Neurobiology

Oligodendrocytes Are a Major Target of the Toxicity of Spongionogenic Murine Retroviruses

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The neurovirulent retroviruses FrCasE and Moloney MLV-ts1 cause noninflammatory spongiform neurodegeneration in mice, manifested clinically by progressive spasticity and paralysis. Neurons have been thought to be the primary target of toxicity of these viruses. However the neurons themselves appear not to be infected, and the possible indirect mechanisms driving the neuronal toxicity have remained enigmatic. Here we have re-examined the cells that are damaged by these viruses, using lineage-specific markers. Surprisingly, these cells expressed the basic helix-loop-helix transcription factor Olig2, placing them in the oligodendrocyte lineage. Olig2⁺ cells were found to be infected, and many of these cells exhibited focal cytoplasmic vacuolation, suggesting that infection by spongionogenic retroviruses is directly toxic to these cells. As cytoplasmic vacuolation progressed, however, signs of viral protein expression appeared to wane, although residual viral RNA was detectable by *in situ* hybridization. Cells with the most advanced cytoplasmic effacement expressed the C/EBP-homologous protein (CHOP). This protein is up-regulated as a late event in a cellular response termed the integrated stress response. This observation may link the cellular pathology observed in the brain with cellular stress responses known to be induced by these viruses. The relevance of these observations to oligodendropathy in humans is discussed. (Am J Pathol 2006, 169:1026–1038; DOI: 10.2353/ajpath.2006.051357)

Mouse retroviruses have co-evolved with their host for several million years and are generally nonpathogenic. Many of those viruses that do cause disease are the oncogenic retroviruses, which induce leukemia/lymphoma by insertional mutagenesis or because they have acquired an oncogene through recombination. There is a small group of murine retroviruses that cause an unusual neurological disease characterized by noninflammatory spongiform neurodegeneration, resembling pathologically the lesions associated with the transmissible spongiform encephalopathies. The prototypic member of this group, CasBrE, was isolated from wild mice¹ and molecularly cloned 10 years later.² The agent is maintained in feral mouse populations as an exogenous virus that is transmitted to neonates in maternal milk. Neonatal infection results in life-long viremia and, if virus gains access to the brain, clinical neurological disease. Mice inoculated with virus after postnatal day 10 are completely resistant to the disease. Since the original description and cloning of CasBrE, a number of other viral strains have been isolated in the laboratory that cause similar diseases both in mice^{3,4} and rats.^{5,6} For each of these viruses, neurovirulence is dependent on the sequence of the envelope protein,⁷ although the nature of the neurotoxicity is unclear.

It has been reported that neurons represent the primary target of toxicity of these viruses,^{8,9} and neuronal dropout clearly occurs in this disease.¹⁰ However, neurons within the spongiform lesions have been found not to be infected.^{11–14} The receptor for these viruses is the ubiquitous cationic amino acid transporter CAT1,¹⁵ rendering virtually all cells in the nervous system susceptible to infection. However, murine retroviruses require cell division to complete their replication cycle,

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and these neurons are postmitotic at the time of virus inoculation. Thus, the neurotoxicity of these viruses has been ascribed to indirect effects caused by virus infection of other cells in the vicinity. Some have suggested that neurotoxic products of activated microglia^{12,16} or astrocytes¹⁷ might be involved. However, using an acute form of the disease induced by a chimeric virus, FrCasE,¹⁸ we found that microglial and astrocyte activation were relatively late events in the disease, occurring after the appearance of spongiform lesions.¹⁹ Others have suggested that these viruses might cause a loss of function in the infected cells, perhaps a loss of trophic support.^{8,12}

To identify specific biochemical pathways that were involved in this disease, we performed transcriptional profiling on the brainstems of mice infected with two co-isogenic viruses, FrCasE and F43,²⁰ differing only in their envelope genes. Both viruses are neuroinvasive and infect the same populations of cells, but only FrCasE is neurovirulent. F43 causes neither clinical disease nor histopathology.²⁰ These studies showed that FrCasE but not F43 induced endoplasmic reticulum (ER) stress in the brains of infected mice²¹ and that this response could be studied *in vitro* in NIH3T3 cells.²² The ER stress response was characterized by the up-regulation of ER chaperones, ER degradative machinery, and the transcription factor C/EBP-homologous protein (CHOP). Because these viruses differed in their envelope genes, this response appeared to be induced by the envelope protein. Subsequently we showed that the ER stress was attributable to a folding instability of the FrCasE envelope protein²² and suggested that this disease may represent a protein-folding disorder caused by a conventional virus.

