative of impairment of particular neuronal function in the presence of rabies virus in various regions of the brain.

However, the presence of virus alone may not be the only factor in determining the clinical symptoms and signs. Once the virus has gained access to the central nervous system, rapid development of symptoms and death do not necessarily ensue. High titers of rabies virus in the brain and spinal cord can be found in animals long before clinical signs of the disease appear (79). Numerous evidence indicate that infection by rabies virus may lead to various outcome ,i.e. inapparent or abortive infection, survival with and without residual signs, and death. The prevalence of abortive dog rabies in northern part of Thailand was found to be 17 percent (80). Abortive rabies and recovery from clinical rabies with or without residue has been repeatedly reported among many species (42,43,81,82,83,84,85,86). The mechanisms mediating this resistance and recovery are certainly complex and probably mediated by combination of factors involving virologic, immunologic and genetic aspects.

Mice of various strains show different susceptibility to "street" rabies infection (87). This difference, especially that of resistant SJL/J mice, was associated with restriction of viral replication within the central nervous system. Limitation of replication correlated with presence of neutralizing antibody which developed only in resistant strains suggesting that the immune response under genetic control when it develops early is protective or able to abort disease (88). Rabies virulence can also be determined by strain of the virus. "Fixed" rabies virus injures neurons more extensively than "street" virus strain thus loss the capability for producing Negri bodies (32). The

biological properties of rabies virus have been suggested to correlate with changes in the antigenic phenotype (89). Rabies virus contains five proteins. The single glycoprotein is responsible for the induction and binding of neutralizing antibodies, and for conferring immunity against lethal challenge with rabies virus (90,91,92,93). Analysis of variants selected in vitro with anti-glycoprotein monoclonal antibodies has revealed that only one amino acid substitution at position 333 of the glycoprotein affects virulence in adult mice (90,94). This apathogenic variant virus has been shown to differ from its pathogenic parental virus in its ability to infect neurons in vivo and in vitro (95). Rate of cell-to-cell spread, number of infected neurons and the degree of cellular necrosis were much lower in the case of apathogenic virus, although the distribution of infected neurons in the brain was similar for both viruses. Moreover, addition of rabies viral neutralizing antibody completely prevented cell-to-cell spread of apathogenic variant virus but not of parental virus. However, non-lethal central nervous system infection with attenuated virus ERA/BHK is not entirely due to inherent properties of the virus that restrict its multiplication in the central nervous system, since suppression of host immune response produces a lethal infection (96). Defective interfering particles may be another factor, in addition to variation in glycoprotein structure that might result in resistance and probably survival (97,98). Natural infection with less pathogenic virus strains and small infectious doses, might allow the host to clear the infection if there is

sufficient time for active induction of immune response and perhaps interferon. A very high level of interferon was detected in rabies virus infected rat brain without beneficial effect. It is, however, possible that interferon production is too late to have any effect (99).

Both humoral and cellular mechanisms are important in successful clearance of rabies virus. Neither can operate in isolation. Depletion of B cells by anti-mu-serum treatment converts an inapparent central nervous system infection in mice with avirulent high egg passage (HEP) strain of rabies virus to one with 60 percent mortality. Further, depletion of T cells with antithymocyte serum or T and B cells with cyclophosphamide potentiates infection (100). Adoptive transfer in these cyclophosphamide treated mice early after infection with immune cells of T or B subsets could reduce the mortality. The best result was observed when unfractionated donor cells were adoptively transferred. Antibody acts by neutralization and lysis of virus-infected cells. T cells exert their protective effect not only through the induction of rabies antibody (101), but also through lymphocytotoxicity effect. The efficacy of post-exposure protection by inactivated tissue culture vaccines correlates with cell mediated cytotoxic response (102). Lethal infection, on the other hand, with street rabies virus strain is always associated with lack of such a response (103). Return of a cytolytic T cell response in immunosuppressed mice correlates with recovery (96,104).

In contrast to the beneficial role of the immune response in rabies infection, it has been suggested that an immune response to rabies virus may also contribute to the disease process (105). Immunosuppressed mice take longer to succumb to rabies virus infection than immunocompetent mice, and the onset of paralysis after experimental immunosuppression is temporally related to the return of immune responsiveness (13,14,15). Passive transfer of rabies immune serum or sensitized cells to infected immunosuppressed animals accelerates the appearance of paralysis and death. Histological examination then reveals marked inflammation and degeneration of central nervous system parenchymatous tissue (14,106). This "early death phenomenon" was first observed in monkeys, mice and later in humans with unsuccessful immunization attempts (16,17,56,107,108). Therefore, immune responses may have dual effects in rabies infections. A precise balance has to be struck between extent of viral infection of the central nervous system and timing of the immune response in order to produce the "early death" or survival with successful clearance of virus. When the brain cells are extensively infected, immune destruction, either by antibody and complement or by cytolytic T cells, contributes to massive cellular necrosis resulting in severe disease or death.

