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Late Virus Replication Events in Microglia Are Required for Neurovirulent Retrovirus-Induced Spongiform Neurodegeneration: Evidence from Neural Progenitor-Derived Chimeric Mouse Brains

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CasBrE is a neurovirulent murine retrovirus which induces a spongiform myeloencephalopathy in susceptible mice. Genetic mapping studies have indicated that sequences responsible for neurovirulence reside within the *env* gene. To address the question of direct envelope protein neuroxicity in the central nervous system (CNS), we have generated chimeric mice expressing the CasBrE envelope protein in cells of neuroectodermal origin. Specifically, the multipotent neural progenitor cell line C17.2 was engineered to express the CasBrE *env* gene as either gp70/p15E (Cas^{E}) or gp70 alone (Cas^{ES}). Cas^{E} expression in these cells resulted in complete ($>10^{5}$) interference of superinfection with Friend murine leukemia virus clone FB29, whereas Cas^{ES} expression resulted in a 1.8-log-unit decrease in FB29 titer. Introduction of these envelope-expressing C17.2 cells into the brains of highly susceptible IRW mice resulted in significant engraftment as integral cytoarchitecturally correct components of the CNS. Despite high-level envelope protein expression from the engrafted cells, no evidence of spongiform neurodegeneration was observed. To examine whether early virus replication events were necessary for pathogenesis, C17.2 cells expressing whole virus were transplanted into mice in which virus replication in the host was specifically restricted by *Fv-1* to preintegration events. Again, significant C17.2 cell engraftment and infectious virus expression failed to precipitate spongiform lesions. In contrast, transplantation of virus-expressing C17.2 progenitor cells in the absence of the *Fv-1* restriction resulted in extensive spongiform neurodegeneration by 2 weeks postengraftment. Cytological examination indicated that infection had spread beyond the engrafted cells, and in particular to host microglia. Spongiform neuropathology in these animals was directly correlated with CasBrE *env* expression in microglia rather than expression from neural progenitor cells. These results suggest that the envelope protein of CasBrE is not itself neurotoxic but that virus infectious events beyond binding and fusion in microglia are necessary for the induction of CNS disease.

The neurovirulent ecotropic murine retroviruses, CasBrE and Moloney ts1, cause progressive, noninflammatory spongiform neurodegenerative disease in motor areas from the spinal cord through the neocortex when inoculated into susceptible neonatal mice (reviewed in references 19, 58, and 60). They provide an appealing model for helping to dissect the poorly understood mechanisms by which retroviruses induce neuropathologic changes in the central nervous system (CNS). The appearance of spongiform lesions can arise as early as 10 days postinoculation (7, 10, 30, 39) and most closely correlates with the level of viral infection in the CNS. Viral infection occurs in a variety of cell types, including vascular, neuroectodermal, and bone marrow-derived elements; however, cytopathology of the infected cells is not a primary feature (2, 3, 23, 29). In contrast, the primary degenerating cell type, the motor neuron, is not infected by the neurovirulent viruses, indicating that murine retroviruses induce spongiform neurodegeneration by an indirect mechanism.

Immunocytochemical and histologic analyses with the CasBrE and ts1 disease models indicate that virus expression in the bone marrow-derived microglia most closely correlates with the appearance of spongiform neurodegeneration (2, 3, 17, 29). Infected microglial cells were directly implicated in the pathogenic process when transplantation experiments showed that CasBrE-infected microglia could induce focal spongiform changes (31). However, that study did not rule out the possibility that the expression of neurovirulent virus by any cell type in close proximity to susceptible neurons was sufficient for the induction of neurodegeneration. In this regard, several groups have suggested that the neurovirulent retrovirus infection in endothelial cells (32), oligodendroglia (36, 37, 39), or astrocytes (47) might play causative roles in the neuropathogenic process (reviewed in reference 58).

Molecular genetic analyses of neurovirulent murine retroviral genomes indicate that the primary viral determinants responsible for neurovirulence reside within the *env* gene (11, 40, 41, 61, 62). To determine whether the CasBrE *env* gene alone was sufficient to induce neurodegeneration, Kay et al. generated CasBrE *env* transgenic mice (22). Although protein expression in these mice could not be detected, low levels of CasBrE *env* mRNA were demonstrated by RNase protection analysis. This expression was associated with the appearance of mild pathologic changes in a significant number of the transgenic mice after 15 to 24 months. These results suggested that *env* gene expression is involved in the induction of neurode-

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generation. However, because *env* expression levels were so low and the incubation period was so extended, the cellular site(s) of *env* expression and whether the Env protein requires interactions with other retroviral proteins could not be ascertained. Thus, questions remain as to whether expression of CasBrE *env* alone is sufficient to induce acute neurodegeneration or whether its toxicity is cell type dependant.

