Rabies virus induces expression of TLR-3 and its associated cytokines in Swiss albino mice

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Article history:
Received: 09 April, 2014
Accepted: 15 April, 2014
Available online: 23 June, 2014

Keywords:
Virus, rabies, cytokine, pathology, innate immunity

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Abstract
The present study was conducted with an aim to investigate involvement of TLR-3 and its associated cytokines (IL1-α, TNF-α, and IFN-α) in the pathogenesis of rabies. A total of 80 adult Swiss albino mice consisting of 20 mice in four groups viz., Challenge Virus Standard (CVS) strain of rabies (GI), poly IC as TLR-3 agonist with CVS (GPI), poly IC (GP) and PBS (GC) as controls were taken. Clinical history score revealed less severe and delayed onset of clinical signs in GPI group. This resulted in better survival of mice on different time points in GPI than in GI. The non-suppurative meningo-encephalitis characterized by perivascular cuffing, focal to diffuse gliosis and neuronal damage (necrosis/ apoptosis) scored less in GPI than in GI. These lesions progressed with time and were more severe on day 8th onwards. The hippocampus, particularly CA1 and CA2 regions, had more severe lesions followed by cerebral hemisphere and the thalamus. The positive apoptotic signals on 12th DPI in GI but not in GPI were supported by the increased expression of caspase 1 in GI on 12th DPI. The TLR-3 and its associated cytokines showed higher expression in GPI than in GI. Further, the presence of virus in brain was detected by both conventional and Real Time PCR using N gene specific primers (533 bp) as early as on 2nd DPI in both the infected groups. The study suggests that TLR-3 induction by its ligand poly IC has some role in reducing the development of disease, through pro-inflammatory cytokines. Further studies are needed to explore the possibility of TLR-3 for therapeutic purposes in rabies and other related diseases.

Citation:

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Photon Ignitor: ISIJN17846372D686323062014

1. Introduction
Rabies is invariably fatal disease of central nervous system (CNS), responsible for approximately 60,000 human deaths annually world-wide (Dietzschold et al., 2005). It is caused by a non-segmented, negative-stranded RNA virus of the genus Lyssavirus under the family Rhabdoviridae. The virus affect all warm-blooded animals including humans and cause disease which progresses to either furious form or dumb form followed by paralysis and death within 3-8 days. In rabies virus infection, both innate and adaptive arms of the immune system play important role, but it is the innate immunity, which plays major role, since there are few MHC molecules for adaptive immunity in the CNS.

Subsequent upon rabies virus infection, the CNS cells initiate protective responses through
production of type I interferons (IFN-α and β), pro-inflammatory cytokines (IL-1α, IL-6, TNF-α), chemokines (CCL-5 and CXCL-10), NK, CD4+ and CD8+ cells (Sen and Sarkar, 2005). A few studies have shown that viral G proteins and dsRNA, generated as an intermediate, are recognized through Toll Like Receptor-3 (TLR-3) (Alexopoulov et al., 2001; Takeda and Akira, 2003; Prehaud et al., 2005). TLR-3 binding in turn triggers interferon (IFN) - regulatory factor 3 (IRF3) phosphorylation and nuclear factor (NF)-κβ activation (Sen and Sarkar, 2005) to induce inflammatory cytokines (tumour necrosis factor (TNF-α), interleukins (IL-6 and IL-1α), chemokines (CCL-5 and CXCL-10) and also activation of the IFN-β promoter for IFN expression. Prehaud et al. (2005) reported enhanced TLR-3 expression with production of IFN-β, chemokines and inflammatory cytokines in response to rabies virus infection in human post mitotic neuron cultures (NT2-N). The enhanced expression of TLR-3 caused better survivability of infected cells by effectively limiting the viral burden mainly through interferon production. Glial cells have also been identified as the major producers of TLR-3 and initiators of inflammation in the CNS (Farina et al., 2005). The brain pathology begins as a consequence of viral induced neuronal death, either by nitric oxide induced neurotoxicity or by apoptosis (Griffin and Hardwick, 1999; Allsopp and Fazakerley, 2000; Fazakerley and Allsopp, 2001). Infiltration of immunological components through the blood-brain-barrier, activation of intrinsic local immune responses under the influence of cytokines and chemical mediators, as stated above, also contribute to neuronal damage. The body of literature suggests that TLR-3 mediated immune mechanism has some pertinent role in antiviral mechanism against rabies but its exact role has not been fully elucidated in detail so far. Therefore, during the present study we studied the role of TLR-3 and other important immune molecules (INF-α, IL-1α, and TNF-α) generated thereof in rabies pathogenesis using mouse as laboratory model. These findings will further explore the mechanism of innate immunity involved in rabies virus pathogenesis.