Host immune response may play a role in determining the clinical presentation of either encephalitic or paralytic rabies in mice (13). Rabies infected immunocompetent mice usually exhibited an ascending paralysis of the limbs, while immunosuppressed mice developed encephalitis with only minor

paralysis. Histopathological changes differed markedly between these two groups in that marked inflammation and degeneration of central nervous system parenchymatous tissues were observed in immunocompetent mice. This was in contrast to limited degeneration and necrosis of individual neurons and mild microglial reaction in immunosuppressed mice. In human rabies, the immune response may also influence clinical manifestations. Patients who had cellular response to rabies virus, as determined by lymphocyte proliferation assay in vitro, manifested clinically as encephalitis rather than paralysis and tended to die faster (109). This finding, however, does not exclude differences in the strain of virus and in viral distribution as determining factors. The less fulminant and slower clinical course in paralytic rabies may be due to failure of viral glycoprotein to be present in large quantities on the neuronal surface membrane. This has been previously shown in the case of "street" virus strain in neural cell lines and laboratory rodents when compared to "fixed" virus strain (110). Correlation with viral distribution other than by distribution of Negri bodies has never been reported with two different clinical presentations, thus unable to exclude viral distribution as a determining factor. In addition, autoimmune phenomenon, i.e. cellular reactivity to myelin basic protein, is observed in encephalitic and paralytic human rabies. Patients with reactivity to myelin basic protein may have a more rapid progression of their diseases (109). The role of myelin basic protein has been previously reported in patients with postinfectious and postvaccinal encephalomyelitis where the

degree of cellular or humoral responses has been correlated with the disease severity (111,112,113,114). In case of human rabies, myelin basic protein reactivity in patients with accelerated death may merely reflect an epiphenomenon associated with widespread damage.

In contrast to patients with postvaccinal encephalomyelitis, antimyelin basic protein antibody has not been detected in the serum or cerebrospinal fluid from rabies patients. This is also true in the case of antirabies antibody. Only 20 percent of rabies patients had seroconversion during the early stage. The attempt to detect antibody to P_2, a neuritogenic antigen, in experimental allergic neuritis, in 3 patients with paralytic rabies also failed (unpublished data). Autoimmune phenomenon similar to those in acute Guillain Barre' syndrome was proposed by Chopra et al. (17), in view of neuropathological changes in these patients. There is need for further detailed studies to provide us with an understanding of the pathogenetic mechanisms involved.

The terminal segment of the natural rabies cycle is the centrifugal spreading from the central nervous system along autonomic nerves to peripheral organs such as salivary glands, adrenal medulla, kidney, lung, liver, muscle, skin and heart. The success of this centrifugal spread, particularly to the salivary gland followed by virus excretion in saliva, is essential to the continuation of the cycle. Due to generalization of the involvement of both neural and nonneural

organs and tissues, viremia was suspected but never confirmed with certainty (61). Rabies virus can be detected in the saliva of dog up to 13 days before development of disease sign (115). Several studies have shown that virus can be recovered intermittently from the saliva of asymptomatic dogs and from dogs which recovered from clinical rabies (116,117). This poses problem in assessing the risk of exposure and is a threatening problem if asymptomatic carrier exists in large numbers in certain area.

NEUROPATHOLOGICAL CHANGES IN RABIES

1. Neuropathological Changes in Human Rabies

Neuropathological changes of the central and peripheral nervous systems of rabies had been previously reported (17,21,62,118,119). The gross examination of the central nervous system in rabies shows mild alterations consisting of vascular engorgement and in brain weight. Gross petichial hemorrhages are rarely observed. Microscopic changes are nonspecific and usually of nondiagnostic value except for the presence of Negri bodies. Despite the severity of symptoms observed, pathological changes do not match the dramatic clinical picture except in a case report which revealed massive laminar necrosis of the brain (120). Furthermore, there is an extreme pathologic variability in human rabies. The frequently noted histologic changes in human rabies are perivascular and parenchymal infiltrations accompanied by neuronal changes primarily in the gray substance. These are not specific and can

