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Sporadic appearance of paralytic spongiform neurodegeneration in a colony of inbred mice is associated with CNS retrovirus expression

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Sporadic appearance of paralytic spongiform neurodegeneration in a colony of inbred mice is associated with CNS retrovirus expression

Magdalene Durbak

Department of Chemistry

Honors Research Project

Submitted to

The Honors College

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Abstract

Exogenous and endogenous retroviruses (RV) have been known to induce vacuolar central nervous system neuropathology. Two mice which were juvenile inbred Rocky Mountain White (IRW), showed signs of spontaneous degenerate motor neuron disease. Signs included wasting, unkempt fur, adduction reflex upon tail elevation, kyphosis, and stilted gait by postnatal day 18 (P18). Brains and spinal cords were examined with H&E staining and stained with a broadly reactive retrovirus antibody. When compared to brains of mice with normal vacuolar pathology as well as mice infected with prototypic non-neurovirulent RV, Fr57E and prototypic neurovirulent RV FrCasE, the subject showed less severity but were clearly distinguishable from the non-virulent RV. Spongiosis was seen in the motor areas in both mice. Immunohistochemical staining showed reactive retroviral antibodies, which is evidence of viral protein expression in glia and endothelia. The type of RV present will take further investigation. The ability for spontaneous appearance of competent RV replication arising from endogenous RV in mice that also can induce neurological disease are highlighted. Similar spontaneous retroviral gene expression in humans have been attributed to diseases such as multiple sclerosis and amyotrophic lateral sclerosis. Understanding the viral re-emergence will shed light on the cause of human and animal retroviral diseases

Introduction

Eight percent of the human genome contains retrovirus-like sequences, human endogenous retroviruses (HERVs), and are believed to be relevant to human diseases. During evolution, germ-cell line infections caused by retroviruses left residual sequences within the genome of mammal ancestors.[1] Retroviruses are single stranded RNA viruses that use its own reverse transcriptase to produce a DNA copy of its genome which is inserted into the host genetic material. Viral genes are then produced by the host itself.[2] The retrovirus consists of a lipid bilayer, envelope proteins within this membrane, and two single stranded RNA molecules. Viral particles also contain other proteins like protease (Pro), polymerase (Pol), and reverse transcriptase (RT).

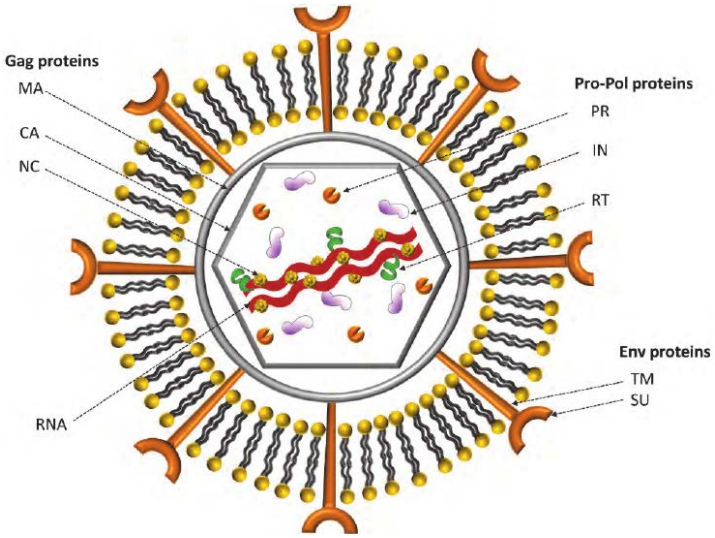

Figure 1. Schematic representation of a retrovirus particle structure, taken from reference three.

Spongiosis due to retroviral spontaneous gene expression has been linked to neurodegenerative diseases in humans including multiple sclerosis and amyotrophic lateral sclerosis.[4] Additionally, retrovirus-induced motor neuron disease is also found in wild mice after infection with the murine leukemia virus, CasBrE.[4] [5] The distribution of spongiform neuropathology in MLV-infected mouse brains has been seen primarily in the motor areas of the

brain.[6] Recently we have observed that mice can spontaneously display similar neurodegeneration symptoms comparable to that of experimentally inoculated with neurovirulent retroviruses.[4 5 6] Mice showed adduction reflex upon tail elevation, kyphosis, unkempt fur and a stilted gait. These mice, arbitrarily labeled mouse one and mouse two, had not been previously inoculated with neurovirulent RV FrCasE; therefore, spontaneous gene expression of retrovirus was suspected.

