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CNS RESPONSE IN A RABID BRAIN: A PATHOLOGICAL VIEW

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ABSTRACT

Animal attacks constitute a huge medical and social problem ending in millions of injuries and thousands of deaths worldwide. Rabies is a dreadful infectious disease that has not been brought under control in many parts of the world even today. Rabies spread by domestic and wild animals particularly by a dog bite, and with the exception of Antarctica, rabies is present on all continents, taking a heavy toll on human lives. Many countries have the status of high-risk areas, but most of the countries around the globe gained the status of rabies free territories. This shows that rabies can be successfully ruled out from the high-risk areas by taking preventing measures and the situation may be better if we continue to untangle the mysterious pathophysiology of this disease. Understanding rabies invasion and its pathophysiology will further enhance the chances of improvement rabies related complications and mortality. Rabies is a prototypic infection of the nervous system in which the virus selectively infects neurons, using retrograde axonal transport to traffic in the nervous system. Neuroinvasiveness, neurotropism and neurovirulence are the major defining characteristics of this virus. The speed of virus uptake, the ability of the virus to spread efficiently from cell-to-cell and the rate of virus replication are the major factors that determine the pathogenicity of rabies virus. Present work tries to understand the different target areas of rabies virus in central nervous system.

KEYWORDS: ***Apoptosis, neuroinflammation, nitric oxide, rabies***

INTRODUCTION

Almost 60000 human deaths occur from rabies globally per year, which is more than that attributed to any other single zoonotic disease (Fooks et al. 2014). The first statement reporting rabies was recorded in the pre-Mosaic Eshmuna Code of Babylon in the 23rd century B.C (Pearce J. 2002). The disease was variously known as lytta or lyssa, coming from the belief that the disease was caused by a worm under the tongue (lytta), or hydrophobia, which describes the thirst and fear of water associated with the disease. The present English name, rabies, comes from the Latin rabere, meaning raging, furious, savage, or madness, whereas the Greek term hydrophobia is now specifically used for rabies in man (Woldehiwet Z. 2002). This disease and the danger connected with the bites of mad dogs have been well known since the time of Aristotle, who, in the fourth century B.C. wrote: "Dogs suffer from a madness which puts them in a state of fury, and all animals that they bite when in this condition become also attacked by rabies."The very extensive investigations of Pasteur and his collaborators proved that the purest and most concentrated virus of rabies was contained in the central nervous system; they also established a method of attenuation of the virus, and solved the important question of protective vaccination against the disease. Negri (1903), through the demonstration of specific cell inclusions in the nervous system of the infected animals, has greatly facilitated the postmortem diagnosis (Dykstra R. 1926). Rabies is an acute encephalitis or meningoencephalitis due to a lyssavirus infection. Eleven distinct lyssavirus species are currently recognized worldwide. Most of which only rarely

