Brain Infection by Neuroinvasive but Avirulent Murine Oncornaviruses

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Received 6 July 1999/Accepted 27 September 1999

The chimeric murine oncornavirus FrCasE causes a rapidly progressive noninflammatory spongiform encephalomyelopathy after neonatal inoculation. The virus was constructed by the introduction of pol-env sequences from the wild mouse virus CasBrE into the genome of a neuroinvasive but nonneurovirulent strain of Friend murine leukemia virus (FMuLV), FB29. Although the brain infection by FrCasE as well as that by other neurovirulent murine retroviruses has been described in detail, little attention has been paid to the neuroinvasive but nonneurovirulent viruses. The purpose of the present study was to compare brain infection by FrCasE with that by FB29 and another nonneurovirulent virus, F43, which contains pol-env sequences from FMuLV 57. Both FB29 and F43 infected the same spectrum of cell types in the brain as that infected by FrCasE, including endothelial cells, microglia, and populations of neurons which divide postnatally. Viral burdens achieved by the two nonneurovirulent viruses in the brain were actually higher than that of FrCasE. The widespread infection of microglia by the two nonneurovirulent viruses is notable because it is infection of these cells by FrCasE which is thought to be a critical determinant of its neuropathogenicity. These results indicate that although the sequence of the envelope gene determines neurovirulence, this effect appears to operate through a mechanism which does not influence either viral tropism or viral burden in the brain. Although all three viruses exhibited similar tropism for granule neurons in the cerebellar cortex, there was a striking difference in the distribution of envelope proteins in those cells in vivo. The FrCasE envelope protein accumulated in terminal axons, whereas those of FB29 and F43 remained predominantly in the cell bodies. These observations suggest that differences in the intracellular sorting of these proteins may exist and that these differences appear to correlate with neurovirulence.

Infection of the central nervous system by murine oncornaviruses results in two distinct forms of chronic neurologic disease (34). Those viruses of the ecotropic host range cause a spongiform encephalomyelopathy primarily affecting the motor centers of the brain and spinal cord. Clinically this disease is manifested by tremor, paralysis, and wasting. The prototype of this group of neurovirulent viruses is ecotropic virus CasBrE (15), originally isolated from wild mice, but other laboratory isolates belonging to the Moloney (47) and Friend (26) strains of murine leukemia virus (MuLV) have also been shown to induce an indistinguishable disease in either mice or rats. Viruses belonging to the polytropic host range group cause a different disease manifested primarily by ataxia and seizures (33, 37, 39). Spongiform degeneration is only rarely noted (33), pathological changes being restricted to astroglial and microglial activation (39). Despite these differences in the character of the diseases, in both models neurovirulence is determined by the sequence of the envelope gene encoding the SU protein (10, 17, 31, 32, 39) and also correlates with the capacity of these viruses to infect microglial cells (39), the resident macrophages of the brain.

During our initial studies of CasBrE we found that after neonatal inoculation this virus infected the brain at relatively low levels, a property which appears to be responsible for the long incubation period of the neurologic disease caused by this virus (3 to 6 months). We reported that another oncornavirus, a strain of Friend MuLV (FMuLV) called FB29 (42), infected the brain at high levels but did not cause clinical neurologic disease (32). When the envelope gene of CasBrE was intro-

465
fragment was ligated into the Ndel- and ClaI-digested FB29 plasmid as described previously (32). The resulting chimeric genome was excised from the plasmid with HindIII and transfected into Mus dunni cells (mouse fibroblasts) (20) by calcium phosphate precipitation as described previously (32). When the infection reached confluence, supernatants were collected as virus stocks and frozen in aliquots at −80°C.

