DETECÇÃO DE S100-β E REAÇÃO DE ASTRÓCITOS DURANTE ENCEFALITE EXPERIMENTAL PELO VíRUS DA ESTOMATITE VESICULAR.

(S100-β DETECTION AND ASTROCYTE REACTION DURING VESICULAR STOMATITIS VIRUS EXPERIMENTAL ENCEPHALITIS)

(DETECCIÓN DE S100-β Y REACCIÓN DE ASTROCITOS DURANTE ENCEFALITIS EXPERIMENTAL POR EL VIRUS DE LA ESTOMATITIS VESICULAR)

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RESUMO

Camundongos foram sacrificados nos dias 2 (Grupo1), 6 (Grupo 2) e 20 (Grupo 3) após a inoculação do vírus (pi) da estomatite vesicular e a proteína glial fibrilar ácida (GFAP), vimentina e S100β foram detectadas no encéfalo através de imunoistoquímica. Os sintomas e lesões mais severos de encefalite foram observados no dia 6. Houve aumento da marcação para GFAP em astrócitos caracterizando astrogliose. A detecção de GFAP e vimentina foram bastante evidentes no dia 6 pi, quando a marcação para vimentina foi mais intensa próxima às áreas de necrose. A proteína S100β foi intensamente detectada no dia 6 pi em neurônios e na micróglia. Observou-se redução da marcação da GFAP em áreas de necrose. Durante a encefalite experimental pelo vírus da estomatite vesicular e, especialmente em camundongos com sintomas sacrificados no dia 6 pi, além de intensa marcação para GFAP e vimentina, detectamos marcação para a proteína S100β em neurônios e na micróglia, mas não em astrócitos onde a S100β é considerada um marcador específico.

PALAVRAS-CHAVE: S100β. GFAP. Vimentina. Astrócitos. Vírus da Estomatite Vesicular.

SUMMARY

Mice were sacrificed at 2 (Group 1), 6 (Group 2) and 20 (Group 3) days post-virus inoculation (pi) and glial fibrillary acidic protein (GFAP), vimentin and S100β staining were studied by immunohistochemistry. Symptoms and severe lesions were observed at day six. There was an increase of GFAP staining in astrocytes characterizing astrogliosis. Detection of GFAP and vimentin was evident after six days post-inoculation and vimentin staining was more intense around injured areas. The S100-β protein was strongly detected at day 6-pi in neurons and microglia. Reduction in GFAP was observed in areas of encephalic necrosis. During VSV encephalitis in the mouse model and especially in those mice with symptoms at day six post-inoculation we showed that besides astrocytes response to VSV infection characterized by upregulation of GFAP and vimentin, we also detected production of the S100β in neurons and microglia, but not in astrocytes, where

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S100β is considered a specific marker.
KEYWORDS: Astrocytes. vesicular stomatitis vírus. GFAP. Vimentin. S100-β.

RESUMEN

Los ratones fueron sacrificados en los días 2 (Grupo 1), 6 (Grupo 2) y 20 (Grupo 3) después de la inoculación del virus (pi) de la estomatitis vesicular. La proteína glial fibrillar ácida (GFAP), la vimentina y la S100b fueron estudiadas por inmunoistoquímica. Los síntomas y lesiones más graves fueron observados en el día 6. Los resultados demostraron aumento inicial de GFAP en astrocitos, seguido por la reducción en aquellos con alteraciones morfológicas degenerativas en algunas áreas del encéfalo que correspondían a necrosis. Hubo elevación de la marcación de GFAP caracterizando astrogliosis. La detección de GFAP y vimentina fue evidente en el día 6 pi, cuando la marcación para vimentina fue más intensa al rededor de las áreas de necrosis. Se observó reducción en la marcación de la GFAP en las áreas de necrosis. La S100b fue intensamente detectada en el día 6 en las neuronas y en la microglia. Durante la infección por el virus de la estomatitis vesicular en ratones con síntomas y sacrificados en el día 6 pi, además de intensa marcación para GFAP y vimentina en astrocitos, detectamos la proteína S100b en neuronas y en la microglia, pero no en astrocitos donde la S100b es considerada un marcador específico.