cause human disease. The etiological agents of rabies encephalitis belong to the Mononegavirales order, the Rhabdoviridae family and the Lyssavirus genus. Lyssaviruses have a 12-kb non segmented RNA genome of negative polarity that encodes five viral proteins: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), aglycoprotein (G) and an RNA-dependent RNA polymerase (or large protein,L). The lyssavirus particle is shaped like a bullet, 100–300 nm long and 75 nm in diameter. It is composed of two structural and functional units: an internal helical nucleocapsid and an external envelope. The nucleocapsid consists of a ribonucleoprotein complex comprising the genomic RNA and tightly bound N protein together with the L and P proteins. The nucleocapsid is active for transcription and replication: the N-RNA template is processed by the L protein,which contains most of the RNA polymerase activities, and its cofactor, the P protein. The lipid envelope is derived from the host cytoplasmic membrane during budding. Knobbed glycoprotein spikes (5–10 nm long and about 3 nm in diameter) consisting of three glycosylated ecto domains, which binds the virions to host cell receptors, protrude through the virion membrane. The M protein forms oligomers that bind to the outside of the nucleocapsid, giving rigidity to the virion structure and providing a binding platform for the viral glycoprotein and the envelope membrane (Graham et al. 2008) The main viral reservoirs include animals of the orders Carnivora and Chiroptera, but all mammalian species are susceptible at any time, and the disease is found on every continent except Antarctica. Rabies virus is a neurotropic virus. Inoculation is generally through a carnivore or bat bite; the virions travel retrograde along axons and across synapses to the central nervous system (CNS). Viral replication and dissemination occur widely throughout the CNS before the virus spreads centrifugally to the salivary glands. Histologically, rabies is characterized by a viral encephalitis with intracytoplasmic Negri bodies in neurons, but these changes can be mild or even absent. Although the presence of Negri bodies has been considered pathognomonic for rabies, they are absent in 20% to 60% of rabies cases. Because the encephalitis of rabies can be challenging to distinguish from other viral encephalitides,the diagnosis of rabies should include ancillary tests such as direct fluorescent antibody test (FAT) and intra cerebral inoculation in suckling mice, which are the standard tests for rabies today.(Stein et al. 2010). Rabies is caused by what is known as a filterable virus (poison), which means that the causative factor is a substance so small that it will pass through a fairly porous porcelain filter. It is present in greatest concentration in the saliva of mad animals. The virus is readily killed by the common disinfectants, drying, sunlight, etc. Fluid saliva is capable of infecting for a period of 24 hours after leaving the gland of the diseased animal, while dried saliva will not infect after 14 hours. Natural gastric juice kills the virus of rabies in 1⁄2 to 5 hours, and bile kills it in a few minutes. The poison of rabies travels only in the nervous tissue, and is eliminated through the nerve supply by glands into the saliva, the tears from the eyes,milk, and the secretions of other glands. The virus or poison of rabies may be present in the saliva of an animal as many as 15 days before it shows any signs of the disease, but 2 to 5 days is the usual period. The virus is transmitted from animal to animal by means of the bite, in which infectious saliva is carried into the resulting wound and into the nerves of the part wounded(Dykstra D. 1926). The rabies virus travels along these axons at a rate of 12-24 mm/d to enter the spinal ganglion. Its multiplication in the ganglion is heralded by the onset of pain or paresthesia at the site of the inoculum, which is the first clinical symptom and a hallmark finding. From here, the rabies virus spreads quickly, at a rate of 200-400 mm/d, into the CNS, and spread is marked by rapidly progressive encephalitis. Thereafter, the virus spreads to the periphery and salivary glands (e medicine). India is reported to have the highest incidence of rabies globally (WHO, 982) In India, about 15 million people are bitten by dogs every year and in 1985, it has been reported that 25,000-30,000 deaths are due to rabies annually, but due to preventive measures, the death rate reduced to 20,585 per year [The Path of the Virus]. Nepal has one of the highest reported per capita rates of human rabies deaths in the world [WHO: global vaccine research forum]. Rabies occurs primarily in Asia and Africa where animal control, vaccination programs and effective human post exposure prophylaxis (PEP) are either not widely available or not effectively applied [Lunney et al. 2011]. From the standpoint of diagnosis and therapeutic opportunities, it is important to understand that rabies does not cause cytotoxicity. Neuronal morphology and lifespan is normal throughout the course of the disease. Death occurs from global neurologic and organ dysfunction. The virion acts in the synaptic space, where homology in amino acid sequences between neurotransmitter receptors for acetylcholine, GABA, and glycine may afford a mechanism for viral binding of these receptors. Thus, its action is neurotoxic, rather than

direct damage. Immune responses and CNS dysfunction are two main factors to be considered during infection(Huang et al.. 2014). In the coming section we try to elucidate the target sites through which virus affects neuronal viability and affect neuronal functions and ultimately proves fatal.

