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Original Article

Natural Infection with Rabies Virus: A Histopathological and Immunohistochemical Study of Human Brains

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ABSTRACT

Objectives: Despite all the efforts and increased knowledge of rabies, the exact mechanisms of infection and mortality from the rabies virus are not well understood. To understand the mechanisms underlying the pathogenicity of rabies virus infection, it is crucial to study the tissue that the rabies virus naturally infects in humans.
Methods: Cerebellum brain tissue from 9 human post mortem cases from Iran, who had been infected with rabies virus, were examined histopathologically and immunohistochemically to evaluate the innate immune responses against the rabies virus.
Results: Histopathological examination revealed inflammation of the infected cerebellum and immunohistochemical analyses showed an increased immunoreactivity of heat shock protein 70, interleukin-6, interleukin-1, tumor necrosis factor-alpha, caspase-3, caspase-9, toll-like receptor3 and toll-like receptor4 in the infected brain tissue.
Conclusion: These results indicated the involvement of innate immunity in rabies infected human brain tissue, which may aggravate the progression of this deadly disease.

Introduction

Rabies virus (RV) is an enveloped bullet-shaped virus belonging to the Rhabdoviridae family, genus Lyssa virus, and is known to be the causative agent in rabies infection in many mammalian species [1]. The widespread dissemination, public-health concern, veterinary implications and economic burdens set rabies as the most important viral zoonosis worldwide [2]. Rabies accounts for over 59,000 deaths annually as reported by the World Health Organization (WHO) and millions more undergo post-exposure prophylaxis [3]. Most of the cases of rabies-infected humans occur in developing countries, where canine rabies remains the main source for human exposure [4]. Despite many attempts toward medical intervention, rabies continues to present as a public health concern worldwide with the highest case fatality rate and the numbers of cases have been increasing, mainly due to the large global rabies reservoirs in both domestic and wildlife animals [5].

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RV is transmitted through the bite from a rabid animal and enters the peripheral nervous system via the neuromuscular junctions or via a sensory nerve through nerve spindles where it infects neurons [6]. The virus moves rapidly from one neuron to another, along the spinal cord, up to the central nervous system (CNS) and the salivary glands [7]. In the CNS, dissemination of the virus occurs rapidly accompanied by serious pathological and pathophysiological changes in the brain, such as neuronal necrosis, neuronophagia, satellitosis and perivascular cuffing [8,9]. Patients eventually die of circulatory insufficiency, cardiac arrest as well as respiratory failure [10]. However, the exact mechanisms underlying RV infection in human and the pathogenesis of rabies are not completely understood [11]. To develop an effective therapeutic approach, it is important to fully understand the mechanism by which rabies causes lethal neurological disease.

Due to the neurotropic nature of RV infection, understanding rabies pathogenesis requires knowledge of histopathological and immunohistochemical characteristics of the disease in the CNS [12]. Studies on RV neurovirulence have been mainly conducted on animal models that were infected with laboratory strains with various degrees of pathogenicity [13]. There is often little or no histopathological evidence of neural destruction in animals dying of rabies and the functional changes in RV-infected neurons in vitro are minimal [14]. Additionally, limited studies have investigated the histological relationship of direct infection of RV with immune responses in human [15]. For a precise understanding of the mechanisms underlying the pathogenicity of RV infection, it is crucial to study tissue from infected humans.

The aim of this research was to study RV-infected human brains histopathologically and immunohistochemically in order to investigate innate immunity against rabies infection.

Materials and Methods

This study was conducted on 9 human cases of rabies with brain necropsy. Post mortem samples (formalin-fixed and paraffin-embedded) were supplied by the WHO collaborating center for Reference and Research on Rabies, Pasteur Institute of Iran and this study was performed in accordance with the national experimental guidelines (Ethic no.: et- 91/0201/4549). The cerebellum tissue blocks were stored at -80°C until analysis was performed. All 9 cases had a history of rabid animal exposure and a clinical history of confusion, hydrophobia, agitation and eventually seizures and coma were present in all cases for a period of 30 to 60 days. All 9 rabies' cases had been previously confirmed by the fluorescent antibody test (FAT), which is the gold standard test in the diagnosis of rabies and is recommended by both the WHO and the World Organization for Animal Health. In the FAT, the presence of specific aggregates of the RV nucleoprotein antigen (N) in brain smears (Negri bodies) is detected by a direct immunofluorescence technique.