To address the issues of cell type specificity and direct toxicity of the envelope, we have developed an alternative approach for expressing the CasBrE *env* gene in the CNS. This involves the introduction of the envelope gene or restricted replication-competent virus into clonal multipotent neural progenitor cells in culture followed by their transplantation into the brains of susceptible mice. These progeneitors have been shown to engraft into the brains of developing mice, where they appear to become functionally and histologically integrated and capable of constitutively expressing exogenous genes (reviewed in reference 49). While to date these cells have been employed to distribute therapeutic gene products (25, 52; reviewed in reference 53), we reasoned that such cells could also be used to resolve certain questions about the biologic significance or toxicity of particular genes. Taking advantage of this unique delivery system, we report that neither the CasBrE envelope protein nor virus expression disseminated from these neural progenitor cells throughout the brains of susceptible mice is sufficient for the acute induction of spongiform neurodegeneration. Our results suggest that neuropathologic changes in the brain are precipitated by CasBrE virus life cycle events beyond virus binding and entry, in endogenous microglial cells.

MATERIALS AND METHODS

DNA constructs and viruses. CasBrE envelope protein expression constructs were generated as diagrammed in Fig. 1A to yield CasBrE SU/TM or SU alone. The 15-1 clone of CasBrE (41) was digested with *Eag*I and *Afl*II to yield a 2.2-kb fragment containing the *env* gene. This fragment was blunted with Klenow fragment and inserted into pSP72 (Promega) digested with *Sma*I. Clones were selected to contain the 3′ end of *env* adjacent to the *Eco*RI site in the polylinker region for subsequent directional cloning into the mammalian retroviral expression vector pSFF (5). pSP72/15-1*env* was cut with *Eco*RI and *Xho*I, and the 2.2-kb *env* region was ligated into pSFF cut with *Eco*RI and *Xho*I. The resulting vector (pCas^{E} [Fig. 1A]) was transfected into PA317 cells (35) to produce virus. The vector spread through the PA317 cell culture as a result of the expression of CasBrE envelope protein conferring ecotropic specificity, as was previously described for another CasBrE *env* construct (28). Viral titers of 10^5 to 10^6 focus-forming units (FFU) per ml were obtained without detectable helper virus. The Cas^{E} virus was then used to infect C17.2 neural progenitor cells as described below.

To generate a secreted form of the CasBrE envelope protein (Cas^{ES}), the p15E portion of the envelope protein was deleted, and two stop codons were placed in frame at the C terminus of the gp70-coding region corresponding to the normal gp70/p15E proteolytic cleavage site. This was accomplished by cutting pSP72/15-1*env* with *Bst*EII and *Eco*RI, isolating the 3.0-kb piece, and reconstructing the C-terminal portion of gp70 and the *Eco*RI site with four overlapping oligonucleotides, as shown in Fig. 1A, as follows: sense strand, oligonucleotides 1 (5′-GTGACCTACCACTCCCCTGACTATGTCTATACTCAGTTTG AACCAGGGGC-3′) (50-mer) and 2 (5′-CAGATTCCGAAGGATAATAAG CGGCCGDG-3′) (28-mer); antisense strand, oligonucleotides (5′-AATTCGC GGCCGCTTATTATCTTCGGAATCTGGCCCCTGGTTCAAACTGA-3′) (50-mer) and 4 (5′-GTATAGACATAGTCAGGGGAGTGGTAG-3′) (27-mer). Clones were sequenced to ensure that the constructs were correct. The p15E-deleted pSP72 15-1 clone was cut with *Xho*I and *Eco*RI, and the 1.3-kb *env*-containing fragment was introduced into the pSFF vector cut with the same enzymes. pSFF containing the 1.3-kb deleted *env* insert (pCas^{ES}) was transfected into a 1:1 mixture of PA317 and ψ2 packaging cells. The cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum and passaged three times, and supernatants from the cells were collected to obtain virus, refered to as Cas^{ES}. These supernatants were used to infect C17.2 cells with the gp70/pSFF construct (see below).

