Ability of Indian street rabies virus isolates to induce apoptosis by in-vitro

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ABSTRACT
An attempt had been made in this study to know the ability of Indian street rabies virus isolates to induce apoptosis in murine neuroblastoma cells. Thirty Indian street virus isolates were tested by fluorescent antibody technique (FAT) among which 20 samples was positive. These positive samples were used to infect murine neuroblastoma (MNA) cells. Four street rabies virus isolates and challenge virus strain (CVS) were used in this study to show their ability to induce apoptosis. Total RNA was isolated and cDNA was synthesized by using gene specific primers pertaining to Caspase-1, BAD (Proapoptotic gene) and Glycoprotein gene of rabies virus. Challenge Virus Standard has been used as control in the amplification as well as in the demonstration of apoptosis. The expression of Caspase-1, BAD and glycoprotein genes in the murine neuroblastoma cells in comparision with CVS was measured by using densitometer. Street virus isolates expressed higher levels of Caspase-1 and BAD in comparison with CVS. Further TUNEL staining method showed higher apoptotic index when compared to CVS. This study is an initial attempt to exhibit the ability of Indian rabies street virus isolates to induce apoptosis in murine neuroblastoma cells (in vitro).

Key words: Apoptosis, CVS, FITC, Housekeeping gene, Murine neuroblastoma cell line, Rabies.

INTRODUCTION
Rabies is a highly neurotropic virus that produces fatal encephalomyelitis in human and an animal. In India, rabies occurs mainly in the urban form and dogs play an important role as reservoir (Palanivel et al., 2011). Each year more than 30,000 people are reported to die from rabies and the WHO estimates that 10-12 million receive post-exposure treatment. More than 90% of all human cases of rabies are believed to be associated with dog and much of the remainder with other domestic animals, particularly cats (WHO 1994).

The genome of the rabies virus is a non-segmented negative sense RNA that consist of five proteins namely the Nucleoprotein (N), Phosphoprotein (P), Matrixprotein (M), Glycoprotein (G) and the virion-associate transcriptase protein (L). The spike like particles are trimeric units of the glycoprotein that enables viral entry into host cells and also responsible for the induction of viral neutralizing antibody. Variation in the antibody binding site and any variation in the genes encoding for the protein may affect the pathogenic and immunogenic properties of the virus. Neuro invasiveness is the major defining characteristics of classical rabies virus infection. Attenuated rabies virus strains differ significantly from street rabies virus strains depends upon the G-associated pathogenic mechanism found in the attenuated rabies virus (Morimoto et al., 2000 and Schnell et al., 2005). Glycoprotein must interact effectively and optimally with cell surface molecule and RNA-N-P-M complex for efficient virus budding (Morimoto et al., 2000).

Pathogenicity of a particular rabies virus variant appears to correlate inversely with rabies virus G expression levels and that increased G accumulation correlated with induction of apoptosis (Galelli et al., 2000). Apoptosis is an active physiological process of cellular self destruction which regulates development and tissue homeostasis. The virus induced apoptosis may play a physiological role in protecting the central nervous system from progression of infection by allowing contact between virus and immune system components. Apoptosis play an important role in producing cell death in rabies virus infected culture cells and ability to induce apoptosis in primary neuronal cultures correlated inversely with its pathogenicity (Morimoto et al., 1999). Thus apoptosis is a part of the host defence response that normally play a protective role in rabies virus infection by restricting viral spread to brain.

Hence the present study was carried out with following objectives to determining the expression of glycoprotein in street rabies virus infected (MNA) cells and assessing the ability of street rabies virus to induce apoptosis.

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MATERIALS AND METHODS
Rabies virus isolates: Rabies suspected calf and dogs brain samples were collected in a sterile container during necropsy as per the routine standard laboratory procedure at department of Veterinary Pathology, Madras Veterinary College, Chennai-7. Impression smears from hippocampus and different regions of brain were taken on clean microscopic slides at the time of sample collection or later. The samples, which were positive by FAT, were stored at -80°C for infecting on to murine neuroblastoma cells.

Direct fluorescent antibody (FAT) test: Direct FAT was carried out as per the method described by (Dean and Abelseth 1973) using Fluorescein isothiocyanate (FITC) tagged rabied anti-nucleocapsid antibody conjugate.

Murine neuroblastoma cell culture: Murine neuroblastoma cell line (MNA) was procured from Diagnostic hybrid Inc, USA were used in this study to propagate the street rabies virus samples. The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). On confluence, the monolayer was detached with 0.25% trypsin and the cells were suspended in growth medium and subcultured with a split ratio of 1:3 as per the manufacturer guide lines. Direct FAT was performed on the cells with anti-nucleocapsid antibody FITC conjugate for the presence of specific fluorescent foci. The positive samples were used to infect MNA in 25 cm² flask and used for RNA extraction.