2. Materials and Methods

2.1 Animals, virus and experimental design

Eighty adult Swiss albino mice, weighing about 20 gm, of either sex were procured from Laboratory Animal Research Section of Indian Veterinary Research Institute, Izatnagar. The mice were kept in polycarbonate cages and provided feed and water ad libitum; and were maintained as per the guidelines of Animal Ethics Committee of the Institute. On day 0, 40 mice were primed with Poly IC (TLR3 agonist) by i/C route @ 25 µg/mouse, of this 20 were further inoculated on the same day with 100LD50/mouse Challenge Virus Standard (CVS) strain of rabies by i/C route and named as poly IC plus infected (GPI), while remaining 20 primed mice grouped as Poly IC (GP). On the same day, 20 mice were inoculated with similar dose of CVS alone and grouped as GI, and another 20 mice were inoculated with sterile phosphate buffer saline (PBS, pH 7.2) by i/C route and grouped as GC (Table 1). The mice were anesthetized with ether inhalation, before inoculation and collection of blood. Dose of inoculums were 0.03 ml/mouse. CVS strain of rabies infected brain tissue, maintained in Biological Products Division of the institute was used for making inoculums. Lethal dose 50 (LD50) of the virus was calculated by mouse inoculation test (MIT) using standard technique (Reed and Muensch, 1938). Aliquots of virus were stored at -70°C for further use. The clinical signs such as ruffled fur, tremors, in coordination, paralysis and prostration were recorded daily for each mouse. Brain, spleen and blood samples were collected at 2, 4, 6, 8, 10 and 12th DPI from 3 mice in each group. The details of experimental design are shown in Table 1.

Table 1: Experimental design showing different groups of mice and important outcomes of experiment

<table>
<thead>
<tr>
<th>Group (No. of mice)</th>
<th>Inoculums (0.03 ml/mouse IC)</th>
<th>Fold change in TLR3 and Cytokine profile on 10 DPI</th>
<th>HP Score on 10DPI</th>
<th>No. of mice survived / inoculated (On 10 DPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI (20)</td>
<td>Poly IC + CVS</td>
<td>0.90±0.36, 0.75±0.15</td>
<td>1.09±0.13</td>
<td>16±0.1</td>
</tr>
<tr>
<td>GP (20)</td>
<td>Poly IC (25 µg/mouse)</td>
<td>0.29±0.19, 0.12±0.03</td>
<td>0.34±0.08</td>
<td>2.66±0.06</td>
</tr>
<tr>
<td>GI (20)</td>
<td>CVS 100LD50/mouse</td>
<td>0.68±0.17, 0.26±0.21</td>
<td>0.76±0.16</td>
<td>34±1</td>
</tr>
<tr>
<td>GC (20)</td>
<td>PBS</td>
<td>0.14±0.04, 0.08±0.11</td>
<td>0.11±0.01</td>
<td>0.02±0.11</td>
</tr>
</tbody>
</table>
2.2 Histology and Immunohistochemistry