PALABRAS-CLAVE: S-100-β. GFAP. Vimentina. Astrocitos. Virus de la Estomatitis Vesicular.

INTRODUCTION

Astrocytes are the most numerous cells within the central nervous system. Early during development they act as guiding structures for neurons; later they become the main source for nutrients and growth factors in the brain, and they are also communication partners of neighboring neurons (KIRCHHOFF et al. 2001, FIELDS e STEVENS-GRAHAM, 2002, PEKNY e NILSSON, 2005). These cells are suspect of being involved in a wide range of CNS diseases, including trauma, ischemia, and neurodegeneration (MUKE e EDDLESTON, 1993). A well-known feature of reactive astrocytes is increased production of intermediary filaments (IFs) and the increased positivity of glial fibrillary acidic protein (GFAP), vimentin and also nestin, two intermediate proteins that are only abundantly expressed in immature astrocytes (ENG et al., 2000).

According to the literature, the morphological changes in glial cells (hypertrophy/hyperplasia) reflect the increase in their functional capacity (PEKNY e NILSSON, 2005). Although the characterization of astrocytic response to brain injuries, neurodegenerative disease and in cell transplantation are common subject of research, we still know little about how astrocytes influence the regrowth or reconstruction of neural network after the developmental period. The overall impact of astrocytic presence on the growth of neurons in the adult CNS needs to be established (GATES e DUNNETT, 2001).

One of the useful astrocyte markers is the intracellular glicoprotein S100-β that belongs to the calmodulin family of calcium-binding proteins. S100-β is produced by astrocytes and acts as a trophic factor for serotonin neurons (WHITAKER-AZMITIA et al., 1990, VAN ELDIK e WAINWRIGTH, 2003) and also regulates the assembly of intermediate filaments by inhibiting GFAP polymerization in the presence of Ca^{+2} (BIANCHI et al., 1994, KLIGMAN e MARSHAK, 1985, MRAK et al., 1995). It has been suggested that S100-β has important autocrine and paracrine action and is considered an intercellular signaling. S100-β may be implicated in the pathophysiology of brain disorders, through increased release by astrocytes consequent to their activation and/or death (ADAMI et al., 2001, ROTHERMUNDT et al., 2003, DONATO et al., 2003, MARENHOLTZ, et al., 2004). S100B stimulates iNOS in rat primary cortical astrocytes through a signal transduction pathway that involves activation of the transcription factor NFκB. The NFκB family of proteins is one of the best characterized transcription factors and regulates expression of many genes involved in peripheral immune and inflammatory responses. In recent years, an increasing body of evidence also supports an inflammatory role for NFκB in the brain (LAM et al, 2001).

Vesicular stomatitis virus (VSV) is a single stranded, negative sense RNA virus, encased in a bullet-shape virion made from only five proteins. Mice experimental infection by the intranasal route results in dissemination by retrograde transport in neurons and ventricular surfaces (HUNNEYCUTT et al., 1994, MACHADO et al., 2003). Disease is dependent on dose, route, and virus and has been used as a model for in vivo and in vitro studies of acute encephalitis (HUNEYCUTT et al., 1994, CHEN et al., 2001, CHEN et al, 2002, CHESLER et al., 2004).

Some studies show rapid response of astrocytes and microglia during acute VSV infection (BI et al.,

1995, VASCONCELOS et al., 2004). The relevance of astrogliosis remains controversial, especially with respect to the beneficial or detrimental influence of reactive astrocytes on CNS recovery from inflammation studies (SILVER e MILLER, 2004). Many studies were able to demonstrate a correlation of traumatic brain injury, hypoxic and ischemic brain damage and upregulation of S100β and few data exists for inflammatory and infectious diseases (ROTHERMUNDT et al., 2003). More knowledge about the correlation of astrocyte activation and S100β expression could help to understand the physiopathology of acute encephalitis and recovery stage using the VSV model. Reports on the VSV infection pathogenesis have been pointed out in acute phase and there are many gaps involving, for instance, aspects about recovery of infected animals and the participation of glial cells in subacute and in late stages.

