Original Research Article

Rabies Virus Severely Influences Host Protein during Terminal Stages of Infection

Mehta Shraddha* and Chowdhary Abhay

Department of Virology & Immunology, Haffkine Institute for Training, Research & Testing, Parel, Mumbai-400012, India
*Corresponding author

ABSTRACT

Rabies, one of the most ancient diseases that cause encephalitis in all mammals is completely preventable provided appropriate post-exposure prophylaxis is initiated on time. Yet, large numbers of rabies deaths are reported each year and currently no effective treatment is available for clinical rabies. In the present study 2-3 week old mice were intracerebrally inoculated with street virus strain to study the effect of rabies virus infection on host protein expression. Brain tissues were harvested at periodic time intervals and analyzed for rabies antigen detection and difference in host protein profiles. N protein antigen remained undetected 48 h pi (post infection), but was found to be positive for rabies N gene by PCR. With progress in infection and development of clinical manifestations, viral antigen concentration was found to be inversely proportional to the total protein content in the brain tissues. By the terminal stages, a ≥40% reduction in protein content was observed and was thought to be a result of inhibition of host translational mechanisms. Studying virus-host protein interactions on a time-point analysis can provide a deeper insight into the disease pathogenesis and help find druggable targets for timely therapeutic intervention or diagnostic biomarkers.

Keywords
Rabies, Host protein, Therapeutic, Biomarkers

Introduction

Rabies is one of the negligible infectious diseases of public health concern and is responsible for over 30,000 deaths in Asia and Africa alone (Mani and Madhusudana, 2013). The disease is 100% preventable provided post-exposure prophylaxis (PEP) treatment consisting of vaccines and immunoglobulins is initiated timely. Despite this, it continues to pose a threat due to ignorance of people in management of animal bites and sparseness of medical resources. Besides, the clinical signs appear only when it reaches the central nervous system (CNS), after which it always proves to be fatal (Wang et al., 2005). Unfortunately, ante-mortem diagnostic tests are most often inconclusive and a negative result does not rule out infection. The problem is further compounded by absence of an effective treatment for rabies (Madhusudana and Sukumaran, 2008; Mani and Madhusudana, 2013). Management of
rabies is usually palliative consisting of heavy sedatives, anti-convulsants, nutritional supplements and intensive care (Jackson, 2009; Nigg and Walker, 2009).

An alternative approach for rabies management, either from diagnostic or therapeutic viewpoint is a worthwhile challenge. This may be achieved through newer techniques such as proteomics or transcriptomics that provide a deeper insight into virus-host interactions. Viruses have a common approach— release its genome into the host cell, replicate and transcribe the genetic information, express encoded proteins, assemble and form mature virions and finally release the viral particle, all of which require the host cell machinery (Lingappa et al., 2013). Rhabdoviruses are no different and several studies have elucidated the role of host proteins such as heat shock protein (Hsp70), Yes-kinase-associated protein (YAP), toll-like receptor (TLRs), etc. in replication/ transcription/ assembly process of Vesicular Stomatitis virus (VSV) and Rabies virus (RABV) (Guleria et al., 2011). The neuroinvasiveness of RABV is attributed to neuronal dysfunction rather than neuronal cell death (Jackson, 2003). Malfunction at any of the four essential stages of neurotransmission: synthesis, storage, release and uptake may contribute to altered neurophysiology (Dhingra et al., 2007). Previous studies show that neurotransmitters such as dopamine typically exhibit upregulation during early stages of rabies, reach its peak on appearance of clinical signs and gradually decline to the level below/equivalent to that prior to infection towards the terminal stages (paralysis) (Fu and Jackson, 2005). Cerebral death prior to failure of vegetative functions has also been reported in experimental rabies (Dhingra et al., 2007).

Therefore tracking the progression of disease is imperative for formulating intervention therapies, especially since street RABV exhibits variable incubation and survival period. The present study was thus designed to understand time-point invasion of RABV and correlate it with the clinical signs and its effect on host protein expression in experimental murine model.