Although these viruses induce ER stress associated with dramatic cell-cycle arrest and apoptosis *in vitro*,¹⁷ curiously no evidence of cytopathology has been reported in the infected cells *in vivo*. Indeed, abundant microvascular endothelial cells and microglial cells,^{12–14} as well as certain populations of neurons that undergo cell division postnatally,^{11,13} are heavily infected by the spongigenic retroviruses and remain undisturbed, even at the ultrastructural level.¹³

It is for these reasons that we decided to re-examine this animal model and address several fundamental questions. What is the identity of the degenerating cells in this disease? What is the connection between virus infection and the cytopathology observed *in vivo*? Is there a link between the protein misfolding observed *in vitro* and the cytopathology observed *in vivo*? In the current study, we found that a major component of the degenerating cells represents oligodendrocytes, not neurons. The loss of these cells appeared to account for a major component of the spongiform pathology induced by these viruses. This is significant because oligodendrocytes proliferate during the first few weeks after birth and should be susceptible to infection. Indeed, infection of oligodendrocytes was readily detected. Furthermore, the degenerating cells expressed CHOP protein, linking cytopathology to ER stress-signaling pathways. These findings suggest that the neu-

rotoxicity of the spongigenic retroviruses may be initiated by a direct toxicity for oligodendrocytes.

Materials and Methods

Mice and Virus Inoculations and Titrations

Inbred Rocky Mountain White (IRW) mice were obtained from the Rocky Mountain Laboratories colony. CHOP^{-/-} and CHOP^{+/-} mice on an FVB/n background, kindly provided by David Ron (Skirball Institute, New York, NY), have been described in detail.²³ Neonatal CHOP^{-/-} and control CHOP^{+/+} mice were generated by interbreeding CHOP^{+/-} mice or crossing CHOP^{+/-} × CHOP^{-/-} mice. All mice were bred and raised at the Rocky Mountain Laboratories and handled according to the policies of the Rocky Mountain Laboratories Animal Care and Use Committee. Genotyping was performed on tail DNA obtained at the time of weaning. The forward and reverse polymerase chain reaction (PCR) primers used for detection of the CHOP wild-type allele were 5'-ATGCCCTTACCTATCGTG-3' and 5'-GCAGGGTCAAGAGTAGTG-3', respectively, straddling the translation-initiation codon and yielding a 450-bp product. For detection of the CHOP-null allele, a reverse P_{gk}.neo primer (5'-ACTTGTGTAGCGCCAAGTG-3') was paired with the CHOP forward primer, yielding a 220-bp product. Reactions were run at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for a total of 35 cycles and were resolved with 1.5% agarose gels.

FrCasE¹⁸ and F43²⁰ are chimeric viruses generated in this laboratory. MoMLV-ts1, a temperature-sensitive mutant of Moloney MLV,²⁴ was obtained from Dr. Paul Wong (M.D. Anderson Cancer Center, University of Texas, Houston, TX). Virus stocks were prepared in either NIH3T3 or *Mus dunni* cells as described previously¹⁸ and infectivity titers determined by a focal immunoassay.²⁵ Mice were inoculated intraperitoneally with 1 to 3 × 10⁵ focus-forming units of infectivity on postnatal day 1. Mice were euthanized when clinical disease, manifested by spasticity, tremor, and paralysis, was clearly manifested. Survival curves were analyzed with InStat GraphPad (San Diego, CA) using the Kaplan-Meier method. For quantification of viremia titers, mice were bled 5 days after neonatal inoculation and the serum was frozen at -80°C. After all sera were collected, infectious virus was quantified by focal immunoassay using NIH3T3 cells.

RNA Quantification

Mice were anesthetized with isoflurane and subjected to cardiac perfusion with ice-cold phosphate-buffered saline (PBS). Brainstems were dissected and stored in RNeasy (Qiagen, Valencia, CA), and total RNA was extracted using the RNeasy mini kit described previously.²¹ CHOP mRNA was quantified by TaqMan real-time reverse transcriptase (RT)-PCR (Applied Biosystems, Foster City, CA) as described previously.²¹

Immunohistochemistry and in Situ Hybridization

Mice were anesthetized with isoflurane and perfused with 3.7% formaldehyde in PBS. Brains were postfixed in the same fixative at room temperature for 48 hours, followed by dehydration and paraffin embedding. Six- μ m sections were subjected to antigen-retrieval in citrate buffer using a Decloaking Chamber (Biocare Medical, Walnut Creek, CA). Primary antibodies were as follows: anti-CHOP (Sc-575; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Iba1 (WAKO, Richmond, VA), anti-HuC/D clone 16A11 (Invitrogen, Carlsbad, CA), anti-NeuN (Chemicon, Temecula, CA), anti-PLP (Sc18529; Santa Cruz Biotechnology), anti-GFAP (DAKO, Carpinteria, CA), and anti-Olig2 (Sc19969; Santa Cruz Biotechnology). The anti-viral staining was performed using monoclonal antibody 697,²⁶ specific for the envelope glycoprotein of FrCasE and nonreactive with endogenous retroviruses, and a rabbit anti-capsid protein antiserum described previously.²⁷

Immunostaining was performed essentially as described previously.²¹ In brief, sections were incubated with primary antibodies overnight at 4 or 37°C. This was followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) and then horseradish peroxidase-conjugated streptavidin (Biogenix, San Ramon, CA). The substrates used were either diaminobenzidine or aminoethylcarbazol, and the sections were counterstained with Meyer's hematoxylin.