The aim of the present study was to investigate the immunoreactivity pattern of astrocytic markers, GFAP and vimentin, correlating and comparing their levels with S-100β localization during acute and in late phase following intranasal inoculation of VSV in mice.

MATERIAL AND METHODS

Virus

The sample of vesicular stomatitis virus, strain Indiana type II was maintained at -80ºC in BHK. Mouse brain suspension used for inoculation was obtained from intracerebral inoculation of mice as previous described by Machado et al. (2003). The VSV titration determined as infecting dose per tissue culture ($TCID_{50}$/0.1 ml), was 10^5 virus/0.1ml.

Experimental infection of mice

Forty male C57BI6 mice, 5 to 7 weeks old (CEMIB-UNICAMP-SP-Brazil), were used for viral inoculation. Mice were mildly anesthetized in a closed container with ether followed by intranasal inoculation with VSV suspension (10^5 virus/0.1ml) in a total volume of 0.03ml administered equally between each nostril according to HUNEYCUTT et al. (1993). Four mice got sterile PBS into the nostril and were used as control. Animals were housed with food and water *ad libitum* and were randomly sacrificed for histological and immunohistochemical analysis (6-12 mice/sacrifice point, Table1). To minimize suffering to the animals they were sacrificed at start of the paralysis symptoms.

Tree groups of inoculated mice were formed: mice sacrificed on day 2 (Group 1), 6 (Group 2) and 20 (Group 3) post inoculation. Non-inoculated mice composed group 4. All the protocols were approved and experiments were carried out in agreement with UNESP-Animal Experimentation Ethics Committee. The animals were housed with food and water *at libitum*.

Histological and Immunohistochemical analysis

Mice were anesthetized with ether and sacrificed with intraperitoneal lethal dose of 5mg of pentobarbital sodium (Nembutal® - Abbott Laboratories) in normal saline solution. Each mouse was perfused transcardially with 30-40ml of phosphate-buffered saline (PBS pH 7.4). After perfusion whole brains were removed and fixed during 8 hours in freshly prepared 4% buffered paraformaldehyde (pH 7.4) and embedded in paraffin according to standard procedures. Five-micrometer brain sagittal sections (1-1.5 mm lateral from bregma) were stained with hematoxylin and eosin in order to detect inflammatory, degenerative or reactive features of neurons. For immunohistochemistry, first endogenous peroxidase activity was blocked by incubating sections in 1% H_2O_2. Sections were treated with Tripsin 1% at 37ºC before blocking of nonspecific binding with powdered skimmed milk (3% in phosphate-buffered saline) for 30 minutes. Sections were then incubated overnight at 4ºC with the anti-GFAP (SIGMA G9269, 1:300), anti-vimentin (Novocastra NCL-VIM-V9, 1:200) and anti-S100β (Novocastra RTU-S100p). Biotinylated antibodies anti-rabbit or anti-mouse (Dako, 1:100) were applied as secondary antibodies and an avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories, USA) served as the third reagent. DAB (3.3-diaminobenzidine-tetrahydrocloride)-H_2O_2 served as chromogen. Sections were counterstained with Mayer hematoxylin. Suppression of primary antibodies incubation was used as control. Because most severe lesions are observed in the olfactory bulb we choose this areas for study, together with frontal cortex, subependimal plate and ependymal layer cells, diencephalon nucleus and neurons at brain stem (HUNEYCUTT et al., 1994, MACHADO et al., 2003). Two independent observers scored the immunohistochemical evaluation. The extent of immunoreactivity for each of antibody used was determined using an arbitrary scale of + to ++++ reflecting the extent and the intensity of immunopositivity.