Mice, virus inoculations, and clinical evaluations. IRW (inbred Rocky Mountain White) mice (32) were bred and raised at the Rocky Mountain Laboratories (RML) and were handled according to the policies of the RML Animal Care and Use Committee. Virus inocula were in the form of tissue culture supernatants containing White) mice (32) were bred and raised at the Rocky Mountain Laboratories (RML) and were handled according to the policies of the RML Animal Care and Use Committee. Virus inocula were in the form of tissue culture supernatants from virus-infected M. dunni cells and contained 2 × 10^6 to 6 × 10^6 focus-forming units of infectivity per ml. Mice were inoculated with 30 μl of virus stock intraperitoneally 24 to 48 h after birth and were observed for appearance of clinical disease beginning at 11 days postinoculation (p.i.) as previously described (32). FrCasS5-inoculated mice all develop signs of severe tremulous paralysis by 14 to 16 days p.i. (32) and were routinely euthanized at that time. Mice inoculated with FB29 or F43 were sacrificed for removal of tissues or were euthanized at 2 to 3 months p.i., when they had developed splenomegaly due to erythroleukemia (42).

Quantification of virus. Virus titrations were carried out by a focal infectivity assay, with anti-gp70 monoclonal antibody 667 (29) to detect foci of infection in M. dunni cells as previously described (5). Viral protein in the brain was analyzed either semiquantitatively by Western blot analysis or quantitatively by enzyme-linked immunosorbent assays (ELISA). Ten percent brain homogenates were prepared in 0.5% NP-40 containing 0.01 M Tris base, 0.15 M NaCl, 0.001 M EDTA (pH 7.4), leupeptin (0.5 μg/ml), aprotinin (1 μg/ml), pepstatin A (0.7 μg/ml), and Pefabloc (24 μg/ml) with 20 strokes of a Dounce homogenizer as previously described (8). For Western blot analysis, homogenates were boiled in 2% sodium dodecyl sulfate–5% 2-mercaptoethanol and resolved by electrophoresis in 9% polyacrylamide gels. Gels were electrophoresed onto Immobilon P membranes (Millipore) probed with antisera to viral capsid protein (g30) of CasBrE (8) or antisera to FMuLV envelope proteins SU (gp70) and TM (p15E). The goat anti-gp70 antisera was a gift from Roland Friedrich (Giesen, Germany), and the anti-TM was a gift from Gerhard Hunsmann (Göttingen, Germany). The use of all antisera in immunoblot analysis has been described previously (8). For Western blot analysis, homogenates were boiled in 2% sodium dodecyl sulfate–5% 2-mercaptoethanol and resolved by electrophoresis in 9% polyacrylamide gels. Gels were electrophoresed onto Immobilon P membranes (Millipore) probed with antisera to viral capsid protein (g30) of CasBrE (8) or antisera to FMuLV envelope proteins SU (gp70) and TM (p15E).

The goat anti-gp70 antisera was a gift from Roland Friedrich (Giesen, Germany), and the anti-TM was a gift from Gerhard Hunsmann (Göttingen, Germany). The use of all antisera in immunoblot analysis has been described previously (29, 39). Immunoblots were developed with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG; Bio-Rad) or HRP-conjugated anti-goat IgG (ICN Biomedicals Inc.) and ECL chemiluminescent substrate (Amersham). Blots were exposed to Kodak XOMAT R film, the images were digitized with an HP 5100 scanner, and bands were quantified with the ImageQuant program (Molecular Dynamics). Antigen-capture ELISA was performed on brain homogenates as described previously (14) with anti-g30 monoclonal antibody 18-7 (4) for antigen-capture and the rabbit anti-p30 described above for detection. Results were standardized with a Triton X-100 extract of sucrose density gradient-purified virus.