Understanding the pathology of neurodegenerative disease starts with the basic cellular components of the human brain, their interaction, and the pathways they use to send signals. Neuroglial cells include astrocytes, oligodendrocytes, ependymal cells, and radial glia. Astrocytes link neurons to blood supply and form the blood brain barrier which allows them to regulate the chemical environment surrounding neurons.[7] Oligodendrocytes form myelin sheaths to provide insulation to increase potentiation of electrical signals down the axon.[7] Ependymal cells are involved in creating cerebrospinal fluid and line the spinal cord and ventricular system of the brain. Radial glia function as scaffold for new neurons.[7] This project follows the steps of maturation of neurons and glia in mice from birth to twenty days of age after infection with a retrovirus that causes a rapid, paralytic neurodegenerative disease. Specifically, the susceptibility of glial cells to viral infection and its impact on the understanding of how glial cells contribute to paralytic diseases was explored. Because retroviruses cause certain human neurodegenerative diseases and have been implicated in other diseases with unknown etiologies, the murine viruses studied are a valuable tool used to imitate responses at different levels of cell maturation relevant to human neurodegenerative diseases.

Mice who have received the virus were compared to mice that were mock-infected. In addition, two mice who appeared to express spontaneous clinical neurological abnormalities were examined for the presence of features of retroviral neurodegenerative disease. First, both infected

and uninfected brains were observed and compared with mice exhibiting a clinical disease of unknown origin. The different aged brains were observed to see when pathology occurs.

Materials and Methods

Mice

All animal protocols were approved by the institutional care and use committee at Northeast Ohio Medical University. Mice were deeply anesthetized with isoflurane and following decapitation the brain was removed and fixed in formalin.

Chemicals Used

The chemicals used for tissue fixation and H&E staining were purchased from Sigma-Aldrich. These chemicals include: eosin, hematoxylin, ethanol, and xylenes.

Tissue Fixation and Paraffin Embedding

Tissue processing was completed using a HistoCore Peloris processing machine. The sample was automatically washed the tissue with several solvents. Solvents were added to dehydrate, clear, and infuse with wax. The dehydration steps included six washes of ethanol at increasing concentrations starting with 70% and ending with 100%. In this case, xylene was used in varying time-sequences beginning with twenty minutes and ending with forty-five minutes. The tissue is infused with wax in three cycles while varying in time (30 min- 45 min). Wax is infused at approximately 60ºC and is allowed to cool to 20ºC to harden. The tissue was embedded using a Histostar™ Embedding Work Station (Thermo Fischer Scientific). The tissue was placed in a cassette designed to be placed in the microtome where slices of 5 -7 µm thick were cut, placed on glass microscope slides and observed.

H&E Staining

Previously made slides were deparaffinized and rehydrated using xylene, ethanol, and deionized water. The slides were stained with hematoxylin make sure to rinse with deionized water

and eosin stained. The slides were dehydrated using alcohol and then xylene. Paramount and coverslips were added.

Immunohistochemistry

Previously mounted slides were heated to allow excess paraffin to be removed and stained immunohistochemically. The slides were rehydrated with xylene, ethanol, and deionized water. The tissue was stained with rat monoclonal 83A25 primary, biotin-anti-rat secondary antibody and streptavidin-HRP. Counterstain was then applied to the slides with H&E staining, taking care to begin at hematoxylin stain due to the rehydrated tissue. Finally, the tissue was dehydrated and observed under a microscope.

Results and Discussion

The activation of RV infection within the central nervous system can lead to alterations in tissue integrity that are observable with standard histological stains.