RABIES PATHOGENESIS AND TARGET SITES

Bites by rabid animals generally inoculate virus-laden saliva through the skin into the muscle and subcutaneou stissues. Other routes of infection are rare (Warrell et al. 2003). During the incubation period the virus can replicate locally in muscle cells or attach directly to nerve endings. Rabies virus enters the body through wounds or by direct contact with mucosal surfaces. It cannot cross intact skin. Rabies virus replicates in the bitten muscle and gains access to motor end plates and motor axons to reach the central nervous system (Ugolini et al.2008; Ugolini et al. 2010; Ugolini et al.2011; Hemachudha et al. 2002; Hemachudha et al. 2013). Host detection of virus intracellularly occurs through the retinoic acid-inducible gene1 pathway (Hornung V2006) through the detection of cap structures on the virus mRNAs and through Toll-like receptors (Rieder M. 2011). This detection stimulates an early interferon response that is antagonised by the virus phosphoprotein. (Chelbi et al. 2006; Brzózka et al. 2006) The mechanism of interferon antagonism seems to be conserved between many of the lyssaviruses. (Oksayan et al.2012; Wiltzer et al. 2012). Once in the CNS, the virus replicates extensively and clinical disease develops (Fooks et al. 2014). The extensive infection of the brain leads to centrifugal dissemination of virus through neurons to distant sites throughout the body. Of particular importance in reservoir species is the spread of the virus to peripheral sites that release the virus into the oral cavity via the salivary glands. Rabies virus antigen staining of the taste buds has also been observed (Murphy et al.1973; Jackson et al. 1999). The salivary glands are innervated from the parasympathetic nervous system via the submandibular ganglion and glossopharyngeal nerves, by sympathetic innervation via the superior cervical ganglion, and by afferent innervation (Emmelin, 1967; Jackson et al. 2013). Ultrastructural studies suggested that the virus is able to travel from the brain to peripheral sites by budding on synaptic or adjacent plasma membranes of dendrites with budding also occurring, albeit less often, from the plasma membrane of the perikaryon(Charlton and Kasey, 1979). Virions are carried in transport vesicles (Klingen et al.2008) and travel to the central nervous system exclusively by fast retrograde transport along motor axons, with no uptake by sensory or sympathetic endings (Ugolini et al. 2008; Hemachudha et al. 2013).Viruses can also enter motor axons in peripheral nerves directly during a penetrating injury. The incubation period varies from 5 days to several years (usually 2–3 months; rarely more than 1 year), depending on the amount of virus in the inoculum, the density of motor end plates at the wound site and the proximity of virus entry to the central nervous system (Ugolini et al. 2011;Hemachudha et al. 2002). The estimated speed of virus migration depends on whether it moves by centripetal retrograde axonal transport or centrifugal spread. In centripetal retrograde axonal transport, migration is fast, with speeds of 5–100mm/day or even faster, because neuronal populations of the same synaptic orderlocated at various distances, e.g. 10 μm to 2 cm, are infected simultaneously (Ugolini et al.2008; Hemachudha et al. 2013). Conversely, centrifugal spread is slow, probably mediated by passive diffusion rather than active transport. The first rapid centripetal phase leads to wide transneuronal transfer within the central nervous system and to infection of dorsal root ganglia via their central connections with the initially infected motor neurons and spinal inter neurons. The virus then moves centrifugally from the central nervous system via slow anterograde axoplasmic flow in motor axons to the ventral roots and nerves and in peripheral sensory axons of the infected dorsal root ganglia,leading to infection of muscle spindles, skin, hair follicles and other non-nervous tissues, such as salivary glands, heart muscle, lung and abdominal visceral organs via their sensory innervation (Ugolini G2008; Ugolini G 2010; Ugolini et al.,2011; Hemachudha et al. 2002; Hemachudha et al. 2013). By the time of clinical onset, the virus is widely disseminated throughout the central nervous system and probably to extra-neural organs (Hemachudha et al. 2006). The first specific clinical symptom is neuropathic pain at the site of the bite. This is caused by virus replication in dorsal root ganglia and inflammation induced by cellular immunity (Mitrabhakdi et al. 2005). Human rabies can manifest as furious or paralytic forms, which cannot be correlated with a specific anatomical localization of rabies virus in the central nervous system (MitrabhakdiE2005; Dumrongphol et al. 1996; Thanomsridetchai et al. 2011). The major clinical signs are probably due to different site-specific responses (Thanomsridetchai et al. 2011). Functional neuronal impairment also

explains coma. Electrophysiological studies with pathological correlates show that peripheral nerve axonopathy or myelinopathy is responsible for weakness in paralytic rabies (Mitrabhakdi et al. 2005). Preferential entry via the motor route explains why subclinical anterior horn cell dysfunction precedes sensory loss in furious rabies and is initially localized at body segments corresponding to the site of the bite, progressively spreading to other locations (Ugolini et al.2011; Mitrabhakdi et al. 2005). The same considerations apply to prodromal symptoms and signs in paralyzed patients (Ugolini et al. 2011; Hemachudha et al. 2013). It is likely that less virus is present in the brain in paralytic rabies (when consciousness is preserved) than in furious rabies. Diffusion tensor imaging in canine paralytic rabies showed that neural tract integrity is compromised at brain- stem level, limiting viral propagation to the forebrain (Laothamatas et al.2008,2011). A viral immune evasive strategy with blood–brain barrier integrity prevents eradication of the virus in the central nervous system (Laothamatas et al. 2011; Roy et al, 2008). There is no evidence of immune suppression or accelerated death in rabies-infected patients (Laothamatas et al. 2008,2011). When the virus reaches the central nervous system, there is massive replication on membranes within neurons. 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. Because morphologic changes in natural rabies are usually relatively mild, it is thought that the severe clinical disease with a fatal outcome must be due to neuronal dysfunction of rabies virus-infected neurons. The precise bases of this functional impairment are unknown, and current knowledge on electrophysiological alterations, effects on ion channels and neurotransmission, and neurotoxicity are reviewed. Rabies virus may induce neuronal death, possibly through apoptotic mechanisms. Neuronal apoptosis has been observed *in vitro* and also *in vivo* under particular experimental conditions (Zhen et al.,2005). Impact of rabies virus on different CNS components include