Double staining was performed as above for the first antibody, the substrate being diaminobenzidine. The sections were then subjected to another antigen-retrieval step that served to remove the reagents from the first stain, leaving the insoluble diaminobenzidine product intact. The second antibody was then added, and staining proceeded as described above. In this case alkaline phosphatase-conjugated streptavidin (Biogenix) was used, and the sections were incubated with the substrate Fast Red.

In situ hybridization was performed on sagittal sections of paraffin-embedded brains from FrCasE-, F43-, and mock-infected mice 12 and 14 days after inoculation. The anti-sense FrCasE envelope-specific RNA probe WM^{XB} was described previously.¹³ It is nonreactive with other exogenous or endogenous murine retroviruses.¹⁸ Synthesis of the digoxigenin-labeled probe as well as the methods used for *in situ* hybridization and immunodetection have been described in detail elsewhere.²⁸

Immunofluorescence Microscopy

For confocal microscopy, 6- μ m sections of paraffin-embedded material were processed as described above except that the slides were incubated overnight at 37°C with a mixture of monoclonal antibody 697²⁶ reactive with viral envelope glycoprotein and goat anti-Olig2. The slides were washed and then incubated with a mixture of AlexaFluor 568 donkey anti-goat Ig and AlexaFluor 488 chicken anti-mouse Ig (Invitrogen) for 30 minutes at room temperature. Nuclei were stained

with the DNA dye DRAQ-5 and slides examined on a UltraView LCI confocal microscope (Perkin-Elmer Life Sciences, Boston, MA). Z-series were acquired using 0.2- μ m optical slices, and images were processed using Metamorph software. For epifluorescence microscopy, mice were perfused with 3.7% formaldehyde in PBS and brains postfixed in the same fixative for 48 hours followed by cryopreservation in 30% sucrose/PBS at 4°C for 48 hours. Brains were frozen over dry ice in optimal cutting temperature (OCT) medium and sectioned on a HM 505E cryostat (Microm, Walldorf, Germany). Sections were dried for 24 hours at 40°C and subjected to antigen retrieval as described above. Sections were then incubated with a mixture of rabbit anti-CHOP and goat anti-Olig2 antibodies overnight at 37°C. After washing in PBS, sections were incubated with a mixture of AlexaFluor 594 donkey anti-goat Ig and AlexaFluor 488 chicken anti-rabbit Ig for 30 minutes at room temperature. Nuclei were stained with the DNA dye 4,6-diamidino-2-phenylindole, and slides were examined on a Microphot SA microscope (Nikon, Melville, NY). Images were acquired with a cooled charge-coupled device camera (C5985 camera; Hamamatsu Photonics, Hamamatsu City, Japan), and images were pseudocolored. All postcapture manipulations were performed with Adobe Photoshop 7 using identical settings for each image. All fluorochrome-labeled secondary antibodies were cross-tested for specificity and were found to be specific for the respective primary antibodies they were used to detect.

Transmission Electron Microscopy

For electron microscopy, mice anesthetized with isoflurane were perfused with Karnovsky's fixative containing 4% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.2 (Electron Microscopy Sciences, Hatfield, PA). Brainstems were dissected and submerged in Karnovsky's fixative and held overnight at 4°C. Samples were then washed twice at room temperature for 30 minutes each in phosphate buffer and then postfixed for 2 hours at room temperature with a freshly prepared mixture of 1% osmium tetroxide and 0.8% potassium ferrocyanide in phosphate buffer. After one wash in phosphate buffer and two washes in water, the samples were treated for 2 hours at room temperature with 1% uranyl acetate in water. The samples were then washed twice in water, dehydrated in ethanol, and embedded in Spurr resin (Ted Pella, Inc., Redding, CA). Silver-colored thin sections were mounted on copper slot grids coated with Formvar and carbon (Ted Pella, Inc.) and examined at 80 kV on a H7500 transmission electron microscope (Hitachi, Tokyo, Japan). Digital images were acquired with a cooled charge-coupled device camera (Advanced Microscopy Techniques, Inc., Danvers, MA) and processed with Adobe PhotoShop version 7.0.

Figure 1. Histopathology of the spongiform lesions in an FrCasE-inoculated mouse, 14 days after inoculation, and the morphology of the degenerating cells therein. **A:** Low-power overview of half of the brainstem from the midline to the cochlear nucleus (Co) and cerebellum (Cb) laterally. This is a merged composite of multiple photomicrographs showing the close spatial relationship between virus-infected cells (red) and the vacuolar pathology. The section was stained with monoclonal antibody 697,²⁶ which is specific for the FrCasE envelope protein, and counterstained with hematoxylin. **B:** Merged composite showing the same area of the brainstem stained with H&E. **Arrows** on the left part of the section point to numerous vacuoles containing nuclei within them. Large numbers of similar vacuoles can be seen throughout the spongiform lesions. **C:** Range of morphologies of the vacuolated cells exhibiting swollen cytoplasm with intact nuclei measuring 6 to 8 μm in diameter. Margination of chromatin was seen frequently, although pyknotic nuclei (**arrowhead**) were rarely encountered. Original magnifications: ×10 (**A**); ×20 (**B**).