All the tissue samples were stored in 10% neutral buffered formalin for 72 hours thenceforth were treated for histological studies. The paraffin blocks were cut into 4-6 µm sections which were mounted on glass slides. The serial sections were stained with hematoxylin and eosin (H&E) for histopathological analyses and immunohistochemical studies. All the samples were examined under a light microscope for histopathological changes including microglial proliferation, perivascular inflammation, neuronophagia and the presence of Negri bodies.

For immunohistochemical analyses, 3 paraffin embedded sections were selected from each sample. The selected sections were deparaffinized in xylene and rehydrated using alcohol series. Endogenous peroxidase activity was inhibited by incubating the samples in 3% hydrogen peroxide in absolute methanol. Antigen retrieval was performed by heating the tissue sections in 10 mM citrate buffer (pH6.0) for 10 minutes at 95°C.

The cooled sections were washed with phosphate buffered saline (PBS)and incubated at 4°C overnight with primary antibody against a series of markers which included; mouse polyclonal antibody against interleukin-6 [IL-6 (Santa Cruz Biotechnology)] diluted to 1:350,mouse polyclonal antibody against interleukin-1beta [IL-1β (Abcam, Cambridge, United Kingdom)] diluted to 1:400, mouse monoclonal antibody against tumor necrosis factor-alpha [TNF-α (Abcam, Cambridge, United Kingdom)] diluted to 1:400, mouse monoclonal antibody against heat shock protein 70 [Hsp70 (Santa Cruz Biotechnology)] diluted to 1:300, mouse monoclonal antibody against caspase-3 (Santa Cruz Biotechnology) diluted to 1:50, mouse monoclonal antibody to caspase-9 (Santa Cruz Biotechnology) diluted to 1:350,mouse polyclonal antibody against toll-like receptor 3 [TLR3 (Santa Cruz Biotechnology)] diluted to 1:50, and rabbit polyclonal toll-like receptor 4 [TLR4 (Santa Cruz Biotechnology)] diluted to 1:200 in a solution containing 1-5% normal goat serum in 0.3% Triton X-100 and 0.1M PBS (pH 7.4).

The primary antibodies were then removed by washing 3 times with PBS. Sections were then incubated with goat anti-mouse (or goat anti-rabbit for TLR4) and rabbit horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) diluted to 1:350 and samples were left to incubate for 1hour at room temperature.

The secondary antibody was then removed by washing the slides 3 times with PBS prior to be incubated in 0.5 µL 3-3′-diaminobenzidine (Roche) and 1.5 µL peroxide buffer for 5-10 minutes at room temperature. The sections were

counterstained with hematoxylin and images were taken using a ×40 objective lens (BX71; Olympus, Japan).

Data were presented as mean ± SEM. Statistical significance between the 2 groups was analyzed by independent samples T test, followed by an appropriate post-hoc test to compare differences among all the data. Differences were considered statistically significant when the $p < 0.05$.

Results

1. RV FAT

Negri bodies were detected by specific fluorescence of bound conjugate in all samples (Figure 1).

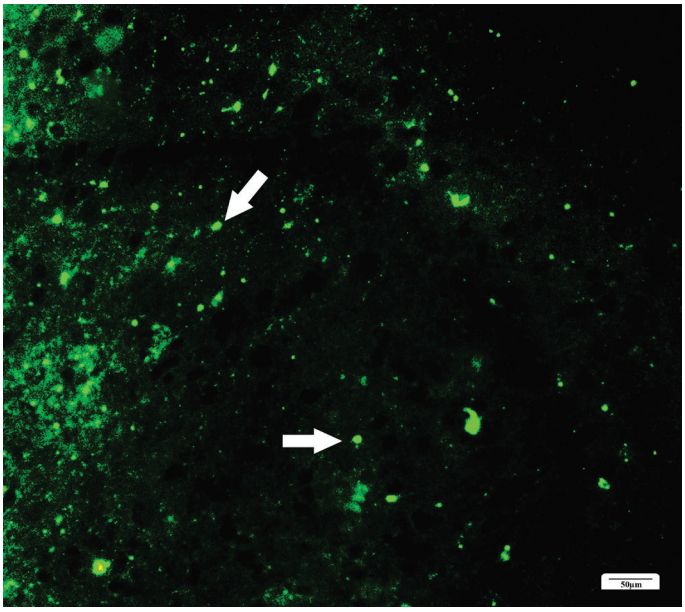

Figure 1. The aggregates of rabies virus nucleocapsid protein viewed as green fluorescence by the fluorescent antibody test.