UP REGULATION AND RELEASE OF INFLAMMATORY MEDIATORS

Microglia are activated in response to a number of different pathological states within the CNS including injury, ischemia, and infection. Microglial activation results in their production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. While release of these factors is typically intended to prevent further damage to CNS tissue, they may also be toxic to neurons and other glial cells (Smith et al., 2012). The cause of death as a result of infection with rabies virus has not been irrefutably established. Overwhelming virus replication in the nervous system leads to many systemic complications, including multiorgan failure. Experimental studies show strong evidence for upregulation of interferons, cytokines, and chemokines in the CNS in response to infection with rabies virus (Hicks et al., 2009; Johnson et al., 2011). Transcriptomic studies have shown the up regulation of many interferon- inducible genes (Wang H2011; Johnson N2008) and this up regulation has been supported by immunohistological demonstration of chemokine production in neurons. Such production drives an influx of immune cells into the CNS, particularly T cells. However, in the case of rabies virus infection, this influx does not control infection and the host invariably dies. In the absence of therapeutic options no mechanisms exist to ameliorate rabies virus replication once the virus reaches the brain (Fooks et al., 2014).

IMPACT ON ION CHANNELS

Dysfunction of ion channels has been shown in rabies virus-infected cultured mouse neuroblastoma NA cells with the whole-cell patch-clamp technique (Iwata et al, 1999). 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 non selective 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 (Jackson et al., 2003).

NEURONAL VIABILITY AND APOPTOSIS

In the recent years, neurotropic viruses in particular have been shown to induce apoptosis within the CNS, and depletion of these non regenerating neuronal cells by apoptosis may result in neurological morbidity [Pekosz et al., 1996]. This association between viral infection of the CNS and apoptosis has spawned a new area of rabies virus research (Suja et al. 2011). Viruses have been shown to induce apoptosis, either as a mechanism for the release and dissemination of progeny virions

or as a defense strategy of multicellular host organisms for the destruction of infected cells and therefore preventing the spread of the virus. Neurotropic viruses spread to the central nervous system, in fact neural cells, and produce clinical illness by causing dysfunction or death of a population of these cells. There is recent evidence that viruses may cause death of cells by apoptosis, which is a tightly regulated energy-requiring process that is orchestrated by a genetic program. Apoptosis plays an important role in producing cell death in rabies virus infection of cultured cells and in the brains of experimentally infected mice (Jackson and Rossiter, 1997). Replication of rabies virus in suckling mouse brain cells resulted in brain cell apoptosis, detected by DNA fragmentation and in situ apoptosis within 25 h after infection and before evidence of intracerebral immune activation. Cell death occurred simultaneously with rabies virus replication. There were clinical signs of illness in infected newborn mice within 24 h after the appearance of DNA fragmentation and before infiltration by lymphocytes. This suggested that onset of illness started independently of the immune function. This conclusion was supported by the occurrence of massive apoptosis followed by paralysis in rabies virus-infected immunosuppressed mice. Direct, viral-induced, neuronal apoptosis was the earliest death mechanism detected in these mice. It was proposed that pathogenesis of this fixed strain of rabies virus in mice begins with the induction of apoptosis by rabies virus replication. Cerebral damage may then be amplified by immunological mechanisms plus an additional unidentified factor. This is followed by increased permeability of the blood brain barrier (Theerasurakarn et al. 1998). A fatal encephalomyelitis developed after intracerebral inoculation of 6-day-old ICR mice with the challenge virus standard (CVS) strain of fixed rabies virus. The brains of CVS-infected mice showed widespread morphologic changes of apoptosis, which were particularly prominent in pyramidal neurons of the hippocampus and in the cerebral cortex. Evidence of oligonucleosomal DNA fragmentation was sought in situ using the TUNEL method. TUNEL staining was observed in many neurons, and rabies virus antigen was usually demonstrated with immunoperoxidase staining in similar regions. Neurons in the dentate gyrus of the hippocampus demonstrated expression of viral antigen, apoptotic changes, and positive TUNEL staining. This region normally demonstrates little infection in CNS-infected adult mice. Double labeling of neurons with TUNEL and viral antigen indicated that infected neurons actually underwent apoptosis. Increased immunoreactivity against the Bax protein was demonstrated compared to uninfected mice. Purkinje cells expressed viral antigen, but did not show significant morphologic changes of apoptosis or TUNEL staining. In contrast, neurons in the external granular layer of the cerebellum did not express viral antigen, but demonstrated greater morphologic changes of apoptosis and positive TUNEL staining than uninfected controls. Apoptotic cell death likely plays an important role in the pathogenesis of rabies virus infection in suckling mice. There was evidence of more apoptosis in the brains of suckling mice than in those of adult mice and this finding explains the greater neurovirulence of rabies virus in younger mice. Rabies virus likely induces apoptosis in vivo by both direct and indirect mechanisms (Jackson and Park,1998).