Materials and Methods

Animals, virus and antibodies: 2-3 week old Swiss Albino mice weighing 10-15 grams were obtained from and housed in our Institute animal house facility under pathogen-free conditions with access to food and water ad libitum. CVS-11, a mouse-brain adapted fixed RABV strain and street RABV (SRV) isolated from a clinical specimen (rabid dog brain) was used in the study. All the procedures were conducted in accordance with guidelines under animal protocols approved by the Institute Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) guidelines. Virus stocks were prepared using protocol similar to that described by Wang et al. (2005) and viral lethal dose 50 (LD$_{50}$) was calculated according to Reed and Muench method (Meslin et al., 1996). Light Diagnostics™ Rabies Polyclonal DFA reagent (Goat IgG FITC Conjugate) specific as anti-RABV nucleoprotein (N), reactive with all lyssaviruses; was obtained from Merck, Millipore, Inc.

Animal infection and tissue collection: Mice were divided into three groups: Group 1 (CVS), Group 2 (SRV), Group 3 (control) and inoculated intracerebrally (ic) with 30µL of 100 LD$_{50}$ of CVS, SRV and PBS respectively. All groups of mice were observed daily twice for clinical findings. Mice were euthanized and brain tissues were harvested at periodic intervals based on appearance of clinical signs. By day 8 post-
infection (pi), mice from all the groups were sacrificed, brain tissues harvested, snap frozen and stored at –80°C until further processing.

**Direct Fluorescent Antibody Test (FAT):**
To detect the presence of rabies antigen in harvested tissue from all the groups, impression smears were made and the slides were stained by polyclonal DFA reagent as per CDC protocol for post-mortem diagnosis of rabies in animals (CDC).

**RNA extraction and nested PCR:**
Harvested mice brains were homogenized and total RNA was extracted from supernatant manually using a QIAamp® Viral RNA mini kit (Qiagen India Pvt. Ltd., New Delhi, India) as per the manufacturer’s instructions. The extracted viral RNA was stored at –80°C until further processing. To confirm the results of direct fluorescent antibody test, nested reverse transcriptase (RT-PCR) specific for N gene was performed on extracted RNA using a protocol by Nagaraj et al. (2006) with minor modifications. The first-round PCR was performed using a One-step RT-PCR kit (Qiagen India Pvt. Ltd., New Delhi, India) as per the manufacturer’s instructions using gene-specific PCR primers (Table 1) at a concentration of 0.6 µM in a 25 µL reaction mixture. The cycling conditions used were: RT step, 50°C/30 min; RT inactivation, 95°C/15 min; 40 cycles of denaturation, 95°C/1 min; annealing, 55°C/1 min and extension, 72°C/1 min 30 s; final extension, 72°C/5 min. The PCR products were then run on an agarose gel electrophoresis (1.5% agarose gel infused with SybrSafe® Invitrogen dye for visualization) for the confirmation. The nested PCR product had a size of 762 bp.

**Total protein analysis:** Snap frozen brain tissues were manually homogenized in 0.5% SDS to give 10% w/v lysis buffer suspension. Further, it was sonicated on ice for 180s (10s pulse on/off) and centrifuged at 15000 rpm/30 min at 4°C. The supernatant was used as protein lysate stock and total protein in all the samples was estimated by Bradford assay using bovine serum albumin as standard. The protein samples (20µg) were loaded and separated on SDS-PAGE with 5% stacking and 10% resolving gel and stained using Coomassie brilliant blue staining.

**Results and Discussion**
In natural rabies, the incubation period is extremely variable and may depend on the initial concentration of the inoculum and the proximity of the wound site to the CNS (Jackson and Wunner, 2002). Evidence suggests that longer incubation periods may also be a result of the virus remaining at the site of inoculation for variably long time. Clinical disease usually develops in about 2 weeks to 1 month, in some cases about a few months and in the rarest cases, over a year. Regardless of the presentation i.e. furious or the dumb form, relatively mild neuropathological changes but severe clinical neurological signs are observed (Jackson, 2002; Jackson, 2003; Fu and Jackson, 2005). In experimental rabies, during the early stages i.e. before the onset of clinical signs, progressive disappearance of sleep is observed while during the terminal stages hippocampal and cortical...
activity slows down. Brain electrical activity is also terminated about 30 min before failure of vegetative functions (Gomme et al., 2012). The present study was therefore designed to evaluate the effect of RABV neuroinvasiveness in correlation with development of clinical signs on neuronal protein synthesis with progress in infection through a time-series analysis in an experimental murine model.