Pathology and immunohistochemistry. Mice were exsanguinated by axillary exsanguination under deep methoxyflurane inhalation anesthesia. Brains were fixed by immersion in 3.7% formaldehyde–phosphate-buffered saline (PBS) for 16 h. Brains were processed for routine histopathology and immunohistochemistry by dehydration and paraffin embedding. For all studies involving F4/80 staining, including dual-color immunohistochemistry, brains were cryopreserved by immersion in 25% sucrose (23), frozen in OCT (Miles) in liquid nitrogen, and stored at −80°C. Paraffin sections were either stained with hematoxylin and eosin or subjected to heat-induced antigen retrieval and stained for viral envelope protein, as described previously (37), with goat anti-gp70 antisera kindly provided by Roland Friedrich and 3-amino-9-ethyl-carbazole as the substrate. F4/80 was stained by a slight modification of a procedure reported by Lawson et al. (21). Briefly, 5-μm-thick frozen sections were stained with rabbit anti-F4/80 antisera, which is kindly supplied by Andrew McKnight (Windesey Institute of Medical Science, London, United Kingdom) and which was diluted 1:1,000 in PBS-0.1% Triton X-100 (PBS-Triton). Sections were incubated for 15 min at room temperature with PBS-Triton and then incubated at 37°C for 2 h with anti-F4/80 followed by biotinylated goat anti-rabbit IgG (Vector) at 37°C for 1 h. Sections were treated with 0.03% H2O2 in PBS for 15 min at room temperature, washed in PBS-Triton, and developed with Elite avidin-biotin complex (Vector) for 45 min at room temperature. After being rinsed in PBS-Triton, slides were exposed to the substrate diaminobenzidine (DAB) (Research Genetics) for 5 to 10 min. Glial fibrillary acidic protein (GFAP) was detected in paraffin-embedded material with a rabbit anti-bovine GFAP as previously described (33). Double staining for viral envelope was done after staining for either F4/80 or GFAP and was performed after sections were developed with DAB. For example, frozen sections were first stained with rabbit anti-F4/80 or GFAP and then bound antibody was detected with HRP-conjugated anti-rabbit Ig. The sections were then incubated with DAB to develop the color reaction (yellow-brown) and afterwards heated to 100°C in citrate buffer, pH 6.0, for 30 min (heat-induced antigen retrieval); this was followed by treatment with 0.03% H2O2 in PBS for 15 min at room temperature prior to staining with goat anti-gp70. The heating step accomplished two things, enhancement of the immunoreactivity of gp70 in the tissues and inactivation of HRP bound to the tissues as part of the first staining procedure. This latter issue is important because the detecting reagent for the anti-gp70 staining procedure was HRP-conjugated anti-goat Ig. Finally the second substrate, blue-colored VIP (Vector), was added. The sections were not counterstained so as to avoid confusion with the colors of the respective HRP substrates. Appropriate controls were used to rule out cross-reactivity of the second anti-Ig antiseraum with the heterologous first antibody (i.e., rabbit anti-F4/80 followed by HRP-conjugated anti-goat Ig; goat anti-gp70 followed by HRP-conjugated anti-rabbit Ig). These controls were consistently negative. Since both of the second antibodies in this study (anti-goat and anti-rabbit Ig) were HRP conjugated, controls were included to rule out HRP carryover. Frozen sections were stained with rabbit anti-F4/80 followed by HRP-conjugated anti-rabbit Ig, but without the addition of DAB substrate. These sections were then heat treated and incubated with H2O2 but were not incubated with the goat anti-gp70. They went directly into the second substrate (blue-colored VIP). These controls were consistently negative, indicating that HRP from the F4/80 stain had been effectively inactivated prior to addition of the anti-gp70 antiserum. It should be noted that the use of frozen sections in this study was
Quantification of F4/80 mRNA in the brain.

Three mice per group were sacrificed at 14 days p.i. by exsanguination under methoxyflourane anesthesia, and the brains were removed and snap frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Life Technologies) and was subjected to an RNase protection assay using $^{32}$P-labeled multiprobe kit mCD-1 (Pharmingen) according to the manufacturer’s instructions. Protected RNA was electrophoretically separated on preformed polyacrylamide gels (Pharmingen), and the gels were dried and analyzed with a STORM PhosphorImager (Molecular Dynamics). The F4/80 band was quantified with the ImageQuant program (Molecular Dynamics).

Statistical analysis.

All quantitations were done at least in triplicate, and the results were expressed as the means ± 1 standard deviation. Data was analyzed by the Mann-Whitney two-tailed $t$ test.