RESULTS

At 2 days post inoculation mild meningitis can be observed in the olfactory bulb area. Symptoms of encephalitis are visible on the 5th day post inoculation. Typical clinical signs observed on 6th day included ruffled fur, conjunctivitis, less activity and decreased consciousness and progressive posterior paralysis. Encephalic lesions at this time included: leptomeningitis, which intensity varied from mild to intense, mostly observed at olfactory bulb and extended to encephalic ventral surface. Nearly all affected mice showed neuronal death in the olfactory bulb and focal malacea. Ventriculitis at laterals, third and fourth ventricles affecting subependymal layer also was

remarkable feature. Perivascular infiltrates and neuronal necrosis were also observed in thalamus, hypothalamus and brain stem. Inflammatory infiltrate was composed of neutrophils and lymphocytes. Mice without symptoms sacrificed on 6th day pi showed mild inflammation restricted to olfactory bulb area. Animals that survive VSV infection do not show any symptom or morphological alteration on 20 days pi.

Fibrous and protoplasmatic astrocytes positive for GFAP were easily identified in animals of VSV inoculated (groups 1, 2, 3) or control (group 4). On the 2nd day p.i., reactive astrocytes were observed in group 1 with moderate staining particularly in white matter areas. The astrocytes were characterized mainly for having large and clear nucleus, clumped chromatin located peripherally, and large nucleoli. The cytoplasm was evident and the thick processes were intensively stained. Cells with those characteristics were observed mainly in the olfactory bulb (Figure 1 A), externally-limiting glia and in the glomerular and mitral cell layers. Six days after VSV inoculation (group 2) we noticed diffuse, strong to intense GFAP immunoreactivity (Table 1), with exception of reduction in the GFAP intensity in some areas of the encephalon that correspond to necrotic regions in mice sacrificed with neurological symptoms. GFAP-positive cells, with large swollen nuclei, evident nucleoli, and processes with a fragmented aspect were observed in areas adjacent to necrotic areas, followed by transition areas where astrocytes showed less marked degenerative morphological aspects, i.e., the processes were short, thick and showed only an initial fragmentation or dissolution (Figure 1B). In mice sacrificed on day 6 without symptoms, the tissue injury was less intense and mainly limited to the olfactory bulb, where GFAP-positive astrocytes with activated morphology were observed. After 20 days (group 3), astrocytes showed typical morphologic pattern and GFAP staining was the same observed in the control group (group 4).

Vimentin expression in astrocytes did not appear in animals of control group except at Bergmann glia and in spare cells in cortex. On day 2 post viral inoculation, no difference in cell staining was observed. Six days post inoculation, strong vimentin immunostaining was detected in round cells with macrophagic appearance in the olfactory bulb (Figure 1C) and in the cytoplasm and thick processes of astrocytes throughout cortex and subpial glia of frontal cortex (Figure 1D). Only few astrocytic cells in the hippocampus, thalamus and brain stem were vimentin positive and Bergmann glia showed a reduction in vimentin stain intensity. Inflammatory cells in the leptomeninges and cerebral ventricles were also vimentin positive. Twenty days after inoculation vimentin, labeling returned to the pattern observed in non-inoculated mice (group 4) and in mice from group 1 (2 days post inoculation).

S100β immunohistochemical staining was faint, diffuse and localized in the neuropil in groups 1, 3 and 4 (control). In these groups just a few cortical neurons showed S100-β cytoplasmatic positivity. S100β protein staining was evidently detected only six days after inoculation (Group 2) and especially in the brain of mice that showed neurological symptoms. The stain was observed in the cytoplasm of neurons in the olfactory bulb (Figure 1E), in microglial cells (Figure 1F), in subependymal plate and ependymal layer cells, in neurons in the frontal cortex, diencephalon nucleus and brain stem. In these brains, the intensity was scored as strong staining and the pattern of distribution and intensity is showed in Table 1. Mice without symptoms on day 6 showed diffuse pattern of positivity in neuropil of the olfactory bulb and periventricular areas.

