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# In situ apoptosis of adaptive immune cells and the cellular escape of rabies virus in CNS from patients with human rabies transmitted by *Desmodus rotundus*

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ABSTRACT

The aim of the current study was to investigate the apoptosis of neurons, astrocytes and immune cells from human patients that were infected with rabies virus by vampire bats bite. Apoptotic neurons were identified by their morphology and immune cells were identified using double immunostaining. There were very few apoptotic neurons present in infected tissue samples, but there was an increase of apoptotic infiltrating CD4+ and TCD8+ adaptive immune cells in the rabies infected tissue. No apoptosis was present in NK, macrophage and astrocytes. The dissemination of the human rabies virus within an infected host may be mediated by viral escape of the virus from an infected cell and may involve an anti-apoptotic mechanism, which does not kill the neuron or pro-apoptosis of TCD4+ and TCD8+ lymphocytes and which allows for increased proliferation of the virus within the CNS by attenuation of the adaptive immune response.



## 1. Introduction

The biological process of apoptosis involves maintaining cellular homeostasis and preventing pathogenesis of many diseases. Activation of caspases, which are in the family of cysteine proteases, is involved in the initiation of apoptosis within cells (Schmitz et al., 2000). Several studies have shown that apoptosis prevents the dissemination of viruses within the body and is an important host defense mechanism against infectious viruses (Dietzschold et al., 2001; Yan et al., 2002; Warrell and Warrell, 2004).

Neurotropic viruses cause cell death by either apoptosis or necrosis. Cellular apoptosis is an energy dependent process that requires the synthesis of macromolecules, and necrosis of the cell is primarily caused by the lack of sufficient energy for the cell to function. Each of these forms of cell death is associated with several distinct morphological features. Cell apoptosis involves a specific sequence of morphological changes within the cell that includes condensation of the cytoplasm and nuclear chromatin. Then the cell ruptures into membrane-bound apoptotic bodies, which contain various cytoplasmic organelles and nuclear fragments that are then removed by healthy neighboring cells and macrophages (Griffin and Hardwick, 1999).

Cell apoptosis occurs through either an intrinsic reaction in infected or damaged cells or by the induction of apoptosis by cytotoxic T cells, which function by secreting cytokines, such as tumor necrosis factor-alpha (TNF-α), releasing perforin and granzymes, or by activating the Fas ligand-dependent apoptotic pathway in apoptosis target cells (Baloul and Lafon, 2003). The control of apoptosis is achieved by a family of genes related to the *Bcl-2* proto-oncogene (Pradelli et al., 2010).

The induction of apoptosis by the rabies virus is a controversial topic within the literature. Some studies have shown that the rabies virus induces cellular apoptosis in infected brain cells of preclinical animal models (Jackson and Rossiter, 1997; Theerasurakarn and Ubol, 1998). However, a study by Morimoto et al. (1999) demonstrated that highly virulent strains of rabies produced less cellular apoptosis than strains that were less virulent. Adle-Biassette et al. (1996) showed that apoptosis of neurons of the hippocampus and brain stem occurred in patients who were infected with rabies and Human Immunodeficiency Virus (HIV), which suggests that apoptotic death of neuronal cells contributes to the pathogenesis of rabies.

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Other experimental studies have shown that apoptosis is the major cause of neuronal death in rabies which is induced by a T cell dependent immune response (Jackson and Rossiter, 1997; Fu and Jackson, 2005; Ubol et al., 2005; Juntrakul et al., 2005). However, Jackson et al. (2008) showed that in humans the neuronal apoptosis does not play a significant role in rabies infections and is not involved in limiting the spread of the virus to uninfected cells or the overall pathogenesis of rabies.

Following a viral infection, the programmed cell death of infected cells by apoptosis is a cellular response that primarily functions to limit viral propagation (Alcami and Koszinowski, 2000). However, studies utilizing new strategies that reduce neuron apoptosis and attenuate the rabies associated immune response of cytotoxic T lymphocyte (CTL) and NK cells have shown there is an increase in viral dissemination (Baloul and Lafon, 2003). Experimental studies have shown that the induction of apoptosis of cells involved in an immune response that occurs during a rabies infection is primarily caused by the rabies virus and reduces the host's ability to fight the rabies infection (Camelo et al., 2000; Lafon, 2005).

Other studies have shown that besides neurons, astrocytes and microglial cells are able to sustain viral replication, which contributes to viral dissemination and persistence of the rabies viral infection within a host (Matsumoto, 1963; Smith et al., 1991). Additionally, it is has been shown that glial cells, which release cytokines and neurotoxins, directly affect rabies pathogenesis by altering the function of neurons (Ray et al., 1997). In the present study, we studied apoptosis of neurons, astrocytes, and several immune cells in the CNS tissue of patients who were infected with rabies transmitted by vampire bats to determine what role cellular apoptosis has during a rabies infection.