The CasBrE molecular clone 15-1 and the 15-1–Friend murine leukemia virus (MuLV) chimeric viruses CasFr^{L} and FrCas^{E} have been previously described (41, 42). The CasFr^{L} and FrCas^{E} viruses have rapid in vivo replication kinetics, which allow these viruses to induce rapid severe neurodegenerative disease. The 15-1 virus induces disease only very slowly and at a very low incidence. The proviral structures of the virus constructs are shown in Fig. 1B. The 15-1 and CasFr^{L} viruses contain N-tropic *gag* sequences, whereas the FrCas^{E} virus contains NB-tropic *gag* sequences from Friend MuLV. The CasFr^{L}, 15-1, or FrCas^{E} virus stocks were produced in Dunni fibroblasts having titers of 1×10^6 to 3×10^6 FFU per ml. These replication-competent viruses and the replication-defective viruses Cas^{E} and Cas^{ES} were used to infect the engraftable neural progenitor cell line C17.2 developed by Snyder et al. (50).

Cells. C17.2 cells were grown on Primaria dishes (Falcon) in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM sodium pyruvate, 2 mM glutamine, and 100 U (each) of penicillin, streptomycin, and amphotericin B (Fungizone) per ml (DM10). Cells were passaged at a dilution of 1:40 when they reached 95% confluence to avoid in vitro differentiation. Cells were infected at an multiplicity of infection of approximately 1 in the presence of 8 μg of Polybrene per ml for 4 h. At the time of passage, cells were evaluated for infection by detection of envelope protein expression by either immunohistochemistry or fluorescence-activated cell sorting (FACS). Immunostaining was carried out by using monoclonal antibody (MAb) 667 or 697, as described previously (28, 29). FACS staining was carried out with cells which were removed from the dish by using 0.05% trypsin–0.53 mM EDTA (Life Technologies), washed with DM10–phosphate-buffered saline (PBS), and either kept on ice or fixed for 20 min in 10% Formalin–PBS. A portion of the fixed cells was permeabilized with 0.1% Trition X-100 in PBS for 5 min for intracellular protein localization. Cells were incubated with either MAb 667 or 697 for 30 min, washed, and incubated with a 1:200 dilution of fluorescein isothiocyanate–goat anti-mouse immunoglobulin G (IgG) (Cappel) in PBS containing 2% fetal calf serum. For transplantation experiments, cells expressing Cas^{E} were sterilely sorted, and the brightest 50% were grown up for transplant experiments. Cells expressing Cas^{ES} were obtained by subjecting C17.2 cells to two rounds of infection with defective virus at a multiplicity of infection of ~10. After the second round of infection, all cells were expressing envelope protein when examined immunohistochemically. No diminution in signal strength was seen with passage of these cells. All CasBrE *env*-expressing cells were tested for superinfection interference by using Friend MuLV FB29 (48) by the focal immunoassay (8) with MAb 720 (45).

Supernatants of virus-infected cultures were taken just prior to passage for evaluation of virus production. The C17.2 neural progenitor cell line was created by the introduction of two retroviral vectors (46, 50). Cellular supernatants were screened to evaluate the incorporation of these original vector RNAs into the newly generated virions. One vector encoded LacZ and neomycin resistance genes, and the second vector coded for avian *myc* and the neomycin resistance gene. Infected Dunni target cells were therefore screened for β-galactosidase expression by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) histochemistry (50) and for neomycin gene expression by selection in medium containing 100 μg of G418 (Life Technologies) per ml. Virus titrations were performed on Dunni cells as previously described (8).

Mice. The mice used in these experiments included the FVB strain (Jackson Laboratories) (*Fv-1^{b}*), which is permissive for B- and NB-tropic virus replication, and the IRW (Inbred Rocky Mountain White) strain (*Fv-1^{n}*), which is permissive for N- and NB-tropic virus replication. Mice were housed and cared for in the specific-pathogen-free animal facility at the Longwood Medical Research Center. All animal procedures were carried out in accordance with American Association for the Accreditation of Laboratory Animal Care guidelines and were approved by the Animal Care and Use Committee at Harvard Medical School.

Mice were evaluated for clinical neurological signs as previously described (10, 29, 41).