CVS and SRV both of which are neurovirulent in nature and behave in the same manner except that the street strain has a relatively longer incubation period were included in the study. Two groups of mice were inoculated ic with 30 µL of 100 LD_{50} doses of CVS and SRV respectively and PBS inoculated mice were used as controls. Mice were euthanized and brain tissue was harvested on days 2, 4 and 6 pi for CVS and days 2, 4, 5, 6, 7 and 8 pi for SRV. The mice were observed daily for morbidity and mortality and clinical signs were noted. All the animals under CVS group became moribund by day 6 pi while those in SRV inoculated group reached terminal stages by day 7/8 pi. Table 2 provides information about the development of clinical manifestations with progression in infection. While ruffling of fur and changes in gait such as toe walking were observed within 48 h in both CVS and SRV infection, hunched posture was evident from day 4 onwards in CVS group and day 5 in SRV group. Hind limb paralysis set in by day 6 pi but was more pronounced on day 7 pi in SRV infection. By day 6 in case of CVS infection and day 7 in SRV infection, all the infected animals had become immobile and mortalities extended up to day 8 in the latter.

The progress of RABV infection was also tracked using conventional nested RT-PCR (Figure 2) specific towards the corresponding N gene. Absence of any prominent clinical signs on day 2 pi in virus infected groups correlated with the FAT results. However, with more sensitive technique such as PCR, a 762 bp band size of the N gene was observed in the inner round. The desired bands were more prominent in both outer and inner rounds of PCR only day 4 onwards. Progressive increase in fluorescent intensity and antigen distribution and therefore viral concentration is clearly evident in figure 1 and is in consensus with previous studies that state that the rate of RABV infection in vivo is extremely low during the first 72 h. but the brain tissues appear to be studded with viral antigen during the terminal stages of infection (Fu et al., 1993).

Several studies indicate that with increase in viral propagation, expression of a large number of host proteins is altered. Therefore, in order to study the effect of RABV infection on total protein in neuronal tissues, proteins were solubilized using 0.5% SDS solution, concentration was estimated using Bradford assay (Table 3) and profiles were analyzed using 1-D SDS PAGE (Figure 3). Protein lysates were loaded at a concentration of 20 µg in each well and electrophoresed at a constant 100 V for 1.5 h.

It was observed that the total protein content decreased with progress in infection. The protein content remained more or less same as that of uninfected controls until day 2, but day 4 onwards it progressively decreased. During the terminal stages, ≥40% reduction in total protein was observed. 96 h pi, RABV replication and therefore RABV RNA levels appear to increase exponentially (Fu et al., 1993). As RABV replication and its trans-synaptic spread are dependent on
the host gene functions that parallel the replication of RABV proteins, the effect on host protein expression may be correlated. Several genomic studies have shown that alterations in host gene expression are a major consequence of RABV infection (Prosniak et al., 2001; Wang et al., 2005), which is therefore subsequently seen as an effect on host protein expression too. Previous studies that have shown that neuronal proteins involved in synapse, neurotransmission, apoptosis and majorly metabolism (in vitro) (Zandi et al., 2009; Kluge et al., 2013) and ion homeostasis (in vivo) (Dhingra et al., 2007) are down regulated. In clinical specimens however, proteins responsible for the maintaining the cytoskeletal architecture in particular were found to be differentially expressed (Mehta et al., 2015). Thus the reduction in host neuronal proteins may either be a consequence of down regulation in protein synthesis or a result of increased protein catabolism or changes occurring at the translational level. However, in vitro studies show that under normal conditions protein synthesis remains uninhibited by RABV for several days after infection (Madore and England, 1975). We may therefore speculate that the reduction observed in the neuronal total protein may be a result of the latter. Further, Komarova et al. (2007) have demonstrated that RABV proteins can inhibit host cellular translation via a protein-protein interaction giving rise to a translational control of gene expression situation.