2. Histopathological findings

The tissue samples were examined for changes, such as microglial proliferation, perivascular inflammation, neuronophagia and presence of Negri bodies. Astrocytic proliferation and swelling associated with enlarged nuclei were observed in stained sections (Figures 2A and 2B). Perineuronal satellite oligodendroglia surrounded degenerated neurons with condensed chromatin and little cytoplasm (Figure 2C). Neuronal cell bodies were red, angular and shrunken. Furthermore, their nuclei were contracted and dense. The necrotic neuron cell bodies were surrounded by macrophages. Perivascular cuffing associated with neuronal degeneration was also prominent (Figures 2D, 2E and 2F). Eosinophilic and sharply outlined inclusion bodies (Negri bodies) were observed in the cytoplasm of certain nerve cells infected with RV (Figures 2G and H).

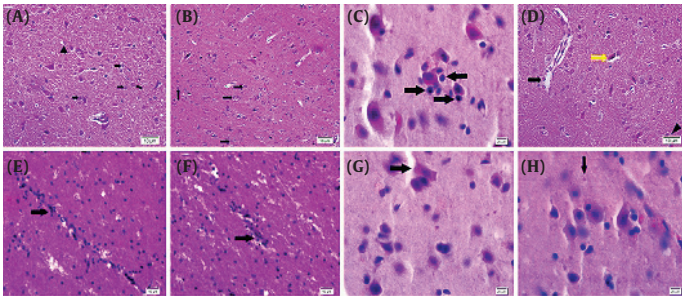

Figure 2. Hematoxylin and eosin staining. (A) and (B) Astrocytic proliferation, astrocyte nuclei are somewhat enlarged and appear more numerous than expected (arrows) associated with astrocytic swelling (arrow head), magnification ×100. (C) Perineuronal satellite oligodendroglia (arrows) surround degenerate neurons with condensed chromatin and little cytoplasm, H&E staining, magnification ×200. (D),(E) and (F) Neuronal cell bodies are red, angular, and shrunken and their nuclei are contracted and dense (yellow arrow). The necrotic neuron cell body is surrounded by macrophages that will phagocytose the cell debris (arrow head). Perivascular cuffing (arrow) associated with neuronal degeneration (arrow head), H&E staining, magnification ×100. (G) and (H) Negri bodies, formed within neurons of the CNS have long been the hallmark of rabies infection (arrows), magnification ×200.

3. Immunohistochemical findings

The immuno-reactivity of inflammatory mediators such as IL-6, IL-1β and TNF-α in RV-infected brain tissue (IL-6 = 21.6 ± 1.16, IL-1β = 18.2 ± 0.9 and TNF-α = 24 ± 1.39) were statistically significantly higher ($p \leq 0.01$) than those detected in normal brain tissue (IL-6 = 2.5 ± 0.5, IL- 1β = 3.4 ± 0.6 and TNF-α = 4.5 ± 1.29). Immunohistochemical examination revealed that Hsp70 immuno-reactivity in RV-infected brain tissue was significantly increased compared to normal brain tissue ($p \leq 0.01$). The mean number of Hsp70 reactive cells, per square millimeter, in RV-infected and in normal brain tissue was 20.6 ± 1.45 and 1 ± 0.28, respectively. Moreover, expression levels of caspase-3 and caspase-9 were significantly increased in RV-infected tissue (caspase-3 = 21.1 ± 1.4 and caspase-9 = 30.3 ± 1.09) compared to those detected in normal tissue (caspase-3 = 1.5 ± 0.5 and caspase-9 = 2.1 ± 1.8) ($p \leq 0.01$ and $p \leq 0.001$, respectively). Furthermore, expression levels of TLR3 and TLR4 were significantly increased in RV-infected tissue (TLR3 = 26.1 ± 1.07 and TLR4 = 24.1 ± 6) compared to those detected in normal brains [TLR3 = 3 ± 1.15 and TLR4 = 6 ± 1.22; $p \leq 0.001$ and $p \leq 0.01$ respectively (Figure 3)].