RNA isolation from infected neuroblastoma cells: The RNA was isolated from the infected neuroblastoma cells as per the method described by (Smith and King 1996).

cDNA synthesis: Complementary DNA was synthesized with the total RNA isolated from the infected MNA cells using first strand synthesis system (Superscript™III) for RT-PCR as per the manufacturer’s instructions. Approximately 1 µg of RNA was denatured with oligo dT (50µM) and Dnntp (10mM) at 65°C for 5 min and snap cooled on ice for 5 min. The cDNA thus synthesized was used to amplify the Glycoprotein gene or stored at -70°C for future use.

Polymerase chain reaction for amplification of the glycoprotein gene: The polymerase chain reaction for amplification of the Glycoprotein gene was performed with the set of primers as described by (Tirumurugaan 2001).Sense primer (Position 546 to 583): 5’-GGAATTGCTCAGGAG TAGCG-3’-Antisense primer (Position 1200 to1181):5’-TAA GAC ATT GCC GTC AGG TC-3

Single step reverse transcription polymerase chain reaction: The single step RT-PCR for amplification of the Caspase-1 gene was performed with the set of primers as described by (Wesselingh et al. 1994)

The following set of primers were used as
5’-TAT GGA CAA GGC ACG GGA CCT ATG-3’
5’-CCA GCA GCA ACT TCA TTT CTC TG-3’

Primers for the amplification of BAD and β-ACTIN: The amplicon of 494bp and 140bp in length was amplified as per the method described by (Hon Kit Wong et al. 1994 and Wesselingh et al. 1994) respectively.

Sense: 5’-GAG GAA GTC CGA TCC CCG GAA-3’
Antisense: 5’-CGG CGC TTT GTC GCA TCT GT-3’

Sense primers: 5’ TGC TGTCTTCCACCTAT CG-3’
Antisense primers: 5’ TTG GTG ACA ATA CCG TCA-3’

Quantity one-1d analysis software: The Gel documentation unit from Bio-rod Laboratories Inc, USA was used to take the images of the agarose gel electrophoresed PCR amplicons. The densitometric values for the PCR amplicons were obtained using quantity one 1D analysis software version.

Tunel assay: The APO-BRDUTM kit was obtained from Invitrogen, USA and used to detect apoptotic cells by flow cytometry as per the manufacturer recommendation.

RESULTS AND DISCUSSION

Out of 30 samples, 20 were found to be positive for rabies by direct FAT using rabies anti-nucleocapsid antibody conjugate (Plate 1). Positive samples revealed areas of brilliant green fluorescence and negative samples were identified by the absence of specific fluorescence.

The positive samples were used for infecting murine neuroblastoma cell line. Three passages were performed in the MNA cells and direct FAT was performed with the anti-nucleocapsid conjugate to detect the infectivity in the cells

Plate 1: Direct Fluorescent Antibody test for the detection of rabies in brain impression smears employing anti-nucleocapsid conjugate labelled FITC conjugate
(Plate 2). Out of the 20 samples four street virus from different species D556, Calf 13/5, D2/5/07 and D17/7/07 were selected for the study to determine their ability to induce apoptosis. CVS strain of fixed virus was used as control in this study and was also used to infect MNA cells. Fluorescent microscopic examination of infected cover slip cultures of the samples revealed multiple fluorescent foci, 48 hours post infection. RNA was extracted from the infected cells and cDNA synthesized to determine the expression of caspase-1, BAD and Glycoprotein (Plate 5 and 6).

Apoptosis or Programmed cell death is a process were individual cells undergo systemic cell destruction in response to wide variety of stimuli (Choi and Benveniste, 2004). The main characteristics of apoptosis are cellular shrinkage, membrane condensation, membrane blebbing and DNA fragmentation.

Apoptosis consists of two major phases namely commitment and execution which are activated by external or internal stimuli. The stimulation of proapoptotic gene results in depolarization of mitochondrial membrane potential or direct activation of caspase (Harada and Grant, 2003). The execution phase includes low caspase activation which stimulates downstream effectors such as caspase-6 and caspase-9 (Arnoult et al., 2002).

Sense RNA virus has been shown to induce apoptosis. Rabies virus induced apoptosis is shown to be dependent on the strain of virus, the route of inoculation and the type of host cell (Reid and Jackson, 2001; Theerasurakam and Ubol, 1998). In natural cases of rabies virus infection,
neuropathological or neurodegenerative changes are not prominent (Iwasaki and Tobita, 2002). Although apoptosis is induced by rabies virus in cellculture, its role in natural infection is conflicting. Hence, this study was undertaken to compare the expression of viral glycoprotein gene with the expression levels of two proapoptotic gene caspase-1 and BAD gene.