NO

Rabies-induced synthesis of inducible nitric oxide (iNOS) in the central nervous system has been suggested as a pathogenetic step in neuronal cell damage in rabies infections (Ubol et al. 2001). Whether this event can be extrapolated to human or not is not clear. Nitric oxide (NO) is a short-lived diffusible molecule, the production of which is catalyzed by nitric oxide synthase (NOS). Two major isoforms of NOS, inducible and constitutive, have been identified. Constitutive NOS, cNOS, is present in endothelial cells and neurons and catalyses low-level NO production, which plays a role in neurotransmission and vasodilatation. Inducible NOS, iNOS, is induced as a response to various stimuli such as endotoxins and in¯inflammatory cytokines. NO synthesis due to iNOS has been reported to be involved in the pathogenesis of neurodegenerative diseases. For example, experimental auto-immune encephalitis in mice is a demyelinating disease associated with an excessive production of NO. Rabies is a disease of the central nervous system which involves significant upregulation of iNOS gene expression and down regulation of neuronal cNOS (nNOS) gene expression (Ubol et al. 2001). NO may play a role in blood brain barrier leakage and may contribute to tissue damage due to the generation of peroxynitrite [Hooper et al.1995; Akaiki et al. 1995). NO by itself has been shown to induce neural cell death via apoptosis and replication of rabies virus in neural cells induces apoptotic cell death in vivo and in vitro [Jackson et al.1997; Theerasurakarn et al.1998), A study (Ubol, 2001) demonstrated that inhibition of NO production by

an iNOS inhibitor significantly increased the survival time of rabies virus-infected mice. Extension of the survival time as accompanied by delayed viral replication, slow progression to apoptotic death and delayed expression of the apoptotic gene, but there was no effect on the intracerebral inflammatory response. The significance of iNOS inhibition, but not nNOS inhibition, on the severity of rabies virus infection is supported by the evidence that only iNOS mRNA is dramatically up regulated during rabies virus infection [Akaikiet al. , 1995]. In summary, the study indirectly demonstrated possible effects of NO on the pathogenesis of rabies encephalitis. This diffusible molecule may directly induce the apoptotic death of neurons (Ubol, 2001). The onset of clinical signs in RV–infected rats and the clinical progression of disease correlated with increasing quantities of nitric oxide in the brain to levels up to 30-fold more than in controls, which was determined using spin trapping of nitric oxide and electron paramagnetic resonance spectroscopy (Hooper et al., 1995)

CONCLUSION

The basis for neuronal dysfunction in rabies is complex, but they may involve degenerative changes in neuronal processes such as dendrites and axons. Permeability of the blood-brain barrier to immune effectors is important for viral clearance and recovery from rabies. No effective therapy for human rabies is available. Hopefully, an improved understanding of rabies pathogenesis will lead to the development of novel therapies for human rabies. There have been recent important advances in understanding of how rabies virus spreads and causes disease in its hosts. Till date, it may be safe to conclude that virus may cause dysfunction of CNS by interaction through ion channels, nitric oxide, and several neurotransmitters beside that apoptosis may have a role to play. However, there is no satisfactory explanation for fundamental issues such as the basis for neuronal dysfunction in rabies. More research is needed in good experimental animal models in order for us to better understand the pathogenesis of this ancient disease.

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