RESULTS

Viruses containing Friend envelope do not cause neurologic disease. In this study we compared brain infection by the three viruses shown schematically in Fig. 1. FB29 is the prototype into which the 3’ pol and env sequences from FMuLV 57 and CasBrE were introduced to generate the chimeric viruses F43 and FrCasE, respectively. Thus, the chimeric viruses share all sequences with FB29 except for 3’ pol and env. Since FB29 and FMuLV57 are both FMuLVs, the sequences of their envelope proteins are >97% identical. In contrast, CasBrE has evolved in wild mice and is only 79% identical to FB29 in the 3’ pol-env region. Neonates were inoculated intraperitoneally with each virus and observed for evidence of tremor and/or paralysis (Fig. 1). All mice inoculated with FrCasE reached the terminal stage of paralysis by 16 to 18 days, whereas none of the mice inoculated with FB29 or F43 exhibited clinical signs of neurologic disease, even by 3 months of age. At that time point the FB29 and F43 mice were euthanized because they had developed erythroleukemia (42).

Pathological evaluation of FB29- and F43-inoculated mice. Mice were sacrificed at 16 days p.i., a time when all FrCasE-inoculated mice exhibited severe clinical disease. Coronal and sagittal sections of the brain at multiple levels from brain stem to olfactory bulbs were evaluated by routine hematoxylin and eosin staining. As described previously (8), FrCasE-inoculated mice exhibited widespread spongiosis in the brain stem, subcortical gray matter, and deep cerebral cortex (Fig. 2). Despite the lack of clinical signs in either FB29 or F43 mice, even at 16 days p.i., the mice inoculated with FB29 exhibited foci of spongiosis primarily localized to the subcortex and brain stem (Fig. 2). Spongiosis was a consistent finding in the six FB29-inoculated mice examined at this time point but was limited in extent compared to that in mice inoculated with FrCasE. The lack of clinical disease in the FB29-inoculated mice likely was a consequence of the restricted and focal nature of the lesions. Clinical disease is seen in FrCasE-inoculated mice only when the spongiosis becomes extensive (8, 24). In contrast, spongiosis was a rare and inconsistent finding in F43-inoculated mice and then was only observed in small foci in the brain stem (Fig. 2). This phenotypic difference between FB29 and F43, despite the high degree of sequence homology, prompted us to continue to include both viruses in this study.

Microglial and astrocytic response. We previously have shown that, despite the high-level infection of microglia by FrCasE, there appears to be no increase in immunostaining of
microglia for Mac-1 (CD11b) or F4/80 (23, 24), both of which are markers of microglial activation. In addition, there appeared to be no noticeable increase in GFAP staining, indicative of astrocytic activation, unless the disease course was slowed by dilution of the virus inoculum (8). Staining of brain sections from FB29- and F43-inoculated mice at 16 days p.i. for F4/80 and GFAP demonstrated a similar lack of evidence of upregulation of F4/80 and only minimal focal increases in GFAP staining in comparison to uninoculated controls (not shown). In order to apply a more rigorous measure of glial activation, GFAP was quantified in whole-brain extracts by Western blot analysis (Fig. 3A) and F4/80 mRNA was quantified by RNase protection assay (Fig. 3B). Although there was considerable variation in the GFAP signal among the infected mice, it was clear that overall levels of GFAP were elevated in the infected mice, analyzed as a group (P = 0.01 compared to uninoculated controls) (Fig. 3A). However, no significant differences in GFAP levels among the infected groups were observed. Despite the increased expression of GFAP, there was no evidence of upregulation of the microglia-specific marker F4/80 in any of the virus-infected groups (Fig. 3B). Thus, there was no measurable difference in the activation of either microglia or astrocytes by either of these viruses, irrespective of whether they induced neurologic disease.

Lack of correlation between clinical disease and viral burden in the brain. Since viral burden has been shown to be an accurate predictor of the incubation period for neurologic disease.

FIG. 3. Quantification of markers of glial activation in mice 16 days after inoculation of FrCasE, F43, or FB29, a time point at which all FrCasE-inoculated mice exhibited severe neurologic disease. Upregulation of GFAP (A) is a marker of astrocytic activation (12) and was measured by semiquantitative Western blotting. Bands developed by chemiluminescence were quantified after digitization with ImageQuant (Molecular Dynamics) software and are expressed in terms of volume as a measure of relative signal strength. There was a significant difference (P = 0.01) between the infected mice (grouped together) and the uninoculated controls, but no significant differences between the infected groups were found. Upregulation of F4/80 (B) is a marker of microglial activation (2) and was measured at the mRNA level by an RNase protection assay. Data is expressed as percentage of the signal from the “housekeeping” gene encoding GAPDH. There was no evidence for upregulation of F4/80 in any of the infected mice.