Four street virus isolates and CVS were used in this study and such an attempt to determine the apoptosis induced by street rabies virus isolates has not been studied before. Although real time PCR is the latest method for quantification of gene expression, several other methods have been used for semi-quantitative analysis of gene expression. One such method of densitometric evaluation of the gel band intensity has been used in this study. The difference in the input DNA was normalized in comparison to the level of gene expression of housekeeping gene (β-actin) (Plate 7 and 8). This method has been used by several authors for quantification of gene expression. A time course study of the proapoptotic and viral protein gene expression would have been ideal. However, the expression levels of glycoprotein and the proapoptotic genes were assessed at a single time point only (48hrs post infection) as an early report by (Ubol et al. 2005) showed increased levels of Bad and caspase-1 from 48 hours post infection (Plate 3 and 4).

The Caspase-1 expression was not evident in the rabies virus uninfected cells, as expected. However, the rabies virus infected cells showed varying levels of Caspase-1 gene expression level from approximately 8 to 50 the relative absorbance units. The lowest level of expression was seen in CVS strain while, the highest levels were with the isolates from dog (D2/5/07). Earlier study with fixed virus strains (Zhang et al., 2003) have shown up regulation of Caspase-1 gene expression from day 2 to 6 post infection. They observed 70% increase in Caspase-1 gene expression at day 2 with peak expression at day 3 followed by reduction on day 4. However this expression has been demonstrated in rabies virus.

Similarly study was carried out with another proapoptotic gene BAD. BAD expression was found to be high in three of the four street rabies virus tested and the lowest level expression was seen with CVS. The isolates D2/5/07 showed highest level of BAD and Caspase-1 expression.

The Glycoprotein of rabies virus has been known to be involved in the receptor mediator uptake of the virus and
also plays a major role in the induction of neutralizing antibody. The pathogenicity of the different rabies isolates has also shown to the largely dependent on the Glycoprotein (Morimoto et al., 1999). The expression levels of glycoprotein have been shown to be negatively correlated with apoptosis. The present study was also an attempt to correlate the expression of glycoprotein with that of two proapoptotic genes. The CVS virus that had the lowest level of proapoptotic gene expression exhibited the highest viral glycoprotein gene expression. To further strengthen this claim the street rabies virus D2/5/07 had the highest expression of Caspase-1, BAD, but a lower expression of glycoprotein gene. The exact mechanism of this negative correlation is not clear; however it can be proposed that the viral glycoprotein gene expression inhibits the proapoptotic genes and the subsequent molecules downstream. This could probably be an evasion (Alcami and Koszinowski, 2000) mechanism by the virus so that the virus infected cells are not killed. This strategy may also enable the virus to persist in the neurons of the host. All the street rabies virus isolates in general had lower levels of glycoprotein gene expression with concomitant increased proapoptotic gene expression. This probably would have reflected in the varying degrees of apoptosis inhibition in the host. This could also be one of the reasons for the huge variation in the incubation period in the case of street rabies infection.

The CVS strain resulted due to several passages in the laboratory host leading to higher glycoprotein expression and lower level of apoptosis. This could be the reason as to why the CVS strain of rabies virus is able to produce consistent symptoms and defined incubation period in the laboratory host.

One of the other features of apoptosis is DNA cleavage that was detected by Tunel assay (Theerasurakarn and Ubol, 1998). In this assay BrDU has been used to label the cleaved DNA and the incorporation of BrDU antibody coupled with FITC. Although difference in Caspase-1, BAD gene expression was seen between CVS and street virus isolates this was not reflected in the apoptotic cell (Plate 9) population. CVS and street rabies virus infection in MNA cells induced similar proportion of apoptotic cells. It was not possible to quantify the cells expressing the viral glycoprotein using flow cytometry due to non availability of glycoprotein specific monoclonal antibody. Hence apparently it appears that the increased or decreased expression level of proapoptotic gene was not reflected in the actual apoptotic cell population. The reason for this is not clear. Further studies are needed to determine the expression levels of other apoptotic genes and established correlation between gene and protein expression using Real Time PCR and Flow Cytometry respectively. However, the result of the study shows that the viral gene expression and apoptotic gene expression are related.

From this study, it concluded that Caspase-1 gene expression in rabies virus infected MNA cells was lowest in CVS strain and varied among the street virus isolates. BAD gene expression in rabies virus infected MNA cells was lowest in CVS strain and varied among the street virus isolates. Rabies virus glycoprotein gene expression was highest with CVS strain and the lower in street rabies virus isolates. There exists a negative correlation between glycoprotein gene expression and apoptotic gene expression.

REFERENCES