be found in other forms of encephalitis. Such inflammatory reactions consist of small and large mononuclear cells, plasma cells and occasionally polymorphonuclear leukocytes around small veins, venules, capillaries in the brain stem, spinal cord, thalamus, cortex, basal ganglia, cerebellum, and hippocampus in decreasing frequency. Extension of the inflammatory response into white matter is also present, especially in the brain stem, cerebellum and spinal cord. There is usually mild evidence of leptomeningeal inflammation. Areas of microglial aggregation in association with neuronal degeneration and neuronophagia (Babe's nodules) are not common, but become prominent in cases with an accelerated course. The distribution of inflammatory infiltrates in paralytic rabies is more prominent in the lumbar and lower thoracic spinal cord and medulla. The inflammation is most marked in the anterior and posterior horns and in dorsal root ganglia. Histopathologic changes of cranial nerve and spinal root ganglia showed capsular cell proliferation and degeneration of neurons with variable degrees of inflammation. There is no difference in the frequency of ganglionic involvement between encephalitic and paralytic rabies. Abnormalities are found in both groups of human rabies but changes are more profound in paralytic cases. In encephalitic rabies, the axons as well as the myelin sheaths of peripheral nerve trunks and dorsal nerve roots usually appear swollen. Vacuolation is sometimes noted in axons. Schwann cells are found to be increased in number. Leucocytic infiltration is noted around veins in one-third of cases. Myelin is noted to be absent in peripheral nerves (119).

In paralytic rabies, the primary abnormality is segmental demyelination and remyelination as well as Wallerian degeneration in teased single fibers from the peripheral and spinal nerves. Infiltration by inflammatory cells is not a constant finding (17).

Lyssa or Negri bodies, which are intracytoplasmic inclusions, are a most helpful diagnostic microscopic finding in rabies. These bodies are dense masses of rabies viral protein and virions as revealed by electron microscopy and have been demonstrated to correspond with sites of viral replication (62,69,70). Negri bodies are found in 75 to 80 percent of human rabies cases. They are best seen in large neurons and much less frequently in glial cells in regions of the brain where there is little inflammatory reaction. They are absent in areas where neuronal degeneration, neuronophagia and intense inflammation are prominent. Cerebellum, hippocampus, brain stem, spinal cord as well as cerebral cortex are the usual sites at which Negri bodies are detectable regardless of the clinical form of rabies. Tangchai et al. (21) reported that Negri bodies were fairly ubiquitously distributed in the gray matter, but were more prominent in the large nerve cells. Dupont and Earle (118) reviewed 49 cases of rabies and found that the cerebellum was the most frequent site of Negri bodies formation (59.5% of cases), followed by the hippocampus (42.9% of cases) and much lower frequency in a number of other sites throughout the CNS. The finding of Negri bodies should not be considered conclusive for a final diagnosis. By using Seller's impression technique, a

substantial proportion of brain specimens from animals as well as human showed structures indistinguishable from Lyssa or Negri bodies but fluorescent-antibody technique and mouse inoculation tests remained negative (121,122). An increasing yield of sensitivity can be achieved in formalin-fixed paraffin-embedded tissue by pretreatment with trypsin digestion, followed by immunofluorescent staining of rabies antigen (123). The use of the avidin-biotin-peroxidase complex method as well as in situ hybridization using radiolabelled DNA probe for nucleocapsid are also highly sensitive and specific.

The centrifugal spread of virus in the nerve during the final stage of infection to various organs usually does not result in significant pathology except in the myocardium. The ultimate event in the course of rabies is cardiovascular disturbance either in the form of abnormal conductivity or contractility. This is likely to be due to myocarditis (124).

2. Neuropathological Changes in Canine Rabies.

Peripheral nerves serve as the pathways for spreading of rabies virus from inoculation sites to the CNS. In experimental infections with "street" virus, rabies antigen have been demonstrated in peripheral nerves by FAT. According to the study of Schneider (125), in 1969, after footpad inoculation of the right hind leg with fox strain virus, virus replication in the CNS of mice could be demonstrated as early as 60 hours in the neurons of spinal ganglion of sacral, anterior and posterior parts of lumbar, and thoracic cord at 2,21,32 and 8%

respectively. Rabies antigen was also seen early in the dorsal root neurons and the dorsal and lateral horns but rarely ventral horn. Infection then spreads rapidly to the entire cord and medulla between 130 and 160 hours, and with appearance of paralysis of hind legs within 196 hours. Early involvement of the brain was demonstrated in the gray matter, and rabies antigen was occasionally found in unidentified structures of the white matter (probably axons), and more prominently in nerve cell processes surrounding the ependyma of the central spinal cord canal. They concluded that virus titers in the different CNS segments increased with time.