Results

Histopathology of the Spongiform Lesions

The spongiosis induced by the two neurovirulent viruses used in these studies, FrCasE and MoMLV-ts1, is localized to gray matter and gray-white matter interfaces of the ventral spinal cord, brainstem, thalamus, and, in the case of FrCasE, deep cerebral cortex. During infection by either virus, however, the lesions were seen only in areas with the highest concentrations of infected cells (Figure 1A), and it has been reported that 40 to 60% of these cells represent microglia.¹²

Within the spongiform lesions induced by all of the spongigenic murine retroviruses can be seen abundant

cells exhibiting a distinctive cytopathology consisting of swollen, watery cytoplasm that often contains large vacuoles (Figure 1, B and C). The cytoplasm of many of these cells was disrupted, leaving empty vacuoles each containing a round nucleus of rather uniform size (6 to 10 μm in diameter) (Figure 1C). These observations suggest that many of the larger vacuoles are caused by the loss of these cells.

Ultrastructurally, these cells were characterized by a uniform watery cytoplasmic matrix (Figure 2A), a remarkable lack of recognizable ribosomes and ER, and only scant remnants of Golgi (G in Figure 2B). Focal cytoplasmic vacuoles were observed (Figure 2A, arrow), although in other cells larger vacuoles were seen as well (not shown). The

Figure 2. Ultrastructure of the swollen cells within the spongiform lesions. These are sections of the brainstems of MoMLV-ts1-inoculated mice 32 days after inoculation. The cells in **A** and **C** exhibit marked swelling and dissolution of the cytoplasm associated with generally intact nuclei (N). A high-power view of the boxed area in **A** is shown in **B**. There is little evidence of cytoplasmic organelles except for occasional remnants of Golgi (G) and rather well-preserved mitochondria (M). A high-power view of the mitochondrion boxed in **C** is shown in the **inset**. A small cytoplasmic vacuole is shown (arrow, **A**), although more pronounced vacuolation is also seen.

sparse mitochondria appeared intact (Figure 2C and inset). Aside from some margination of chromatin, the nuclei of these cells appeared intact as well. There was no evidence of viral particles either intracellularly or budding from the plasma membrane. These cells have been described in detail in the original ultrastructural study of this disease,²⁹ although their lineage was uncertain.

Identification of the Vacuolated Cells

Although we and others have considered these cells to represent degenerating neurons, their lineage has not been explored in any detail. We therefore used lineage-specific markers to identify these cells (Figure 3). These studies were performed on MoMLV-ts1-inoculated mice 28 to 33 days after inoculation, a time when spongiform lesions in the brainstem were extensive. The vacuolated cells (Figure 3, white arrowheads) failed to stain with the astrocyte-specific marker glial fibrillary acidic protein (GFAP) (Figure 3A), the microglial marker Iba1 (Figure 3B), or the oligodendrocyte markers proteolipid protein (PLP) (Figure 3C) and myelin basic protein (MBP) (not shown). HuC/D (Figure 3D)³⁰ and NeuN (not shown) were used as pan-neuronal markers. In the case of NeuN, only subpopulations of neurons were positive, making the results difficult to interpret. HuC/D, on the other hand, stained a majority of neurons throughout the neuraxis. A relatively small fraction (Table 1) of the vacuolated cells were found to express HuC/D (Figure 3D, inset), the majority being negative for this marker (Figure 3D, arrowheads). In contrast, more than 90% of these cells (Table 1) expressed the basic helix-loop-helix (bHLH) transcription factor Olig2 (Figure 3E), a protein involved in lineage commitment of oligodendrocytes³¹ and expressed by these cells at all stages of their differentiation. As described by others,³² Olig2 staining localized exclusively in the nucleus, though there was clearly a range of staining intensities (Figure 3E) with some (~10%) (Table 1) appearing negative (Figure 3E, right). Thus, although they did not express markers of mature oligodendro-

cytes, a majority of the vacuolated cells appeared to belong to the oligodendrocyte lineage.

The Vacuolated Cells Express the Transcription Factor CHOP

Both FrCasE^{21,22} and MoMLV-ts1¹³³ induce ER stress manifested by the up-regulation of ER chaperones as well as the transcription factor CHOP. FrCasE-infected mice exhibit the first signs of clinical disease at 14 days after inoculation and are preterminal by 17 to 18 days after inoculation. *CHOP* mRNA was up-regulated in the brainstem at 10 days after inoculation, during the preclinical period, and increased progressively thereafter (Figure 4A). This was not the case for mice inoculated with the nonpathogenic virus F43, which infects the brain at even higher levels than FrCasE,^{20,22} indicating that this response was disease-specific. A similar increase in *CHOP* mRNA was observed in MoMLV-ts1-inoculated mice (Figure 4B).