Samples from brain tissue were fixed in 10% buffered formalin. After fixation, the samples were processed for paraffin embedding sections as per the standard procedure (Luna, 1972) to obtain 4-5 µm thick paraffin sections. These sections were stained with haematoxylin and eosin (HE). The grading of lesions was done by developing histological score for different parameters in different neuroanatomical sites. Each parameter was assigned grade on scale 0-3 i.e. 0= no change, 1 = mild change, 2 = moderate change, 3 = severe change. The score for each mouse at each time point was aggregated and divided by number of mice to obtain mean value. The higher score was taken as the criteria for severe pathological changes over the lower score. Cerebrum, superficial laminae, middle laminae and deeper laminae were examined for neuronal degeneration and gliosis. Similarly grey matter and white matter were observed for perivascular cuffing and gliosis. Hippocampus, dentate gyri, CA1 (Cornua Amonis) and CA2 were observed for neuronal degeneration and gliosis, the grey and white matter for perivascular cuffing and gliosis. The meninges in both the sites were scored for meningitis.

Duplicate sections were processed for avidin-biotin-peroxidase complex (ABC) technique to detect rabies viral antigen using primary anti-rabies monoclonal antibody (Biological Products Division, IVRI) diluted 1:16. After dewaxing and dehydration, the endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide in methanol for 20 min at room temperature. The antigen retrieval was done by microwave heating with 0.1M citric acid (pH 6.0) for 15 min. Tissue sections were then rinsed in PBS (0.01 M, pH 7.2) and incubated with 5% goat serum for 20 min at room temperature. Primary antibody, diluted in 5% goat serum was incubated overnight at 4°C. Sections were washed thrice with PBS for 5 min each. These were covered with biotinylated secondary mouse antibody and kept for 30 min at 37°C in an incubator. Sections were washed with PBS as above, and VECTASTAIN ABC reagent (Cat no. PK4002, Vector Biologicals, USA) was added, left for 30 min at 37°C in incubator. It was further incubated with AEC (Amino ethyl carbazaine) chromogen for 10 min until the dark red stain developed. The slides were then counterstained with Meyer's hematoxylin for 10-15 sec, dehydrated and mounted. Specific primary antibodies were replaced by PBS, normal mouse serum, in negative control sections. Direct fluorescent antibody technique (dFAT) was also used to detect rabies viral antigen in paraffin embedded brain sections as per the standard procedure. The slides were processed as before and were incubated with anti-nucleocapsid fluorescein conjugated rabies polyclonal antibodies (1:16) (Central Research Institute, Kasauli, India), at 4°C overnight. Slides were then washed in PBS thrice for 5 min each, mounted with aqueous mountant and viewed under the fluorescent microscope (Leitz Leica, Germany) in 10X and 40X objectives for the presence of specific apple green fluorescence signals.

2.3 In situ detection of apoptosis

Paraffin embedded brain sections were processed as described in previous step. The sections were permeabilised by adding 100 µl of cytonin (R&D Biologicals, USA) for 20 min at room temperature and then washed twice for 5 min each in DNAse free water. The terminal deoxy nucleotidyltransferase-mediated dUTP nick end-labelling (TUNEL) kit (Cat no.TA4625, R&D Biologicals, USA) was used for detection of apoptotic cells as per the manufacturer’s instructions, keeping suitable controls.