Johnson (20) proposed that the early site of replication depends on the inoculation site. The viral pathway to the salivary glands is through the nerve originating near vital nerve centers.

Ito et al. (126) studied in naturally infected dogs, and observed virus in all over the segments of Ammon's horn, cerebellum, cervical, thoracic, lumbar and sacral cord by FAT which corresponding with MIT.

Lastly, Umoh and Blenden (127) demonstrated rabies antigen in the cranial nerves, skin at the face and other tissues excised from experimentally inoculated goats and dogs, and naturally infected skunks. They found that the cranial nerves and at points close to the brain of experimentally inoculated animals contained only limited amounts of rabies viral antigen. This was in contrast to naturally infected skunks that both

cranial and peripheral nerves were found to contain large amounts of antigen. Further, lack of uniform distribution throughout the CNS was occasionally reported. Silva (128) reported a dog with rabies virus in salivary glands but no virus could be recovered in the brain by MIT. Rabies virus in several cases of naturally rabies infected horses could be isolated from spinal cord, but no virus could be found in other parts of the CNS (129).

Study of rabies virus distribution either in human or animals as mentioned above, however, lack a precise correlation between its distribution and clinical forms of the disease. Most of the studies in human only relied on routine histological examination (H & E) or fluorescent study. Therefore, it is very intensity to see whether virus localization may explain the diversity of clinical symptoms of the disease.

LABORATORY DIAGNOSIS OF RABIES

The diagnosis in human depends on classical manifestations and definite history of exposure. In endemic area, not only the dog or cat, but any biting of mammals including rodents (130) should be seriously considered as potentially rabid. However, the diagnosis is sometimes missed especially in non-endemic areas. A failure to obtain a history of animal exposure was noted in 13 to 40 percent of patients who died of rabies (109,118,131). The major reasons are due to the long incubation period than the bite, usually not severe, is forgotten and the patients came in unconscious and sometimes, rabies is not included in the differential diagnosis. The

clinical recognition can be extremely difficult for a physician who has never seen a case of rabies, or almost impossible in the case of paralytic rabies. A high degree of awareness of the various clinical presentation of rabies as well as confirmation of the diagnosis by appropriate laboratory studies are required for early diagnosis.

Rabies may not be easily diagnosed on clinical grounds alone. Laboratory diagnosis is often required especially in paralytic rabies or in cases without history of definite exposure. Antemortem diagnosis in human cases may be achieved by several techniques. Isolation of the virus from the patients' saliva, tear, cerebrospinal fluid, or urine by mouse inoculation is possible but requires at least 1 week. Demonstration of rabies antibody in blood and cerebrospinal fluid, a definite evidence of rabies infection in nonvaccinated individuals, is not helpful in differentiating vaccination induced complications from rabies. The presence of rabies viral antigen, as demonstrated by immunofluorescent technique, in salivary or corneal smears (132) and in nuchal skin biopsy (133) are also considered diagnostic. These procedures are less invasive than a brain biopsy. Samples of brain, if obtained, should be subjected to mouse inoculation, fluorescent antibody staining or immunoperoxidase staining and histologic and/or electron microscopic examinations. Excluding brain biopsy, immunocytochemical examination of skin biopsy remains the test of highest sensitivity but it also depends on what stage of disease the skin has been taken (134). Biopsy performed during late stage yields more positive results. Enzyme

immunoassay has also produced satisfactory results in rabies antigen detection in brain and salivary gland specimens (135) and it is hoped that this technique as well as immunoblot assay may replace the time consuming mouse inoculation test.

The followings are routine techniques commonly used in postmortem diagnosis of rabies.

1. Seller's Rapid Technique (Impression or Smear)

Seller's rapid staining technique appears to be the simplest and most economical (136,137). Staining requires two dyes; methylene blue and basic fuchsin. Sensitivity of Seller's rapid technique on dog samples was in the range between 59 and 99%, while it was 75 to 80% in the case of human rabies (138,139). Although this technique is the most rapid and simplest, its sensitivity is still not satisfactory.