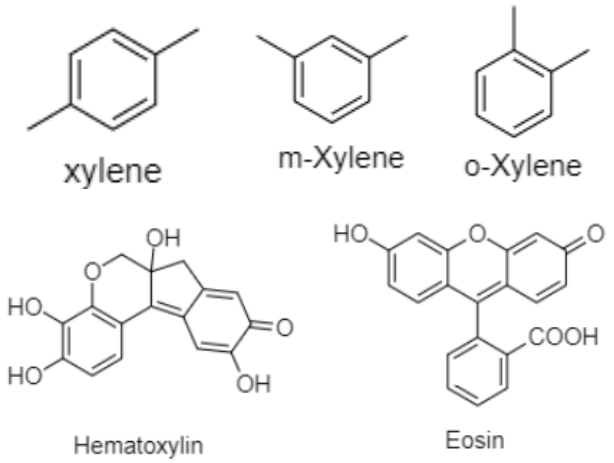

Figure 2 Compounds used for histological analysis of brain tissue

Prior to staining, tissue architecture must be preserved through fixation. The goal of tissue fixation is to remove all water from the tissue and replace it with paraffin wax. To do this, ethanol used to replace water in the tissue with ethanol. The hydrophobic wax will not mix with water. Ethanol and water are still immiscible therefore ethanol must be cleared from the tissue as well. The middle solvent, xylene, must be miscible with wax and ethanol and is considered a clearing agent. Once this tissue is incorporated with xylene, it can be washed with liquid wax until the xylene is completely replaced with wax.

Hematoxylin and eosin staining is a common histological staining technique. Cytoplasmic proteins are acidophilic due to their basic properties and nucleic acids are acidic and therefore basophilic. H&E takes advantage of these properties to stain the nucleus and counterstain the other parts of the cell. Eosin is an acidic dye and stains basic structures pink,

including the cytoplasm of the cell. Hematoxylin stain is basic and dyes acidic parts of the cell purple. Due to nucleic acid found in the nucleus, the nucleus is stained the purple color.

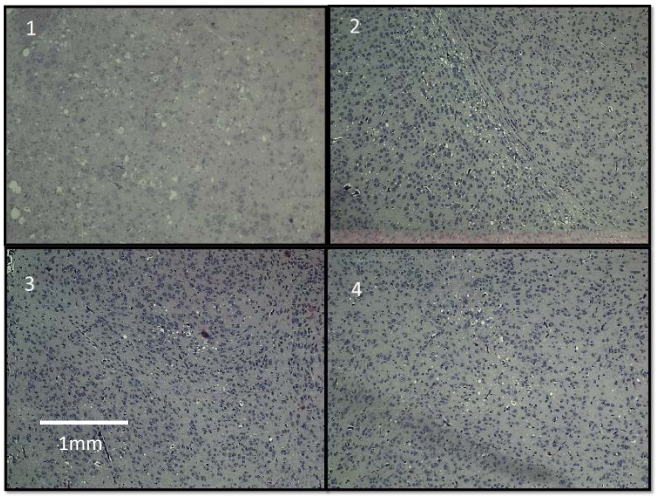

Figure 3 H &E Staining of 1. Medulla, 2. Cortex, 3. Pons, and 4. Thalamus from the first mouse sampled.

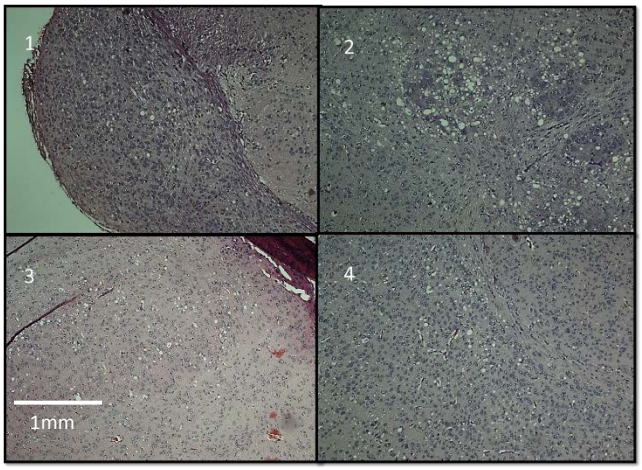

Figure 4 H&E Staining of 1. Hypothalamus, 2. Medulla, 3. Thalamus, and 4. Cortex of the second mouse sampled.

The H&E staining show motor areas of the medulla, cortex, pons, hypothalamus, and thalamus. Within these areas spongiosis is seen, especially in mouse two in the medullary areas as seen by the destruction of tissue integrity within this area. This spongiosis is most likely the cause of the neurodegenerative behavior observed in these mice. The spongiosis observed in the

two sampled mice is similar to the spongiosis observed in brains of mice infected with FrCasE. This suggests the mice studied are infected with a RV.