FIG. 4. Measurements of viral burden in the brain. Mice were infected intraperitoneally as neonates and sacrificed at 14 days p.i. when clinical signs of tremor and paralysis had appeared in the FrCasE-inoculated mice. Ten percent brain homogenates were analyzed for capsid protein (p30) either by polyacrylamide gel electrophoresis and immunoblot analysis with rabbit anti-p30 antiserum (A) or by antigen capture ELISA (B). Immunoblots were performed on two mice per group and are shown with positive controls on the right from extracts of M. dunni cells infected with either FrCasE or F43. ELISA was carried out on larger numbers of mice, and results were expressed as means ± standard deviation in units of nanograms of p30 per milligram of wet brain. Results indicated that both of the nonneurovirulent viruses (FB29 and F43) exhibited higher viral burdens in the brain than the neurovirulent virus (FrCasE). Immunoblot analysis (C) of brain extracts probed with anti-p30 antiserum compares the signal strengths for samples from mice inoculated with F43 at 2 weeks p.i. with that at 4 weeks p.i. Bands were quantified after digitization as described in the legend to Fig. 3.
ease induced by viruses carrying the CasBrE envelope gene (7), it was of interest to examine this parameter in mice inoculated with FB29 and F43. Mice were sacrificed at 14 days p.i., a time when FrCasE-inoculated mice were beginning to exhibit signs of clinical disease. Both Western blot analysis (Fig. 4A) and ELISA (Fig. 4B) for viral capsid protein revealed, surprisingly, that the average viral burdens of FB29 and F43 were two- to fourfold higher than that of FrCasE. Furthermore, Western blot analysis for capsid protein indicated that viral burdens of both F43 and FB29 (not shown) continued to increase between 2 and 4 weeks p.i. (Fig. 3C), suggesting continued virus spread within the brain. These results have been confirmed by Western blot analysis using antisera specific for the envelope proteins gp70 (SU) and p15E (TM) (not shown). Thus, the lack of clinical signs of neurologic disease and the absence or restricted nature of the spongiosis in mice infected with F43 and FB29 viruses, respectively, were clearly not due to lower levels of virus in the brain.

Identification of infected cells in the brain. To determine if the difference in the neurovirulence of these viruses was a consequence of differences in cell types infected in the brain, immunohistochemistry was performed. Mice neonatally infected with F43, FB29, and FrCasE were sacrificed at day 16, when all the animals infected with FrCasE virus were showing signs of severe clinical disease and widespread spongiosis. FrCasE has previously been shown to infect cells of the central nervous system microvasculature (endothelial and perivascular microglial cells), parenchymal microglia, and certain populations of neurons which divide postnatally (23). We compared the distribution of the viruses F43, FB29, and FrCasE by immunohistochemistry with anti-gp70 antiserum.

The distribution and morphology of the infected cells in mice infected with these three viruses were indistinguishable (Fig. 5 compares FrCasE and F43) and included cells associated with the microvasculature (Fig. 5) as well as highly arborized cells resembling parenchymal microglia (Fig. 5) and neurons in the cerebellum (Fig. 7), hippocampus, and olfactory bulbs (not shown). The identity of the highly arborized glial cells infected with F43 was demonstrated by two-color immunohistochemistry (Fig. 6) with rabbit anti-F4/80 antiserum, which has been shown to stain all microglia, since it detects even the low constitutive levels of F4/80 (Fig. 3B) expressed by resting cells (21). It was clear that in F43-infected mice, gp70 staining (blue color in Fig. 6) colocalized with F4/80 (brown color in Fig. 6), indicating that the predominant glial element infected by this virus was the parenchymal and perivascular microglial cell. Similar results were obtained with FB29 (not shown). Thus, the nonneurovirulent viruses F43 and FB29, like FrCasE, infected large numbers of microglia throughout the brain including regions known to be vulnerable to the spongiosis induced by the neurovirulent virus FrCasE.