2. Animal Inoculation

Webster and Dawson (140) reported the inoculation of rabies virus into weanling white mice. Greater sensitivity is obtained by the use of suckling mice. Mouse inoculation test (MIT) serves as a standard technique in comparing the efficacy to the other tests. By MIT, rabies virus can be isolated from brain, salivary glands and saliva specimens (141). However, in diagnosis of rabies, brain specimen is routinely used. Mice are kept for observation for at least 3 weeks. Disease sign in mice usually develops at the end of first week. Confirmative tests require demonstration of Negri bodies as well as the presence of

viral antigen by immunofluorescence test on brain impression smear.

Disadvantages of this technique are time consuming, potential hazards to technicians, and false negative reaction when decomposed tissues are used.

3. Fluorescent Antibody Test (FAT)

The FAT enables detection of rabies antigen in the brain before the development of Negri bodies. It can also be used to detect viral antigen in other tissues than the brain with a high degree of correlation with MIT (126,142). Besides its sensitivity, the test also gives negligible false positive result when appropriately controls are used in parallel and takes only 45 minutes to know the result, thus supplanting older staining methods, i.e. Seller's stain, and can also be used in conjunction with animal inoculation test. FAT can be used either on brain impression specimen or formalin-fixed histological sections (123,127). Trypsin and pepsin digestion has been reported to increase the sensitivity and amplify the reaction (123,127,143).

MIT is slightly more sensitive than FAT for rabies diagnosis. When these two tests are performed by competent and experienced laboratory workers, agreement between them is usually 99% or higher (144). The disadvantages of FAT are impermanence of stained slides, nonspecific reactions and only fluorescent microscope be available.

4. Immunoperoxidase Staining (IP)

Immunoperoxidase methods, presently are accepted widely in clinical and research laboratories. Various modifications to peroxidase techniques have been described such as direct method, indirect method, peroxidase-antiperoxidase method (PAP), protein A-peroxidase method, hapten labeled antibody method and avidin-biotin-peroxidase complex method in order to increase the sensitivity (145,146).

Levaditi et al. (147) and Atanasiu et al. (148) recently introduced peroxidase technique in diagnosis of rabies. The purified antiserum was conjugated to horseradish peroxidase by glutaraldehyde (149) and substrate used was 3,3′-diaminobenzidine (DAB) plus hydrogen peroxide (H_2O_2). This technique is subsequently proved to be sensitive, highly specific and required only an ordinary light microscope.

In 1985, Kotwal and Narayan (150) had developed the direct IP test for increasing the sensitivity. The smears of mice brains infected with CVS rabies virus were performed in 0.5% H_2O_2 in methanol for blocking of endogenous peroxidase before applying the horseradish peroxidase conjugated antirabies. The result was found to be satisfactory. The direct IP test was able to identify rabies viral antigen in overall percentage sensitivity of 95.3% compared to 99% of FAT. In this recommended procedure, IP can be used as an alternative to FAT for providing prompt laboratory diagnosis because of certain advantages this test has over FAT.

Anjaria and Jhala (151) compared the three methods of Seller's stain, FAT and IP on impression smears and on paraffin-embedded sections of "street" rabies virus infected mouse brains. Results showed that sensitivity of Seller's method was definitely less than FAT (76.2% VS 92.5%) on impression smear, and direct IP yielded the same result as FAT (91.36%). In the case of paraffin-embedded sections, the sensitivity were comparable 96.71%, 98%, and 97.2% by these three methods respectively.

Discovery of avidin, a glycoprotein with four binding sites, with high affinity for biotin, a vitamin, led to avidin-biotin system in immunoperoxidase techniques by Guesdon and colleagues (152). The use of this avidin-biotin interaction in immunoenzymatic technique provides a simple and sensitive technique to localize antigens in either formalin-fixed or fresh frozen tissues.

Hsu et al. (153) in 1981, proposed that the avidin-biotin-peroxidase complex (ABC) method is more sensitive than the techniques described above. These authors used a three-stage technique in which biotinylated secondary antibody was used as a bridge between unlabeled primary antibody and an ABC. The optimum staining using an ABC conjugate prepared at a ratio of 4:1 of avidin to biotin-peroxidase (10 µg/ml avidin to 2.5 µg/ml biotin-peroxidase). They suggested that the high sensitivity of this method might be due to the formation of large complex containing multiple peroxidase molecules.

Based on reasons mentioned above, we then used avidin-biotin peroxidase technique in the study of viral distribution. The technique is also not difficult to perform since the secondary antibody that needs to be conjugated to biotin and most reagents, including ABC, are commercially available. Advantage of this method to FAT is that structure of the neuronal tissue can also be studied in detail and it is also possible to see which cell type is predominately infected.