2.4 Quantification of TLR-3 mRNA, its associated cytokines and β-actin

The brain samples collected at different time points in RNA later (Cat. No. 127143807, Qiagen) were used to quantify the mRNA expression. Briefly, 100 mg of brain tissue was taken in 1 ml TRIZOL (Life Technologies Ltd., USA) in teflon glass homogenizer and homogenised until clear suspension was obtained. The homogenate was transferred into eppendorf tubes, incubated for 10 min at room temperature and 200 µl of chloroform was added and mixed vigorously. The tubes were then incubated for 10 min over ice and centrifuged at 12000 rpm for 10 min at 4°C. Following centrifugation, the upper phase containing RNA (approximately 600 µl) was transferred to new eppendorf tube, added with isopropanol (900 µl) , incubated at -20°C for 2 hours and centrifuged (12000 rpm,10 min, 4°C). Supernatant was carefully removed and the precipitated RNA was recovered by centrifugation with 1 ml of 70% ethanol (12000 rpm, 10 min, 4°C). The RNA was dissolved in nuclease free water and stored at -20°C until further use. For making total cDNA the reverse transcription reaction was primed with random hexamer primers, wherein whole length mRNA was converted to cDNA. Briefly, 5µl of total RNA was taken and incubated (70°C, 10min),
Ph cDNA samples were diluted in nuclease free α DPI. Later, signs progressed to inco-ordination α DPI, USA) using specific primers and standard mice) and tremors (1 mouse) as early as on 3 µl of Reverse transcriptase 10X buffer, 2.5 µl of chilled on ice and 25 µl of reaction mixer was prepared by using 5 µl of MgCl₂ (25 mM), 3 µl of Reverse transcriptase 10X buffer, 2.5 µl of dNTP mixture, (10 mM), 15 IU of AMV Reverse Transcriptase (20 IU/µl) (Cat no. A3500, Promega), 1.2 µl of Random Primers (100 pmol), and 0.7 µl of Rnasin in 6.6 µl of nuclease free water. The reaction mixture was incubated at 42° C for 30 min and then heated at 95° C for 5 min and samples of cDNA obtained were stored at -20° C until further use.

Quantitative assay was performed by Real Time PCR (MX 3000 P System, Stratagene, USA) using specific primers and standard protocols as described by earlier workers (Table 1). For mRNA expression of TLR-3 and its associated cytokines such as IL-1α, IFN-α and TNF-α and housekeeping gene β-actin, cDNA samples were diluted in nuclease free water and used for PCR amplification with Qiagen QPCR Master mix (Cat no. 204054, SYBR Green Quanitafact PCR kit; Qiagen, USA) using an adapted protocol according to manufacturer’s instructions. Briefly, a reaction mix was prepared: 12.5 µl master mix, 1.0 µl of forward primer (10 pM) and 1.0µl of reverse primer (10 pM), 2 µl of cDNA (approx. 500 ng) and 8.5 µl of nuclease free water. The optimised runs were as follows: 1 cycle of denaturation (95° C for 10min), 40 cycles of amplification: denaturation (95° C during 30 s), annealing temperature depending on the cytokine (Table 1) for 60 seconds and extension (72° C for 60 s). Quantification results of mRNA expression of TLR-3 induced cytokines and β-actin in brain are shown as the average ratio (± standard error) between the number of molecules of each cytokine and β-actin. The data obtained were analysed using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). Finally, the mean relative expression for each group were statistically analysed by one way ANOVA [least significance difference (LSD) and Duncan’s tests] fort’ distribution (P value) using SPSS (7.5) software. Rabies viral genome was detected by both conventional and Real Time PCR using N gene specific primers. (Table.2)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Annealing Temp</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 3</td>
<td>F 5’GAAGCAGGCGTCCTGGACCT 3’ R 5’TGTGCTGAATTCGAGATCCA 3’</td>
<td>330</td>
<td>62</td>
<td>McKimmie et al., 2005</td>
</tr>
<tr>
<td>IFNα</td>
<td>F 5’AGGACAGGAAAGATTGGA 3’ R 5’GCTGCTGATGAGGAGTCATT 3’</td>
<td>200</td>
<td>62</td>
<td>McKimmie et al., 2005</td>
</tr>
<tr>
<td>IL1α</td>
<td>F 5’GATGTCCAATTACCTACCTCCA 3’ R 5’ACAACCTTGCTGCTGAGCA 3’</td>
<td>250</td>
<td>62</td>
<td>McKimmie et al., 2005</td>
</tr>
<tr>
<td>TNFα</td>
<td>F 5’CCCTTTACTGCGACCTTCTT 3’ R 5’AACCTGACACCTTCCTCCTTT 3’</td>
<td>310</td>
<td>62</td>
<td>McKimmie et al., 2005</td>
</tr>
<tr>
<td>β actin</td>
<td>F 5’TCTAGGCACAGGTTGGTG 3’ R 5’TCTAGGAGTGGTCGTCGAGG3’</td>
<td>460</td>
<td>56</td>
<td>McKimmie et al., 2005</td>
</tr>
<tr>
<td>RabN</td>
<td>F5’ACTGATGTAGAAGGGATGATG 3’ R5’GAACGGAAGTGGATGAAATA 3’</td>
<td>533</td>
<td>50</td>
<td>Gupta et al., 2005</td>
</tr>
<tr>
<td>Caspase 1</td>
<td>F 5’ TATGGACAAAGGCACGGACCTATG 3’ R 5’CCAGCAGCAACTTCATTCTCTG 3’</td>
<td>430</td>
<td>55</td>
<td>Sukathida et al., 2005</td>
</tr>
</tbody>
</table>