DISCUSSION

Previous studies showed that two days after inoculation VSV antigen was detected in the olfactory bulb of mice, and on day six post inoculation, the viral antigen was disseminated in areas such as periventricular ependyma, hippocampus, thalamus, hypothalamus, mesencephalon nuclei, and brain stem nuclei (HUNEYCUTT et al. 1994, MACHADO et al., 2003, MACHADO et al., 2006). These areas showed correspondence with those where we observed, on six days post inoculation, neuronal degeneration and more intense astrocytic reaction characterized by intense GFAP reactivity. The semi-quantitative results of GFAP, vimentin and S100β detection are showed on Table 1. Astrocytic response is commonly seen where tissue damage has occurred (PEKNY e PEKNA, 2004, PANICKAR e NOREMBERG, 2005). We noticed decrease in GFAP detection in astrocytes together with fragmentation on their processes in surrounding areas of necrotic tissues. At the same areas, vimentin staining was stronger and most evident than GFAP. In areas where initially viral replication takes place (olfactory bulb) we detected positive cell, to both GFAP and vimentin. Vimentin is a mesenchimal intermediate filament that characterizes immature astrocytes (BIGNAMI et al., 1982). After birth, vimentin is progressively replaced by GFAP as a key step in the differentiation of astrocytes (BOVOLENTA et al., 1984). The co-expression of vimentin and GFAP is, however, retained is some astroglial population in normal adulthood (LAZARIDES, 1982) and is upregulated in response to injury in the CNS. This explains why the less positive vimentin stain was observed in animals after 2 and 20 days of VSV inoculation (groups 1 and 3) and also in normal mice (group 4) and the strong detection observed during the acute inflammatory phase.

In addition to astrocytes, round cells with macrophage morphology were vimentin positive. YAMADA et al. (1992) described similar microglial behavior in pathological human brain. Round microglia stained with OX-42

antibody was described by VASCONCELOS et al. (2004), in necrotic areas during VSV encephalitis, confirming the presence of those cells in VSV induced lesion.

Because astrocytes function as a syncytium of interconnected cells both in health and in disease, rather than as individual cells, any disturb at a specific region could reflect in the whole astrocyte population (PEKNY e NILSSON, 2005). Both fragmentation and reducing of GFAP observed in astrocytes during VSV encephalon infection may also represent a signal for structural deficit of mature astrocytes, including blood brain barrier breakdown, as observed by BI et al. (1995). These morphological changes also represent disruption of astrocyte modulating functions and probably contribute to the worsening of the lesions caused by inflammation in agreement with MUKE e EDDLESTON (1993) observations.

The S100β protein was strongly stained in the brains of mice mostly during the acute phase of VSV encephalitis. Although it has been described as an exclusive marker for astrocytes, S100-β has also been localized in neurons and other cells of different areas in the brain of some mammals including humans (RICKMANN e WOLFF, 1995, STEINER et al., 2007). In infected symptomatic mice, we observed neuronal cells with positive stain against S100-β in areas that match with those where viral dissemination that was previous described (HUNEYCUTT et al., 1994, MACHADO et al., 2003, MACHADO et al., 2006) and where astrocytes are activated, contiguous to necrotic areas. As reported in advance, depending on the disease context and on the concentration, S100-β can be viewed as beneficial to promote neuronal survival or it can be seen as detrimental for neuronal function. Serum protein S100-β determination is considered a sensitive marker of brain injury (SAVOLA et al., 2004), and reflects the degree of blood-brain barrier opening (KLEINDIENST et al., 2004). A beneficial effect of S100-β intraventricular infusion was observed on recovery of rats with brain traumatic injury (KLEINDIENST et al., 2004) and in a biaxial strain model *in vitro* injury (ELLIS et al., 2007). At the low levels normally found in the brain extracelullar space, S100-β acts as a neurotrophic factor protecting neurons against noxious stimuli and stimulating neurite outgrowth (UEDA et al., 1995, BARGUER et al., 1995, IWASAKI et al., 1997). We detected faint extracellular staining in encephalons of mice in groups 1, 3 and 4 suggesting a constitutive basal production in mice brain. Low concentrations of extracelullar S100-β induce astrocytic proliferation *in vitro* (SELIENFREUND et al., 1991). However, elevated concentrations (i.e. micromolecular) of S100-β induce the expression of iNOS in astrocytes (HU et al., 1996), that can directly induce neuronal death via apoptosis (KRONKE et al., 1997). As the most severe neuronal lesions are detected in the olfactory bulb of symptomatic mice at day 6p.i., we believe that the strong neuronal detection of S100β at this day might be associated with induced neuronal death.