Discussion

Although rabies is inevitably a fatal disease and presents a horrifying clinical picture, little is known about the

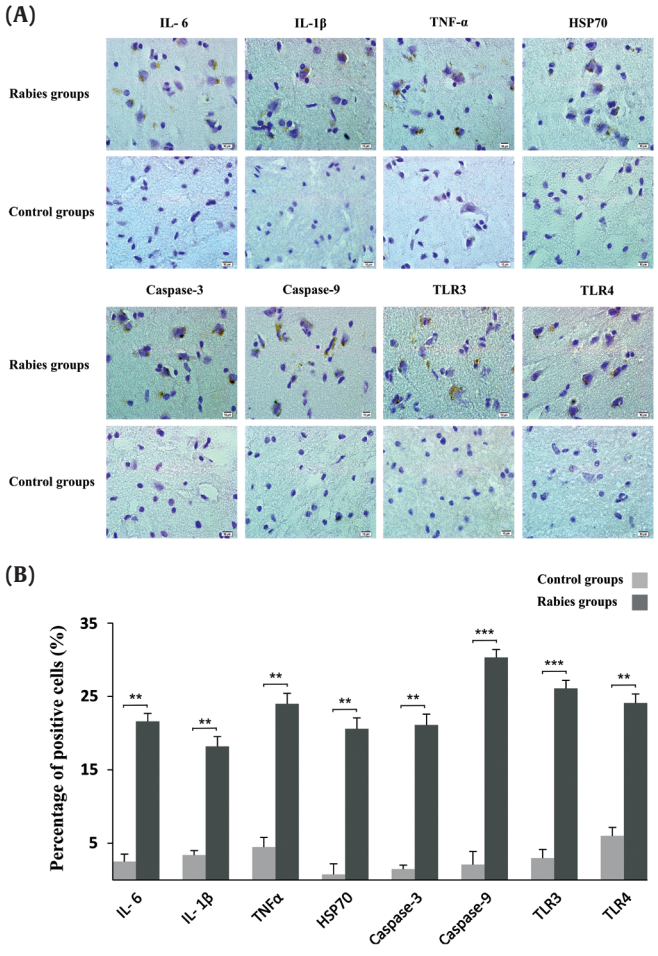

Figure 3. Immunohistochemical staining of cerebellum tissue sections. (A) Increased expression of apoptosis markers (caspase 3 and caspase 9) and inflammatory markers (IL-6, IL-1β, TNF-α, HSP70, TLR3, TLR4) in rabies groups compared to control groups are shown. Nuclei are stained with hematoxylin (blue), whereas apoptosis markers and inflammatory markers are stained in dark brown. (B) Bar charts showing the percentage of positive cells.
Each value indicates the mean ± SEM.
** $p < 0.01$.
*** $p < 0.001$.

exact mechanism of infection and mortality due to rabies-associated encephalitis [12]. The involvement of the inflammatory response in the pathogenesis of the virus is yet to be well characterized [15,16]. Some studies have demonstrated that virus distribution alone cannot explain the pattern of symptoms or the clinical diversity of rabies [15]. Therefore, other mechanisms such as immune responses other than direct viral infection may be involved in CNS pathogenesis [15]. To date, no detailed histopathological and immunohistochemical study on human brain tissue infected with RV has been published. Previous studies have contributed to understanding of the pathogenesis and diagnosis of rabies, which highlights the importance of conducting these types of study in humans. In this current study, histopathological and immunohistochemical examinations of human brain tissue were performed on individuals infected with RV. All the rabies-infected tissue analyzed in this study presented with Negri bodies as the main marker of RV infection, as well as an inflammatory response as evidence. Although the presence of Negri bodies in neurons is a pathologic hallmark of rabies, these inclusion bodies in the infected neurons may also be absent. The main purpose of this study was to assess the distribution of rabies viral antigen and inflammatory changes within the human brain.

The results in this study showed that astrocyte nuclei are somewhat enlarged and appear more numerous than expected. A long-held belief is that when neurons are injured or some kind of perturbation of the perineuronal microenvironment is present, oligodendroglia around the neurons hypertrophy and proliferate in a process referred to as satellitosis [17]. Microscopic lesions of the CNS including perivascular cuffing with lymphocytes, macrophages and plasma cells, are typically lymphomonocytic (non-suppurative), such as microgliosis which is sometimes prominent and variable but often not severe neuronal degeneration and ganglioneuritis. These findings support the notion that RV could induce inflammation in the CNS and is usually involved in the infection.