3. Results and Discussion

3.1 Clinical signs

The infected group (GI), showed ruffled fur (3 mice) and tremors (1 mouse) as early as on 3rd DPI. Later, signs progressed to inco-ordination (4th DPI, 1 mouse), paralysis (6th DPI, 3 mice) and prostration (9th DPI, 2 mice). By 10th DPI, only 4 mice survived, which died on 12th DPI. With the passage of time, the numbers of mice exhibiting the above clinical signs were kept increasing till all the mice succumbed. On the contrary, the mice in poly IC plus infected group (GPI) although had similar pattern of onset of clinical signs, but developed symptoms a little late, involving less number of mice. The number of mice survived in GPI was more (6 mice) than the GI (4 mice) on 10th DPI. Although, all mice in both the groups died on 12th DPI. The control (GC) and poly IC (GP) treated mice did not show any clinical signs throughout the experimental period.
3.2 Histopathology and Immunohistochemistry

The GC and GP groups of mice didn’t reveal any specific histopathological changes in brain as evident by their HP scores (2±0.1 and 0.66±0.06, respectively). The mice in GI, on 2nd DPI, showed mild non suppurative meningo-encephalitis. The cerebral and cerebellar neurons were pyknotic; the neuropil and white matter had glial reaction and engorged vessels. The neurons of hippocampus (both dentate gyrus and CA1 and CA2 layers) appeared normal, similar to that in controls (GC and GP). On 4th day, the inflammatory changes in meninges and the brain substance were more pronounced. The neurons of dentate gyri and CA2 regions were degenerated and neuropil had diffuse mild microgliosis. On day 6th, the neuronal degeneration was more prominent in CA1 and CA2 layers along with diffuse microgliosis and perivascular infiltration with mononuclear cell.

The reactive microglia cells with foamy cytoplasm (gitter cells) were further increased by 8th day. On 10th day, there was extensive degeneration of the neurons in cerebral hemisphere and in the CA1 and CA2 regions of the hippocampus admixed with many apoptotic bodies and gitter cells. The white matter in the thalamic/hypothalamic areas had similar reaction. The HP score in cerebral hemisphere showed increasing trend from 4th DPI (2.33±0.04) onwards and reached maximum (16.6±0.2) on 10th DPI and almost similar trend was observed in hippocampus with mean HPS of 17.33±0.33 on 10th DPI. On the contrary, the GPI group showed mild degree of non suppurative meningo-encephalitis in different anatomical sites on days 2, 4, 6 and 8. The neurons, however, did not show any specific changes. However, on 8th and 10th day, in addition, there was mild degeneration of neurons in CA1 and CA2 regions with little glosis. On 12th day, only mild