Although generally assumed as an astrocyte marker S100β was not detected by immunohistochemistry during acute phase of VSV encephalitis. The fail in detect S100β staining in astrocytes could be related with the cell compromise of production of another cytokine as TGFβ1 (MACHADO et al., 2006) or as have been suggested by OHTAKI et al (2007) the viral infection may impair astrocyte function via a downregulation of S100β and interference with inflammatory response.

In conclusion, during VSV infection in the mouse model and especially in those mice with symptoms at day six post inoculation we showed that besides astrocytes response to VSV infection characterized by upregulation of GFAP and vimentin, there is also S100β production detected in neurons and microglia, but not in astrocytes, where S100β is considered a specific marker.

Table 1 - Summary of general immunohistochemical staining patterns of GFAP, vimentin and S100-β in brain of mice inoculated and sacrificed on 2, 6 and 20 days post inoculation and normal control. Semi-quantitative results of observations in the olfactory bulb, frontal cortex, subependymal plate and ependymal layer cells, diencephalon nucleus and neurons in brain stem are showed.

Days post VSV inoculation	**Astrocytes markers**			
	GFAP	**Vimentin**	**S100-β**	**Symptoms**
2 (n=12)	++	+	+	No (n=12)
6 (n=12)	++++	+++	+++	Yes (n=5)
	+++	++	++	No (n=7)
20 (n=12)	+	+	+	No (n=12)
Control (n=4)	+	+	+	No (n=4)

faint staining, ++ moderate staining, +++ strong staining, ++++intense staining

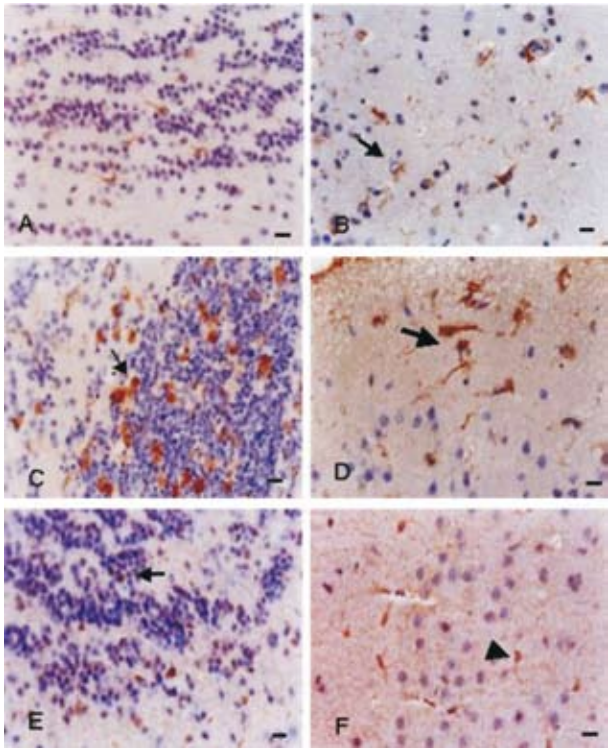

Figure 1: A - Olfactory bulb of mice from control group showing GFAP positive astrocytes with normal morphology. Note small nuclei and thin processes and compare with astrocytes in **B**. From B to F - GFAP, vimentin and S100-β immunostaining in mouse encephalon observed at 6 days post VSV inoculation. **B -** Astrocytes in the olfactory bulb has vesicular nucleus (arrow) and fragmented processes surrounding necrotic area where GFAP staining intensity is faint. **C –** Phagocyte round cells within olfactory bulb with intense staining for vimentin (arrow) **D** – Strong vimentin immunoreactivity in astrocytes at frontal cortex. **E** – Olfactory neurons showing S100-β positive stain (arrows). **F** – Microglial cells in frontal cortex with positive staining for S100β (arrow). Sagittal section, ABC method, A-F: Bar =12.5 μm

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