It was reported previously that in MoMLV-ts1-infected mice, cells within the spongiform lesions expressed CHOP protein.³³ We have confirmed this result in mice infected with MoMLV-ts1 (not shown) and FrCasE (Figure 4C). CHOP⁺ cells were not found in mice inoculated with F43 or in the mock-inoculated controls. These distinctive cells appeared morphologically indistinguishable from the vacuolated cells described above (Figures 1, 2, and 3). Indeed, cell counting revealed that ~90% of the vacuolated cells expressed detectable CHOP protein (Table 1). Furthermore, co-localization of CHOP and Olig2 was confirmed by two-color immunofluorescence microscopy (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

Although CHOP was concentrated in the nucleus,³³ many cells also exhibited surprisingly intense cytoplasmic staining (Figure 4D), suggesting the possibility that this protein may have leaked from the nucleus. Interestingly, CHOP protein was also detected in many of the larger vacuoles that contributed to the spongiform pathol-

Figure 3. Identification of vacuolated cells using lineage-specific markers. Sections were taken from the brainstem of a MoMLV-*ts1*-inoculated mouse 32 days after inoculation and stained for the astrocyte marker GFAP (A), the microglial marker Iba1 (B), the mature oligodendrocyte marker PLP (C), the pan-neuronal marker HuC/D (D), and the pan-oligodendroglial marker Olig2 (E). Only Olig2 stained these cells (arrowheads). Arrows point to cells staining positive for the respective marker.

Table 1. Expression of CHOP and Lineage-Specific Markers by Vacuolated Cells

Protein*	Number of mice†	Total cells counted‡	% Positive
CHOP	6	1661	90.5 ± 3.1
Olig2	5	716	93.7 ± 4.3
HuC/D	6	611	8.3 ± 3.4

*Parasagittal sections of the brain through the cerebellar nuclei, thalamus, and hippocampus were stained with antibodies to CHOP, Olig2, or HuC/D and counterstained with hematoxylin.

†Brain sections were from two FrCasE-inoculated and three to four MoMLV-ts1-inoculated mice manifesting signs of advanced neurological disease.

‡Only vacuolated cells were counted. Sections were viewed with a ×40 objective lens and each section was examined in its entirety.

ogy induced by both FrCasE (Figure 4E, left; arrowheads) and MoMLV-ts1 (Figure 4E, right; arrowhead and inset). This supports the idea that these vacuoles were remnants of CHOP⁺ cells.

Because the up-regulation of CHOP is disease-specific and expressed only in the vacuolated cells, it was of interest to ask whether CHOP was involved in the pathogenesis of this disease. The genetic background of the CHOP^{-/-} mice (FVB/N) renders them susceptible to MoMLV-ts1.³⁴ As expected the virus-infected CHOP^{-/-} mice expressed no detectable CHOP mRNA in the brainstem (Figure 5A). We compared the clinical course in virus-infected CHOP^{+/+} and CHOP^{-/-} mice (Figure 5B). There was a small, but statistically significant, delay of ~4.5 days in the incubation period of the disease in the CHOP^{-/-} mice ($P < 0.05$). However, this delay correlated with a small, but significant ($P < 0.05$), decrease in early virus replication as measured by viremia titers 5 days after inoculation (Figure 5C). Early viremia kinetics have been shown previously to represent a sensitive indicator of neuroinvasiveness.³⁵ This suggests that slower tempo of the clinical disease in CHOP^{-/-} mice was not attributable to a direct effect of CHOP expression in the brain but instead was a consequence of its effect on virus replication in peripheral organs.

Are the Vacuolated Cells Infected?

Studies by us and others have shown clearly that neurons within the spongiform lesions are not infected. Because the vacuolated cells now appear to belong to the oligodendrocytes lineage, this places them in a proliferating pool that should be susceptible to productive infection. Thus, we thought it important to revisit this issue. Infection of oligodendrocytes has been reported previously,^{36,37} but the methods of detection were not specific for the inoculated virus, leaving open the possibility that this represented the activation of endogenous viral sequences. When more specific methods were used, the identification of oligodendrocytes was based on morphology alone.¹² We therefore used a monoclonal antibody, 697,²⁶ that reacts specifically with the envelope protein of FrCasE but is nonreactive with endogenous retroviruses. Immunofluorescence microscopy (Figure 6A) revealed the presence of FrCasE envelope protein (green) in

Figure 4. A: Up-regulation of CHOP mRNA in the brainstem as a function of time after inoculation of FrCasE. Mice were inoculated intraperitoneally as neonates. Mice inoculated with FrCasE exhibit focal spongiform lesions at 10 days after inoculation, mild tremor, and spasticity at 14 days after inoculation and preterminal paralysis and wasting at 17 days after inoculation. F43 is a highly neuroinvasive, but avirulent, virus carrying an envelope gene differing from that of FrCasE. B: CHOP up-regulation of a similar magnitude was observed in preterminal mice 35 days after neonatal inoculation of MoMLV-ts1. C: CHOP protein was detected within the spongiform lesions in the brainstem of an FrCasE-inoculated mouse 17 days after inoculation. D: CHOP protein was concentrated in the nuclei of the vacuolated cells, although one could frequently also find the protein in the cytoplasm. E: Anuclear vacuoles within the spongiform lesions induced by FrCasE (left) and MoMLV-ts1 (right) also contained CHOP⁺ material (arrowheads), suggesting that these represented remnants of CHOP⁺ cells.