Transplantation of C17.2 cells. For transplantation, preconfluent control or infected C17.2 cells were detached from the plates by treatment with trypsin-EDTA and washed two times with medium containing 10% fetal calf serum and then two times with 4°C Dulbecco's PBS (Ca^{2+} and Mg^{2+} free). The cells were resuspended at 3×10^7 to 9×10^7/ml in Dulbecco's PBS containing 0.2% trypan blue and kept on ice prior to injection.

Resuspended C17.2 cells were injected into cryoanesthetized neonatal (P0) pups essentially as described previously (52). Approximately 2 μl of a well-triturated cellular suspension was injected into each lateral ventricle. The mice were warmed on a heating pad until their activity levels returned to normal and then returned to their mothers. No significant mortality or morbidity was associated with the anesthesia or the intracerebral injections.

Detection of engrafted cells and virus and envelope protein expression. Mice were evaluated for engraftment of C17.2 cells between 2 and 4 weeks after intracerebral inoculation of engrafted cells. Mice were lethally anesthetized with Metofane and decapitated or perfused with PBS containing 2 mM $MgCl_2$ and 1.25 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] followed by 2% paraformaldehyde in 50 mM phosphate buffer (pH 7.2) supplemented with 2 mM $MgCl_2$ and 1.25 mM EGTA. Perfused brains were removed from the cranium, sectioned into 2- to 3-mm coronal slices, and post-fixed for an additional 4 h. The sections were cryopreserved overnight in 30% sucrose–PBS with $MgCl_2$ and EGTA at 4°C, embedded in OCT (Miles), and frozen in plastic molds. Brains from decapitated mice were dissected from the cranium and immediately frozen in the vapor phase of liquid nitrogen and stored at −70°C for later frozen sectioning. Alternatively, some of these brains were immersion fixed in 4% paraformaldehyde and then subjected to paraffin embedding and sectioning.