Although the vascular and microglial infections caused by these three viruses appeared morphologically indistinguishable, there was a notable difference in the neuronal infection (Fig. 7). It has been previously shown that FrCasE infects the granule neurons of the cerebellar cortex (23), a population of neurons which was also found here to be infected by F43 (Fig. 7, left) and FB29 (not shown). The distributions of the respec-
tive envelope proteins in these cells, however, appeared to be strikingly different. Previous studies found that the envelope protein of FrCasE accumulates in the cell bodies of granule cells (Fig. 7, left) as well as in their distal axons, which make up the parallel fibers of the molecular layer of the cerebellar cortex (Fig. 7, right). In contrast, the envelope proteins of F43 and FB29 (not shown) were restricted primarily to the cell bodies in the granule layer with only focal radial staining in the molecular layer (Fig. 7, right), likely representing the proximal axons of granule neurons (see cartoon in right panel of Fig. 7). This suggested a difference in the transport of these envelope proteins in neuronal cells and that this difference appeared to correlate with neurovirulence.

**DISCUSSION**

Much of the work on the nature of the neurovirulence of murine oncornaviruses has focused on the neuropathogenic viruses. Here we have characterized the brain infection by viruses which do not cause clinical disease in order to begin to understand elements of the infection which are disease specific. At a time when the FrCasE mice were in the terminal stage of paralysis, viral burden in the brain was actually two- to threefold lower than that of the clinically unaffected mice which had been inoculated with FB29 and F43. Despite the continued spread of virus and progressively increasing viral burden over a 1- to 2-month period of observation, these mice never exhibited signs of tremulous paralysis or wasting seen in FrCasE-inoculated animals. These observations clearly indicate that neurovirulence can be uncoupled from viral burden, an observation which has also been made in the polytropic murine retrovirus model (37).

Since infection of microglial cells is required for FrCasE neurovirulence (24, 25), it is possible that the sequence differences within the envelope genes of FB29 and F43 altered the tropism of these viruses for microglial cells. The results of the present study provide convincing evidence that this is not the case. The infection of microglia by all three viruses was verified by showing colocalization of the viral envelope protein and the microglia-specific marker F4/80. We specifically focused on regions of the brain known to be susceptible to the neuropathogenicity of FrCasE, namely, the deep layers of the cerebral cortex and thalamus as well as the vestibular nucleus of the brain stem (not shown) (8). All three viruses infected abundant microglia within these regions of the brain, indicating that differences in viral tropism for these cells do not explain the striking differences in neurovirulence of these viruses. Furthermore, since the sequence differences between the neurovirulent and avirulent viruses reside primarily within the envelope gene (Fig. 1), the neurotoxicity which appears to
be induced by the expression of this protein in microglial cells must be sequence specific.

Despite the use of the same receptor by both viruses in vivo (6), the envelope proteins of FrCasE and FB29 have only 75% homology (Fig. 1). Unfortunately, the differences are scattered throughout the envelope protein including variable region A (VRA), which encompasses the receptor binding surface of the molecule (13). This makes predictions of structure and function impossible, and, to date, there have been no fine-mapping studies reported for this model, which would further localize the relevant sequences within the envelope protein which are involved in neurovirulence. It is, however, worth considering the sequences which might be responsible for the difference in the neuropathogenicities of F43 and FB29, viruses which exhibit 97% amino acid sequence identity of their envelope proteins. Unlike F43, FB29 consistently induced focal spongiosis, particularly in the subcortex and brain stem (Fig. 2). Of the 19 differences within the envelope proteins of F43 and FB29, 3 are shared between FB29 and FrCasE (serine80→valine, serine84→alanine, and threonine171→serine). Interestingly, serines 80 and 84 are located within VRA and threonine 171 is located in the α helix of the VRB domain, which may be involved in stabilizing the structure of the receptor binding domain (3). If one extends this comparison to another strain of FMuLV (PVC211) (27), which also causes a spongiform encephalopathy and whose envelope protein is 96% identical (38) to that of F43, one finds that the serine84→alanine change is seen in this virus as well. Thus, one might speculate that the induction of spongiosis by FB29 as well as PVC211 may be a consequence of their containing a subset of critical envelope sequences which are also involved in the neurovirulence of FrCasE. The location of these sequences in a region of the protein involved in receptor interaction suggests the importance of this interaction in the neurovirulence of these viruses. It should be noted that the receptor for these viruses functions as a transporter for cationic amino acids (1, 44). Although the evidence suggests that binding of the envelope protein of Rauscher ecotropic virus to the receptor interferes only marginally with arginine transport in fibroblasts (43), this appears to be a consequence of the limited accessibility of the receptor for the envelope protein due to glycosylation of the receptor (45). It is not known whether more-dramatic effects on arginine transport might perhaps be caused by the envelope protein of FrCasE when expressed in microglial cells.