Olig2⁺ cells (red nuclei). This method, however, does not allow examination of these cells in the context of the spongiosis.

Two-color immunohistochemistry revealed abundant 697⁺/Olig2⁺ cells both within the spongiform lesions of the gray matter (Figure 6, B–D; black arrows) as well as within adjacent white matter tracts such as the corpus callosum (Figure 6E, black arrows), in which spongiosis was sparse. Although the fraction of total Olig2⁺ cells that were 697⁺ varied depending on the region of the brain examined, cell counting within those areas exhibiting the highest concentration of lesions (Figure 1A) indicated that ~15% of Olig2⁺ cells also stained with 697 (total of 1289 Olig2⁺ cells counted). The 697⁺/Olig2⁻ cells, often seen in these areas as well, exhibited the morphology of microglia (Figure 6C, white arrow). Curiously, the scat-

Figure 5. Effect of the CHOP gene on the neurovirulence of MoMLV-ts1. CHOP^{+/+} and CHOP^{-/-} littermates were inoculated as neonates with MoMLV-ts1 and observed for signs of clinical neurological disease. **A:** Some mice were sacrificed when they exhibited clinical signs and their brainstems assayed for expression of CHOP mRNA using real-time RT-PCR. No CHOP mRNA was detected in those mice genotyped as CHOP^{-/-}. **B:** Kaplan-Meier survival curves of the clinical data from two independent experiments (28 CHOP^{+/+} and 33 CHOP^{-/-} mice) indicate that there was a 4.5-day delay in the incubation period (time to hindlimb paralysis) in the CHOP^{-/-} mice ($P < 0.05$). **C:** However, viremia titers assayed 5 days after inoculation were slightly lower in the CHOP^{-/-} than in the CHOP^{+/+} mice ($P < 0.05$) likely accounting for the delay in appearance of clinical signs of disease in these mice (see text). Each point represents a different mouse.

tered Olig2⁺ cells having an appearance consistent with the CHOP⁺ cells (ie, with extensive cytoplasmic swelling and vacuolation) either stained weakly or not at all for viral envelope protein (Figure 6D, white arrowhead). Using differential interference contrast microscopy to examine cellular morphology in more detail, it became clear that 697⁺/Olig2⁺ cells within the spongiform lesions often contained focal paranuclear cytoplasmic vacuoles (Figure 6, F–H), although little if any viral protein was detectable in the cells with more extensive vacuolation (Figure 6G, white arrowhead).

To explore further whether the cells exhibiting extensive cytoplasmic vacuolation were infected, we used an anti-sense RNA probe (WM^{XB}),¹³ which is highly specific for FrCasE envelope sequences, to look for viral RNA (Figure 7). The probe was titrated on mouse brains infected with FrCasE and F43 and was used at the highest concentration that produced no cross-hybridization with the latter virus (Figure 7D). Viral RNA was detected in both vascular (Figure 7, black arrows) and parenchyma cells (Figure 7, white arrowheads). Many of the parenchymal cells contained paranuclear cytoplasmic vacuoles (Figure 7A) that sometimes filled the cytoplasm (Figure 7B). Cells exhibiting cytoplasmic dissolution, but with relatively intact nuclear chromatin structure, expressed clearly detectable, although low, levels of viral RNA (Figure 7C, white arrowhead). In contrast, cells exhibiting effaced cytoplasm and nuclei were consistently negative for viral RNA (Figure 7C, white arrow). Collectively, these results suggest that the spongiform retroviruses are directly toxic to oligodendrocytes, although as the cytopathology progresses, signs of virus infection wane.

Discussion

Here we have presented evidence that a major fraction of the cells that degenerate in both FrCasE- and MoMLV-ts1-infected mice are not neurons but instead belong to the oligodendrocyte lineage. Surprisingly, neuronal de-

generation appeared to account for only a minor component of the neuropathology induced by these viruses. The degenerating oligodendrocytes were identified by virtue of their expression of the bHLH transcription factor Olig2. Olig2 is an early marker of the oligodendrocyte lineage³⁸ that is expressed throughout oligodendrocyte development.³⁹ In the embryo, the expression of Olig2 is detectable in common multipotential progenitors of oligodendrocytes and neurons⁴⁰ as well as in a common progenitor of oligodendrocytes and astrocytes.⁴¹ However, during commitment to the neuronal or astrocyte lineage, Olig2 expression is extinguished. Thus, the expression of Olig2 by the majority of the vacuolated cells in this disease represents strong evidence that they belong to the oligodendrocyte lineage. The level of differentiation of these cells is not clear. These cells did not express PLP or MBP (markers of mature oligodendrocytes). However, both are cytoplasmic proteins and could have been lost because of the extensive cytoplasmic damage exhibited by these cells. It is also possible that these cells represent more immature forms. Although frank demyelination does not occur in this disease, ultrastructural evidence of hypomyelination has been reported,²⁹ having also been observed by us (not shown). This would appear to be more consistent with the loss of precursors than with the death of fully differentiated myelin-producing oligodendrocytes, as occurs in multiple sclerosis. We are currently examining the transcriptional profile of these cells, which may resolve this question.