Figure 1: Hippocampus region (CA1, CA2 and dentate gyrus) in GI showing extensive loss of CA1 neurons and microgliosis compared with GPI. Subsequent on inoculation with 100LD50/mouse of CVS, samples were collected on days 6, 8 and 10 and stained with H&E. 10X and 40X
vascular infiltration in meninges, mild degeneration of neurons in CA2 layer and moderate degree of gliosis were observed when compared with GI. However, in both GI and GPI all mice died by 12th DPI. The changes were slightly more in hippocampus than in cerebral hemisphere. The overall HP score was significantly low on 4, 6, 8, 10 and 12th DPI in GPI as compared with GI (Figure 1). The brain sections subjected to immunostaining. Both GI and GPI groups showed reddish colour signals, mainly confined to neuronal cytoplasm of cerebral cortex as early as 4th DPI onwards. On 6th DPI, the signals were detectable in neurons of CA1, CA2 regions of the hippocampus and the thalamus (Figure 2). The controls showed no detectable signals.

In TUNNEL assay, the cerebrum in GI showed a few apoptotic cells on 12th DPI, while other groups did not show apoptosis in any part of the brain examined (Figure 3).

3.3 Confirmation of virus by direct FAT and PCR assay
The brain sections were subjected to direct fluorescent antibody test. Both GI and GPI showed apple green signals as early as on 4th DPI in cerebrum and the hippocampus. The signals were, however, more intense on subsequent time intervals, but without much difference between the groups. The brain sections from controls did not show any signal (Figure 4).

Rabies viral nucleoprotein gene (N gene) in part was detected in brain samples as early as on 2nd DPI in GI and GPI groups. The expected PCR amplicon of 533 bp was observed in 1% agarose gel as a confirmatory result (Figure 5).

The similar amplification was further confirmed in real time PCR by dissociation curve at the end of cyclic reaction.

3.4 TLR-3, its associated cytokines and Caspase 1 expression
A significantly enhanced of TLR-3 expression was observed on 2nd DPI, which persisted till 8th DPI in GPI followed by GI, GP and GC. The values in other groups remained low throughout the experimental period. The same trend was noticed in expression of IL-1α in these groups. However, IFN-α mRNA expression was found significantly increased on 2nd DPI in GI followed by GPI, GP and GC groups and remained high till 4 DPI. Later on, it was reversed, higher in GI and lower in GPI till the end of the experiment. The expression of TNF-α was higher at all time points in GPI than in GI and other controls. The expression of Caspase 1 mRNA in GI was marked on day 2nd and remained high till peaked at 12th DPI. Whereas, in GPI it remained depressed throughout the period. GP and GC group of mice showed low levels of caspase 1 than GI and GPI, except on day 6th, where GP showed higher expression (Figure 6).

Figure 2: Immunohistochemical demonstration of viral antigen in cerebrum and hippocampus on day 6 in Swiss albino adult mice
Figure 3: Cerebrum showing a few apoptotic cells in GI (arrow), while GPI and GC did not show any signals using TUNNEL, paraffin section, 40X

![GI (40x), GPI (40x), GPI (10x)]

Figure 4: Cerebrum showing positive fluorescent signals both in GI and GPI groups of mice at 6DPI. dFAT. 40X, Day 6

![Brain GI, GPI, Spleen Control, GC]

Toll like receptors (TLRs) are members of the interleukin -1 receptor (IL-1R) super family which play a crucial role in the innate host defence by recognizing pathogen-associated molecular patterns (PAMPs) (Takeda et al., 2003; Akaria et al., 2004). Amongst various toll like receptors, TLR-3 usually expressed on neurons, glia cells and macrophages,