It is hypothesized that the induction of inflammatory responses, on one hand can lead to the clearance of RV from the CNS when the virus dose is low. However, extensive inflammation in the CNS can result in disease and death when large fixed doses of virus are administered to infect mice [18]. Nevertheless, there are limited data as to whether RV could induce an inflammatory response in the CNS [16]. Consistent with pathological evidence of inflammation in the CNS, immunohistochemical examination showed an increased immuno-reactivity of IL-1β, TNF-α, IL-6, TLR3 and TLR4. Upregulation of these combined factors may play an important role in coordinating the dramatic inflammatory responses associated with rabies-encephalopathy [19]. These factors can modify the hippocampus and other limbic-system functions, including electrical cortical activity, hypothalamo-pituitary-adrenal axis and serotonin metabolism [20]. These factors may also trigger astromicroglial activation and the development of inflammation of the brain in the CNS [21]. Upregulation of IL-1β and TNF-a, are two important pro-inflammatory cytokines that have been shown to be associated with meningitis and damage to the blood-brain barrier [22]. These factors also prime macrophages and microglial cells to release reactive nitrogen intermediates, which are associated with CNS inflammation and the severity of rabies disease [23]. Solanki et al who examined 25 brains of patients with either furious orparalytic rabies, showed no correlation between inflammation or viral antigen distribution , or expression of IL-1 β and TNF-α (in microglia, macrophages and lymphocytes)

[20]. Increased modulation of microglia/macrophages and lymphocyte infiltration in the brain has been suggested to play a role in the treatment of neurological disorders (such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis) by preventing neuronal damage, improving repair, and eliminating toxic protein [24-26]. In addition, Miao et al [27] described the innate immune response in the brain during rabies infection using wild type (street) and attenuated rabies strains in a mouse model and concluded that either intracerebral or intramuscular infection of mice with street strains, failed to induce both the innate and adaptive immune responses in the CNS, leading to death.

It has previously been demonstrated that human T-cell immunity to RV, where high concentrations of serum IL-6 have been detected, cannot reduce encephalitic rabies, whereas those without this response survive longer and present with paralytic rabies [16].

Hsps are involved in several normal cellular functions (ex. Innate immune responses and apoptosis) and also involved in the replication process of several viruses [28,29]. Over-expression of Hsp70 in this study confirmed the efficiency of RV replication. This protein was shown to be upregulated as early as 4 hours post-infection and has been shown to bind specifically with the RV N protein in the nucleocapsid, resulting in a dramatic increase in P protein synthesis and increased viral production [30].

TLR3, a Type I intracellular transmembrane protein is a negative regulator of axonal growth that has been found in glial cells and neurons in brain disorders, neurodegenerative diseases and viral infections [31,32]. Some studies have shown that TLR3 plays a crucial role in the core of Negri body formation surrounded by a ring of viral N and P proteins [33]. Induction of such factors by RV infection may be involved in the CNS inflammation which consequently could contribute to the subsequent process of pathological changes. Préhaud et al [34] reported that human postmitotic neurons (NT2-N cell line) expressed TLR-3 and could mount an innate immune response that was characterized by the production of IFN, chemokines, and inflammatory cytokines, in response to RV and dsRNA [poly (I:C)]. They demonstrated for the first time that human neurons constitutively express TLR-3. It was proposed that viral components other than dsRNA trigger the innate immune response. They also suggested that in the absence of glia, these viral components have the intrinsic machinery to trigger a typical innate immune response, including the 2-step interferon response during RV infection [34].

Apoptosis plays an important biological role in the development and homeostasis of cell populations, as well as in the pathogenesis and expression of many disease processes [35]. Apoptosis has been proposed to be the principal cause of neuronal death [11], limiting the spread of viruses [36-38]. An important regulatory event in the apoptotic process is the activation of caspases, a family of cysteine proteases [39]. Activation of caspase-9 and -3 was detected in this study, suggesting that apoptosis induced by RV was also involved in the activation of a caspase-dependent pathway as observed previously [40].

Conclusion

The results observed in this study through detailed histology of human cerebellum following infection with wild-type street rabies, showed insight into the immunology behind the infection indicating innate immunity may aggravate disease progression. These findings further our understanding of viral pathogenesis, and may aid the development of novel strategies for rabies treatment.

Conflicts of Interest

The authors declare that there was no conflicts of interest associated with this paper.

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