The vacuolated oligodendrocytes in mice infected with both of the viruses studied here expressed the bZIP transcription factor CHOP, the up-regulation of which is a late event in what has been termed the integrated stress response (ISR).⁴² The ISR is initiated by at least four different protein kinases, each activated in response to different cellular stresses.⁴³ These kinases include GCN2, which is activated by amino acid deprivation; PERK, activated by ER stress; PKR, activated by the presence of the double-stranded RNA often associated

Figure 6. Infection and vacuolation of Olig2⁺ cells. **A:** Confocal images of the brainstem of an FrCasE-inoculated mouse at 16 days after inoculation stained for Olig2 (red) and viral envelope protein (green). 4,6-Diamidino-2-phenylindole (blue) stains nuclei. **B–E:** Two-color immunohistochemistry to illustrate the frequency of infected Olig2⁺ cells and proximity to spongiform pathology. Nuclei were not counterstained in these sections. **Black arrows** point to cells in which 697 (red) stains the cytoplasm, and anti-Olig2 stains the nucleus (brown). Infected Olig2⁺ cells were seen in both gray matter of the brainstem (**B–D**) and white matter of the corpus callosum (**E**). **C:** A 697⁺/Olig2⁺ cell, having the morphology of a microglial cell, is marked by a **white arrow**. **D:** An extensively vacuolated Olig2⁺ cell (**arrowhead**) is essentially negative for 697 staining. **F–H:** When sections were viewed by differential interference contrast microscopy, 697⁺/Olig2⁺ cells were observed with focal cytoplasmic vacuoles. **G:** However, as seen in **D**, 697 staining was weak, at best, in cells with more extensive coalescent vacuolation (**arrowhead**).

with virus infection; and HRI, activated in erythroid cells by heme deficiency.⁴⁴ The substrate for these kinases is the α subunit of the eukaryotic translation initiation factor 2 (eIF2 α). The phosphorylation of eIF2 α down-regulates protein synthesis and qualitatively alters the translational and, ultimately, the transcriptional profile of the cell, resulting in activation of the effector arms of the ISR, which restore homeostasis. Thus, the expression of CHOP in the degenerating cells, as well as the detection of increased phosphorylation of eIF2 α by Western blot in the brainstem of MoMLV-ts1-infected mice,³³ suggests that an ISR had been activated in these cells.

The nature of the cellular stress that activated the ISR is open to debate. On the one hand it is conceivable that cellular stress could be initiated by amino acid deprivation. The receptor for these viruses is the amino acid transporter CAT1,¹⁵ a cell surface protein that is down-

regulated by virus infection.⁴⁵ Nevertheless, there is little evidence that virus infection has an effect on amino acid transport that would be functionally meaningful.^{45,46} The activation of PKR by the presence of double-stranded RNA might also be considered. However, the highly neuroinvasive but nonpathogenic virus F43 infects the same populations of cells as does FrCasE, making PKR an unlikely candidate.

In vitro both FrCasE^{21,22} and MoMLV-ts1⁴⁷ express unstable envelope proteins that misfold in the ER and induce a classical unfolded protein response, associated with the up-regulation of ER chaperones, activation of ER-associated degradative machinery (ERAD), and the brisk up-regulation of CHOP. Although it is not feasible to assess protein folding in the ER *in vivo*, we found that F43 expresses higher steady-state levels of viral envelope protein than does FrCasE both *in vitro* and in the brains of

Figure 7. Viral RNA was expressed in vacuolated cells. Sections of FrCasE-infected brainstems were hybridized with a digoxigenin-conjugated envelope-specific anti-sense RNA probe¹³ and developed with alkaline phosphatase-conjugated anti-digoxigenin followed by Fast Red as substrate. **D:** F43-infected brainstem served as a control for specificity. Cells associated with the microvasculature (**arrows**) as well as parenchymal cells stained positive for viral RNA. **A:** Infected cells often exhibited focal cytoplasmic vacuoles (**arrowheads**). **B:** Vacuoles in some cells were extensive, filling much of the cytoplasm (**arrowheads**). **C:** A cell (**arrowhead**) containing large coalescent cytoplasmic vacuoles and also a clearly detected signal for viral RNA. Note that the nucleus of this cell still exhibits some chromatin structure, whereas the neighboring cell, which is negative for viral RNA (**white arrow**, C), shows complete cytoplasmic and nuclear effacement. Slides were viewed using differential interference contrast microscopy.

infected mice.²² This has suggested that the FrCasE envelope protein engages ER quality control systems in both settings. It is therefore reasonable to suggest that ER stress is a likely source of the activation of the ISR in the brain.