recognizes viral glycol-proteins and dsRNA during the virus replication process. The stimulation of TLR-3 in turn causes release of pro-inflammatory cytokines, chemokines and other cellular mediators to help reduce the virus burden (Alexopoulov et al., 2001; Takeda and Akira, 2003; Prehaud et al., 2005). In the
Figure 5: Pattern of TLR-3 expression, expression of associated cytokines and caspase-1 in Swiss albino mice inoculated intracerebrally with 100 LD50/mouse of CVS. Relative levels of β-actin, TLR 3, associated cytokines, and Caspase 1 were assayed by Real Time PCR: (a) GPI showed increased expression of TLR 3 than GI and other controls throughout the investigation, (b) On 2nd, 4th DPI, GI showed increased expression of IFN-α but later GPI showed higher expression than GI. (c) On 2nd, 4th DPI, GI showed increased expression of IL-1α but later GPI showed higher expression than GI. (d) GPI showed increased expression of TNF-α than GI and other controls, (e) GI showed significant higher expression of Caspase 1 than GPI and other controls. The data are expressed as mean ± SD of 3 mice at each time point. (GP-Poly IC group, GPI - Poly IC + CVS infected group, GC - PBS control group)

(a)

(b)

(c)

(d)

(e)

Figure 6: Detection of nucleoprotein gene in brain by conventional PCR. Rabies viral nucleoprotein (N) gene was detected in brain samples as early as 2nd DPI in GI and GPI groups. The expected PCR amplicon of 533 bp was observed. Lane M: 100bp Marker, Lane L1 : GI, Lane L2 : GPI, Lane L3 : GC present study, we used poly IC as TLR-3 agonist with the hypothesis that it would additively cause up regulation of TLR-3 and its associated cytokines in CVS infected mice to delay the rabies virus pathogenesis.

During the investigation it was found that the clinical signs developed at relatively later stage in the group primed with poly IC as compared to CVS infected group (GI) alone. Similarly, the higher proportion of mice also survived on day 10 post infection in GPI than the GI. All the mice succumbed to infection in both the groups on 12th day of infection. The reason of late commencement of clinical signs and better survivability in GPI could be due to the effect of significantly (p<0.05) higher expression levels of type I IFNs, which play antiviral role, over others. It is appropriate to mention that the findings could have been clearer with a relatively lower dose of challenge virus (50LD50 in place of 100LD50). Further, mice in both GI and GPI showed emaciation (though not recorded) which corroborated well with high amount of TNF-α.
in these groups. Similar findings in mice inoculated with CVS by intra-cerebral route have been documented (Jackson and Park, 1998).

The microscopic brain lesions in the infected and poly IC treated groups (GI and GPI) were more or less similar in nature but differed in their severity/intensity and pattern of distribution as demonstrated by IHC and dFAT. The HP score revealed lesser pathology in GPI than in GI involving hippocampus particularly CA1 and CA2 regions followed by the cerebellar hemisphere and the thalamus. The findings were further supported by demonstration of fewer antigens in GPI than in GI by immunohistochemistry. Further as the disease progressed, the lesions were very intense and heightened on days 8th and 10th in GI compared with GPI. There was wide spread involvement of neurons in cerebral cortex, hippocampus and the cerebellum. The neurons in cornu ammonis layers were severely affected than the neurons of other locations in both GPI and GI, as evidenced by localization of antigen by IHC and dFAT. Many workers have demonstrated similar pathology in above anatomical sites in natural cases of rabies as well as in mice infected with CVS (Jackson and Park, 1998; Park et al., 1998; Jackson, 2003; Pamini et al., 2005). The lesser pathology in GPI over the GI could be ascribed to increase expression of TLR-3 owing to additive effect of Poly IC which caused more production of type I interferon. Kinetics of INFα and IL-10α in brain of rabies infected mice during experimental period shows that Interferon is an early defence mechanism against virus. Its level declined by day 8, where as the cytokines like IL-1 increased gradually reaching to peak on day 8. The previous studies have shown that during rabies viral replication in neurons, the G proteins and dsRNA, generated as intermediates, are being recognized through TLR-3 which in turn trigger the signalling pathway, via interferon (IFN) - regulatory factor 3 (IRF3) phosphorylation and nuclear factor (NF)-kβ activation to induce inflammatory cytokines (TNF-α, and IL-1α), chemokines (CCL-5 and CXCL-10), and also activation of the IFN-β promoter for IFN expression (Alexopoulov et al., 2001; Takeda and Akira, 2003; Prehaud et al., 2005). And the same mechanisms might hold true in the present study.