We next used immunohistochemistry to determine if retrovirus was present (indicated with brown staining in Figures 7-9). This protocol uses a primary antibody specific that recognizes specific viral proteins and a secondary antibody containing biotin for detection. The primary antibodies for RVs usually recognize one or more of three major proteins encoded within the retroviral genome: Gag, Pol, and Env. Gag, acronym for Group Antigen, forms the viral core structure. Env is the envelope protein and Pol is the reverse transcriptase. The primary antibody was αEnv and targets a seventeen amino acid long immunosuppressive region present in many retroviral envelope proteins ($SDGGGX_2DX_2R$).[8] These residues are conserved among retroviruses and therefore used in creating antibodies against them. The monoclonal antibody 667 or monoclonal 83A25 used in this experiment binds to twelve of those amino acids located in the variable region of the receptor binding domain.[9] The secondary antibody, Biotin-anti-rat, reacts to the primary antibody and does so by reacting to rat IgG. The goat IgG was the conjugated with biotin protein. The protein streptavidin is tetrameric and binds tightly to biotin. This protein is conjugated with horseradish peroxidase (HRP) which contributes to the brown color in seen in the immunohistochemical stain. When streptavidin binds to biotin on the secondary antibody this causes HRP to produce a colored complex.

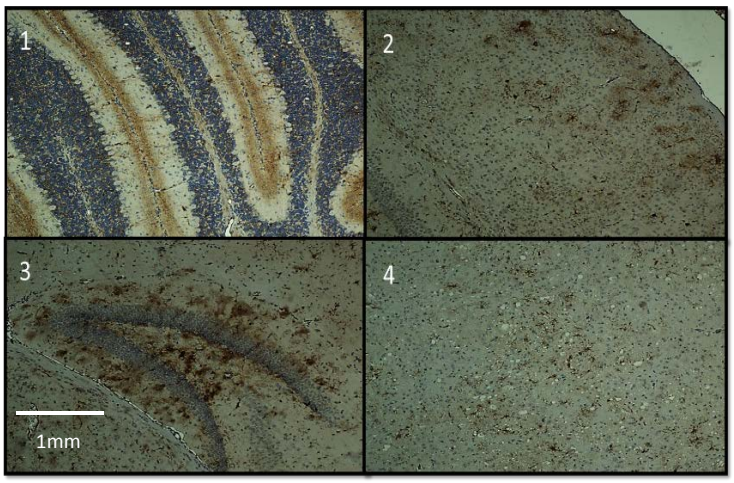

Figure 5. Immunostaining with rat monoclonal 83A25, Biotin-anti-rat secondary antibody and streptavidin-HRP, from the first mouse in 1. Cerebellum, 2. Cortex, 3. Dentate Gyrus, and 4. Pons

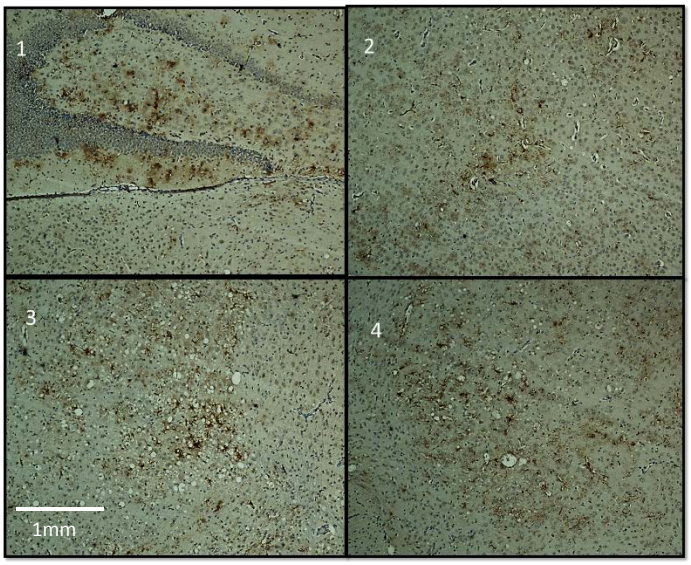

Figure 6. Immunostaining with rat monoclonal 83A25, Biotin-anti-rat secondary antibody and streptavidin-HRP, from the second mouse in 1. Cerabellum, 2. Cortex, 3. Dentate Gyrus, and 4. Pons