The lack of upregulation of F4/80 mRNA in the brains infected by any of the viruses examined in this study confirmed previous immunohistochemical studies which showed a similar lack of upregulation of either Mac-1 (CD11b) or F4/80 pro-
proteins (23, 24) coincident with the appearance of spongiform lesions. The upregulation of this membrane protein is a sensitive marker of microglial activation (2), and recent studies of mouse scrapie indicate that increased expression of F4/80 protein is associated with increased levels of mRNA as well (9, 46). It is possible that the lack of microglial activation induced by the viruses examined in this study was a consequence of the early time points at which the brains were analyzed (16 days p.i.). We have examined brains from mice inoculated with F43 at 4 weeks p.i. with similar results (not shown). In addition, mice inoculated with polytropic murine retroviruses and examined at 15 to 18 days p.i. consistently demonstrated microglial activation spatially coincident with the sites of virus infection in the brain (39). Thus, the lack of response seen in the present study appears to be virus specific. Despite the lack of response by microglial cells, astrocytes did appear to respond to all three viruses studied here, as revealed by the increased steady-state levels of GFAP detectable in brain homogenates. Immunohistochemical studies, however, indicated that the increase in GFAP-positive astrocytes in the brains of infected mice was minimal (not shown). At present, the relative transparency of these viruses in the mouse brain is unexplained. These results, however, confirm our previous contention that neither microglial nor astrocytic activation is a correlate of neurovirulence in this model (24).

There was one feature of the infection by FrCasE and the nonneurovirus proteins which was strikingly different. We have previously shown by immunohistochemistry, in situ hybridization, and electron microscopy that FrCasE infects granule neurons in the cerebellar cortex (23). The envelope protein is detectable both in the cell bodies and the distal axons of granule neurons in the cerebellar cortex (23). The envelope proteins were found to localize primarily in the cell bodies and virus assembly occurred in those cell bodies and dendrites of the granule neurons. These observations indicate that the accumulation of the envelope protein of FrCasE in the distal axons must be a consequence of protein transport to that site. F43 and FB29 (not shown) also infected these neurons, but the respective envelope proteins were found to localize primarily in the cell bodies and proximal axons, not in the distal axons (parallel fibers). Although this difference in localization of envelope proteins was striking, it should be emphasized that there is no evidence that infection of these cerebellar cortical neurons is involved in the pathogenesis of the spongiosis induced by FrCasE (24). While the infection of granule neurons is widespread and highly productive (23), degenerative changes are not observed, even when viewed ultrastructurally. The neurons which do undergo degenerative changes are not infected (16, 19, 23). It is infection of neighboring microglial cells which appears responsible for the neuronal damage (24, 25). On the other hand, since the sequence of the envelope protein appears to determine neurovirulence, the difference in the distribution of this protein in cerebellar cortical neurons may be telling us something important about differences in the way the respective proteins interact with cellular proteins, a feature which correlates with neurovirulence.

It is the expression of the FrCasE envelope protein in microglial cells, not neurons, however, which appears to be responsible for the spongiosis induced by this virus (24, 25). Interestingly, nonpolarized cells also recognize these signals and sort apical and basolateral proteins in distinct vesicular compartments (48), and in some cases, it has been shown that these proteins are delivered to distinct domains in the plasma membrane (40). Thus, the striking difference in the distribution of the envelope proteins of F43 and FrCasE in cerebellar granule neurons may reflect differences in the way the respective proteins are handled by nonpolarized cells such as microglia. Whether this putative difference in protein sorting is relevant to neurovirulence is being examined genetically.

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