Many neurotropic viruses result into neuronal cell death by either apoptosis or by necrosis (Griffin and Hardwick, 1999; Allsopp and Fazakerley, 2000; Fazakerley and Allsopp, 2001). CVS strain causes apoptotic cell death in many neuronal (Theerasurakarn and Ubol, 1998; Morimoto et al., 1999) and non-neuronal cells (Jackson and Rossiter, 1997). Besides, experimental infection of rabies in adult, suckling, and neonatal mice with CVS was found to cause marked neuronal apoptosis in multiple brain regions (Jackson and Rossiter, 1997; Jackson and Park, 1998; Theerasurakarn and Ubol, 1998). In present study, we could demonstrate a few apoptotic cells in the cerebral hemisphere in GI only on 12th DPI, which is supported by increased expression of caspase 3 on same day but not in other groups. Caspase 1 promotes apoptosis by activating IL-1 which inturn stimulates caspase 3 through IL-6 up regulation. Enhanced expression of TNF-α also mediates apoptosis mainly through engagement with TNFR1, and promotes inflammation through the activation of macrophages and NK cells and the induction of other cytokines and adhesion molecules (Tracey and Cerami, 1994). The higher TNF-α and more number of NK cells and macrophages in GI support this view. However, the reasons for the absence of apoptosis in GPI could not be explained.

In the present study, we found significantly higher increase in TLR-3 mRNA expression on day 2 and onwards in GPI than in GI and the controls. This clearly indicates that Poly IC and CVS strain of rabies virus in GI have some additive effect to enhance expression of TLR-3 that might have triggered the intracellular signalling pathways to induce type-I IFN synthesis. Similarly other cytokines (IL-1α, TNF-α and IFN-α) registered higher levels of expression in GPI than in other groups including GI, except at some time points. These findings further support our view that TLR-3 and its associated cytokines have some role in reducing the brain pathology. Similar findings have also been reported by earlier workers in rabies infected brain using semi-quantitative RT-PCR, in rabies infected human post-mitotic neuron culture (Ware et al., 1996, Prehaud et al., 2005) and in CNS by injecting Poly IC into the brain (Park et al., 2006). These studies point out possible role of TLR-3 and its associated cytokines in the pathogenesis of rabies and the same holds true in experimental model as well.

TNF-α and other cytokines such as IL1α, and IFN-α mediate their protective activity through different mechanisms, such as
(i) Direct inhibition of virus replication in neurons.
(ii) Accelerated clearance of RV infection via the induction of brain inflammatory processes, which open the blood–brain barrier (BBB) to allow access of immune effectors such as RV-neutralizing antibodies and T cells to the infected neurons.
(iii) Enhanced microglial activation, which likely contributes to the immune defence (Lokensgard et al., 2001).

It is possible that the outcome of RV infection is predominantly controlled by both leukocyte infiltration and microglia activation. There is evidence that CNS inflammatory processes contribute to the clearance of virus from the CNS (Morimoto et al., 2001). TNF-α which links adaptive and innate immunity is produced by T cells and macrophage lineage including microglia cells (Bette et al., 2003). We could demonstrate rabies viral nucleoprotein (N gene) in brain samples as early as on 2nd DPI in both the infected groups (with and without poly IC) by both conventional and Real Time PCR. This was in contrary to IHC and dFAT, where we could be able to detect the viral antigen on 4th DPI. It is possible that N gene in case of virus like rabies is expressed at an early stage due to its location at 3’ end and the corresponding proteins are translated at later stage.

Conclusion

The study indicates that TLR-3 and its associated cytokines have role in rabies pathogenesis. The investigations with variable dose of TLR agonists and variable virus load may provide better information in this important area of rabies pathogenesis.

Acknowledgements

The authors are thankful to the Director, IVRI and Joint Director, CADRAD for providing necessary facilities for conducting research.

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