**Table.1** Outer and inner primer sequences for nested RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction (5’-3’)</th>
<th>Sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer</td>
<td>Forward</td>
<td>gccctacag acc tct aca atg gat gcc gac aa</td>
<td>1500</td>
</tr>
<tr>
<td>Outer</td>
<td>Reverse</td>
<td>gga ttg ac(at) aag atc ttg ctc at</td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>Forward</td>
<td>ttg t(at)g a(tc)ca ata tga gta caa</td>
<td>762</td>
</tr>
<tr>
<td>Inner</td>
<td>Reverse</td>
<td>ctg gct caa aca ttc ttc tta</td>
<td></td>
</tr>
</tbody>
</table>

**Table.2** Correlation of clinical manifestations with antigen detection

<table>
<thead>
<tr>
<th>dpi</th>
<th>Clinical Manifestations</th>
<th>Antigen detection results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change in gait</td>
<td>Hunched back</td>
</tr>
<tr>
<td>CVS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SRV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
Table 3: Total protein concentration estimated using Bradford method; C-Control

<table>
<thead>
<tr>
<th>dpi</th>
<th>C</th>
<th>CVS</th>
<th>SRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>45.00</td>
<td>42.59</td>
<td>45.20</td>
</tr>
<tr>
<td>4</td>
<td>40.00</td>
<td>41.00</td>
<td>35.00</td>
</tr>
<tr>
<td>5</td>
<td>----</td>
<td>36.60</td>
<td>32.00</td>
</tr>
<tr>
<td>6</td>
<td>30.00</td>
<td>27.50</td>
<td>20.00</td>
</tr>
</tbody>
</table>

Figure 1: Tracking the RABV infection progression pattern using FAT analysis at [A-C]: 2, 4 and 6 dpi respectively in CVS infected brain tissue and [D-I]: 2, 4, 5, 6, 7 and 8 dpi respectively in street virus infected brain tissue.
**Figure 2** Representative picture showing the presence of the 100 bp DNA ladder (lane 1), outer PCR product of 1500 bp (lane 2) and inner PCR product of 762 bp (lane 3) specific towards the RABV N gene.

**Figure 3** Graphical representation of total protein estimation

**Figure 4** 1-D SDS PAGE results on 10% resolving gel (Left to right): C- control, D2 to D6 (CVS), D2 to D8 (SRV) and L-ladder
Thus, though the present study clearly demonstrates a decrease in the total protein concentration with an increase in virus concentration, some proteins appear to be upregulated at certain time points. For example those at (i) ~12 kD on day 7/8 in SRV, (ii) ~31 kD on day 7 in SRV (iii) ~52 kD on day 4 in CVS and days 6/7 in SRV (Figure 2). In general, an increase in protein expression at time points just before paralysis i.e. at day 4 in CVS and day 6/7 in SRV was observed. Prosnaik et al. (2001) have demonstrated that proteins involved in cell-to-cell trans-synaptic spread of the virus such as neuroleukin are more likely to be activated during the later stages of infection.

The preliminary data generated through this time-point experiment was able to correlate the onset of clinical signs with the changes observed in protein expression during infection. Tryptic digestion followed by mass spectrometry can yield sequencing related information that may be used for identification of protein of interest. Since our study included whole brain analyses, it is difficult to prove if the protein expression changes particularly during the intermediate stages (from onset but before paralysis) are restricted to only infected neuronal cells or other non-infected neuronal/non-neuronal cells. Further studies are therefore warranted both time point-wise as well as region-wise using more advanced techniques such as two dimensional gel electrophoresis to determine the appropriate time for initiating a therapeutic intervention and/or help identify proteins that may prove to be potential drug targets or diagnostic biomarkers.

Acknowledgement

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Reference

CDC, Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing. A minimum standard for rabies diagnosis in the United States, Atlanta.