The finding that CHOP^{-/-} and CHOP^{+/+} mice were equally susceptible to the disease is not surprising, because CHOP appears not to be directly involved in the activation of cell-death pathways. Through transcriptional up-regulation of its downstream target, the eIF2 α phos-

phatase GADD34, CHOP effects a net decrease in phosphorylated eIF2 α .⁴⁸ Because CHOP^{-/-} cells express lower levels of GADD34 than CHOP^{+/+} cells, they maintain higher levels of phosphorylated eIF2 α , the effect of which will depend on the context. Perk^{-/-} cells⁴⁹ or eIF2 α mutant cells,⁵⁰ both of which are impaired in phosphorylation of eIF2 α , are rendered exquisitely sensitive to cell death driven by ER stress. On the other hand, GADD34^{-/-} cells, in which high levels of phosphorylated eIF2 α are maintained, are also hypersensitive to the lethal

effects of ER stress.⁵⁰ Thus, although CHOP expression is useful as a marker of cells undergoing ER stress, the net effect of CHOP deletion is unpredictable and could be neutral.

One issue that has been puzzling is the relative paucity of viral protein and RNA in the vacuolated oligodendrocytes. As mentioned above, ER stress is associated with the down-regulation of protein translation and an increase in protein degradation, the net effect of which is to decrease the client protein load on the folding capacity of the ER. The observation that steady-state levels of viral envelope protein are lower in the brains of FrCasE than in F43-infected mice²² is consistent with this effect. In addition, temporal studies of FrCasE-infected mice revealed that the steady-state levels of viral envelope protein in the brain decreases with time as the disease progresses.²⁷ These observations initially appeared counterintuitive, suggesting an inverse relationship between the level of neurotoxicity and levels of expression of the putative neurotoxic protein. This relationship now appears to be explained in the context of the folding instability of this protein, and its interactions with ER quality control systems. In the current study, this inverse relationship appeared also to be detected at the cellular level. Oligodendrocytes expressing high levels of viral protein exhibited focal cytoplasmic vacuolation, whereas those cells with the most extreme cytopathic effects appeared to be essentially virus-negative. *In situ* hybridization indicated that viral RNA was detectable, albeit at low levels, in cells exhibiting extensive cytoplasmic vacuolation. Thus, we postulate that the loss of viral protein and ultimately viral RNA likely reflects the impact of ER quality control combined with the progressive dismantling of the protein synthetic and, ultimately, the transcriptional machinery of the cell. The detection of CHOP and Olig2 in these cells would appear to be inconsistent with this hypothesis. It is possible, however, that the complexing of these transcription factors to DNA could provide a mode of protection from degradation.

Oligodendrocytes are not the only cells in the nervous system that are infected by these viruses. Endothelial cells, microglial cells, and astrocytes as well as some populations of neurons are also infected. Yet cytopathology has not been observed in these cells. This suggests that oligodendrocytes may be hypersensitive to the cellular stress induced by these viruses. Indeed, oligodendrocytes, especially in the developing brain, are known to be particularly vulnerable to ER stress⁵¹ as well as to the reactive oxygen intermediates⁵² that are overproduced during ER stress.⁴² It is thought that this may be a consequence of the requirement for synthesis of large amounts of myelin, which places a heavy burden on their secretory system as well as to their relatively high content of free iron,⁵³ potentially promoting the generation of extremely toxic oxidation products. Whatever the nature of the insult, not all oligodendrocytes appeared to be equally susceptible, because some infected oligodendrocytes, particularly within white matter tracts, appeared to be unaffected by these viruses. This suggests that factors in addition to virus infection influence cellular vulnerability. Such factors might include the stage of

differentiation of infected cell or perhaps the local environment in which it resides.

The toxicity of these exogenous retroviruses to oligodendrocytes suggests the possibility that retroviral envelope proteins of endogenous origin might have similar effects. The vast majority of human endogenous retroviruses are immobile replication-defective elements.⁵⁴ Because these elements do not go through an extracellular phase, their envelope genes have not been subjected to the selective pressure driving folding stability and thus may be more prone to engaging ER quality control systems. Although an association has been reported between the expression of certain human endogenous retrovirus genes and inflammatory lesions of multiple sclerosis,^{55,56} endogenous retroviruses are known to be activated in response to inflammatory mediators. Thus, this could represent an epiphenomenon. However, in very early lesions observed in patients with relapsing-remitting multiple sclerosis, extensive death of oligodendrocytes has been observed⁵⁷ in the absence of signs of inflammation. This study has raised questions about the role of inflammation in multiple sclerosis and has suggested that, at least in some forms of the disease, the death of oligodendrocytes may represent the initial insult, with inflammation coming later. The cause of cell death in these early lesions is unknown but might be fertile ground in the search for a role of human endogenous retroviruses in this disease.

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