## 2. Materials and methods

### 2.1. Cases

Eight patients that had been fatally infected with rabies virus by vampire bats in the state of Pará, Brazil in 2004 and 2005 were used for this study. Diagnosis of these rabies cases was confirmed by correlation of clinical history with histology, direct immunofluorescence reaction, virus isolation in suckling mice, RT-PCR and antigenic characterization showed variant 3 (AgV3), the primary reservoir of which is the vampire bat *Desmodus rotundus* (GenBank DQ097075.1, DQ097076.1, DQ097077.1, DQ097078.1, DQ097079.1, DQ097080). These confirmatory diagnoses were carried out by another group of researchers at the Evandro Chagas Institute, Pará, Brazil and/or at the Pasteur Institute, São Paulo, Brazil (da Rosa et al., 2006).

Of the eight cases of rabies were elected six fragments of brain regions: frontal cortex, parietal cortex, hippocampus, basal ganglia, cerebellum and oblongata medulla that were harvested and then set in paraffin. The control group ($n = 8$) was selected by the cause of death and included individuals who died from any disease that did not involve the CNS and had no report of an autoimmune disease. Control CNS tissue samples were harvested from the same regions as from the rabies infected patients and were used as internal controls for tissue preparation and normal cell phenotype. So, for this study we performed immunohistochemistry for specific antibodies in 48 brain fragments of rabies cases and in 48 brain fragments of control group.

This study (protocol 057/05) was approved by Research Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Brazil.

### 2.2. Immunohistochemistry

Brain tissue samples were removed from the paraffin blocks and fixed in saline-treated slides. The wax was removed by treatment of the slide with xylene, and then re-hydrated by treating the tissue slide with a series of increasing ethanol gradient washes. Endogenous peroxidase were blocked by treating the tissue samples with 3% hydrogen peroxide and residual antigens were removed by incubating the tissue slide in 50 mM Tris buffer, pH 9.0, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.05% Tween-20® (ICI Americas, Inc., Bridgewater, NJ, USA) for 25 min at 95 °C. Immunohistochemical staining was completed by incubating the specific primary antibodies that were diluted in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) overnight at 4 °C. Table 1 shows all primary antibodies used for immunohistochemistry, with their trademark, their dilutions and detection systems. The tissue samples were then treated with the specific secondary-antibody and then detected using streptavidin–biotin–horseradish peroxidase following the manufacturer's instructions (LSAB+ system-HRP, code K0690, DakoCytomation, Carpinteria, CA, USA). This method was used to detect TCD4 and TCD8 lymphocytes (anti-TCD4 and anti-TCD8 antibodies), NK cells (anti-CD57 antibody), macrophages (anti-CD68 antibody), astrocytes (anti-GFAP antibody) and apoptosis in neurons (anti-caspase 3 antibody). The presence of apoptosis in neurons was confirmed by treating the tissue slides with anti-caspase 3 antibody and then inspecting the cell morphology.

Each reaction was visualized by treating the stained tissue sample with 3,3′-diaminobenzidine-tetrahydrochloride (DAB) (code D-5637, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) under light microscopy.

To determine if cells other than neurons were in the state of apoptosis, after applying the DAB and rinsing in running water, distilled water and PBS was applied to samples anti-caspase 3 antibody overnight at 4 °C. After washing with PBS, bound antibody was revealed with Envision alkaline phosphatase (AP) (code K-4018, DakoCytomation, Carpinteria, CA, USA). All slides were counterstained with Mayer's hematoxylin (Lillie's modification) and then mounted for light microscope.

The expected result would be double labeling of cells undergoing apoptosis with staining brown and red. Cells that only have a

**Table 1**
Primary antibodies used in immunohistochemistry.

| Antibody | Mark/code | Exhibition antigenic | Dilution | Kit used |
|---|---|---|---|---|
| Polyclonal mouse anti-rabies | Evandro Chagas Institute | Damp heat | 1:150 | LSAB-HRP[a] |
| Monoclonal mouse anti-human CD4, T cell | Dako/M834 | Damp heat | 1:1000 | CSA II[b] |
| Monoclonal mouse anti-human CD8, T cell | Dako/M7103 | Damp heat | 1:30 | CSA II[b] |
| Monoclonal mouse anti-human NK cell, CD57 | Immunotech/1166 | Damp heat | 1:100 | LSAB-HRP[a] |
| Monoclonal mouse anti-human CD68 | Dako/M0876 | Damp heat | 1:30 | LSAB-HRP[a] |
| Monoclonal mouse anti-human glial fibrillary acidic protein (GFAP) | Dako/M0761 | Damp heat | 1:200 | LSAB-HRP[a] |
| Monoclonal mouse anti-human caspase 3 | Cell signaling/9661S | Damp heat | 1:100 | Envision-AP[c] |

[a] Immunohistochemistry kit for peroxidase.
[b] Immunohistochemistry kit for peroxidase signal amplification.
[c] Immunohistochemistry kit for phosphatase.