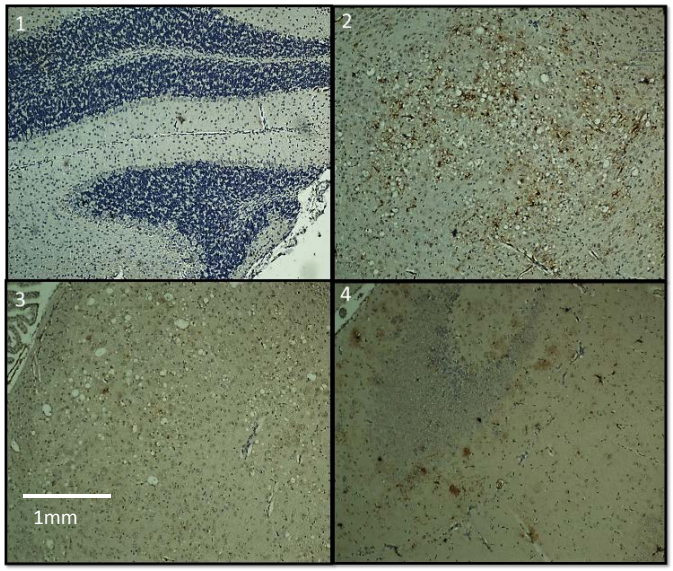

Figure 7. Picture 1 shows the immunostaining done on mouse cerebellum with rat monoclonal 83A25, Biotin-anti-rat secondary antibody and streptavidin-HRP on a control mouse which was not infected with virus. The second, third, and fourth pictures show immune stain from a mouse infected with FrCasE virus (positive control) in2. Pons, 3. Medulla, and 4. Dentate Gyrus

From H&E staining we can see that spongiosis is seen in parts of the motor areas of the brain. Those regions can be found in the hypothalamus, medulla, thalamus, and cortex. Looking at Figure 9 one can see a lack of spongiosis in the first picture which depicts a mouse who had not been given virus. Figure 5 and 6 clearly show much spongiosis compared to the negative control and show similar characteristics of the positive control (also depicted in Figure 9).

Immunohistochemistry showed presence of Env protein. This is seen in the brown staining in Figures 7-9. Positive controls in Figure 9 show similar staining patterns as seen in Mouse One and Mouse Two. This experiment is difficult to replicate due to its spontaneity. It could be that these mice were somehow mixed up and were subjected to the virus. Unless more mice with similar clinical pathology are observed, this analysis can not be known for certain.

Conclusion:

Clinically-ill mice showed signs of motor neuron disease and were euthanized in order to compare to mice with induced motor neuron disease. Overt spongiosis was observed in brain areas consistent with retrovirus induced neurodegeneration. Immunohistochemical staining showed strong retroviral Env protein expression, though further studies involving more specific antibodies will be needed to identify the retrovirus involved. Due to this spontaneous occurrence, only two mice could be observed. It is hypothesized that the mice acquired the virus from their mother during breastfeeding done early in the postnatal period.

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Appendix 1: Safety (Found Using Safety Data Sheet [SDS])

Xylene:

Xylene is classified as harmful and should be used under the chemical hood. Xylene's hazard statements include: flammable liquid and vapor, harmful in contact with skin, causes skin irritation, and harmful when inhaled. Precautionary statements include: keep away from heat/sparks/open flames/ hot surfaces, keep container tightly closed, take precautionary measures against static discharge, wear protective cloves/ protective clothing/ eye protection/ face protection. If swallowed it is recommended not to induce vomiting and poison control be contacted immediately. If contact with skin occurs, it is recommended that contaminated clothing be taken off immediately and to rinse the skin with water. Storage should include a well-ventilated place. If inhaled it is recommended to move to fresh air.

Isoflurane:

Toxicity from a single exposure is considered category 3, meaning substances produce transient (short duration or temporary) target organ effects such as narcotic effects or respiratory tract irritation. When being handled, it should be handled under a chemical hood using proper protective equipment such as protective clothing, gloves, and googles. Hazard statements included: may cause drowsiness and dizziness. Precautionary statements include: avoid breathing dust/fume/gas/mist/vapors/spray, use only outdoors or in well-ventilated area. If inhaled move person to fresh air and call poison control if one feels unwell. Store in well0ventilated place and keep container tightly closed.

Other Safety Precautions:

Mice were kept in the infectious area of the CMU and personal protective equipment was worn at all times to ensure safety. This included surgical mask, yellow isolation gown, and nitrile gloves. Bleach was used to sterilize mice containment units as well as surgical tools. Training is required for all personal working in the CMU. Standard practices of the Institutional Animal Care and Use Committee (IACUC) were followed