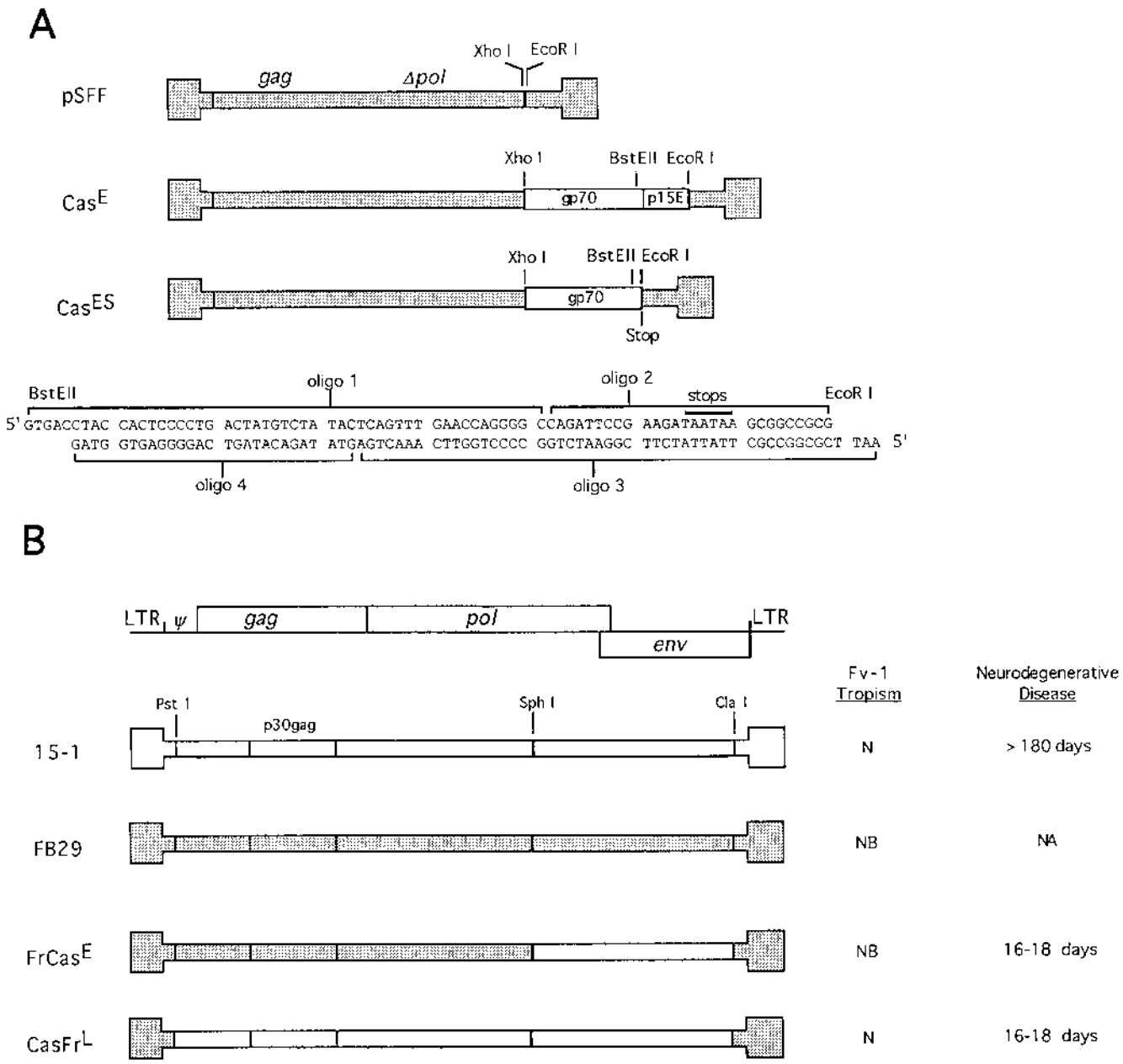

FIG. 1. CasBrE envelope protein expression constructs and viruses. (A) Outline of how the envelope gene from CasBrE clone 15-1 was introduced into the pSFF expression vector (5) by using the *Xho*I and *Eco*RI cloning sites. Cas^E was generated by using the entire envelope gene, which was previously cloned into pSP72 (see Materials and Methods) and is bounded by an *Xho*I site and an *Eco*RI site. To generate Cas^{ES} (SU only), the pSP72-15-1*env* clone was cut with *Bst*EII and *Eco*RI, and the gp70 (SU) portion was reconstituted to the SU-TM cleavage junction by ligating in the overlapping oligonucleotides shown at the bottom. This pSP72-15-1SU clone was cut with *Xho*I and *Eco*RI and introduced into pSFF by using the same sites. (B) Derivation of the chimeric viruses $FrCas^E$ and $CasFr^L$ from the CasBrE molecular clone 15-1 and the Friend virus clone FB-29 (41, 42). The kinetics for neurological disease induction in IRW mice after neonatal injections are given for each virus (41, 42) along with Fv-1 tropism. The location of p30 Gag is indicated to show the derivation of the *Fv-1* genotype. LTR, long terminal repeat; NA, not applicable.

Cells expressing β-galactosidase were identified in fresh frozen or in fixed, cryopreserved sections by X-Gal histochemistry as described previously (43, 50). For optimal detection of engrafted cells, fresh frozen 20-μm sections were collected on cellophane sheets, air dried for 10 to 20 min, fixed for 5 min in 0.5% glutaraldehyde in PBS, rinsed in PBS, and then incubated in the X-Gal solutions. Alternatively, β-galactosidase was detected in paraffin-embedded, fresh frozen, or fixed frozen sections by immunohistochemistry with rabbit anti-β-galactosidase (Cappel) diluted 1:2,000 followed by a peroxidase- or alkaline phosphatase-coupled goat anti-rabbit IgG diluted 1:1,000 (Bio-Rad) as described previously (29).

The levels of C17.2 cell engraftment were also determined by Western blotting (immunoblotting) of 20-μm frozen tissue sections. Sections were extracted in 50 μl each of extract buffer (0.1% Triton X-100, 150 mM NaCl, 50 mM Tris HCl [pH 7.4], 1 mM $MgCl_2$, 1 mM EGTA, and 0.2 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 × *g* for 5 min at 4°C, and supernatants were made 1× in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. One half of each extract was separated on 9% SDS gels and transferred to Immobilon (Millipore), and β-galactosidase protein was detected with rabbit anti-β-galactosidase (1:2,000; Cappel) followed by peroxidase-coupled goat anti-rabbit IgG (1:1,000; Bio-Rad) and detection by ECL (Amersham). CasBrE envelope protein expression was determined by evaluation of the remaining half of the extract by similar Western blotting with MAb 697 (10).

Virus expression in the CNS was determined with crude tissue lysates of 20-μm fresh frozen tissue sections in 100 μl of Dulbecco's PBS by trituration.

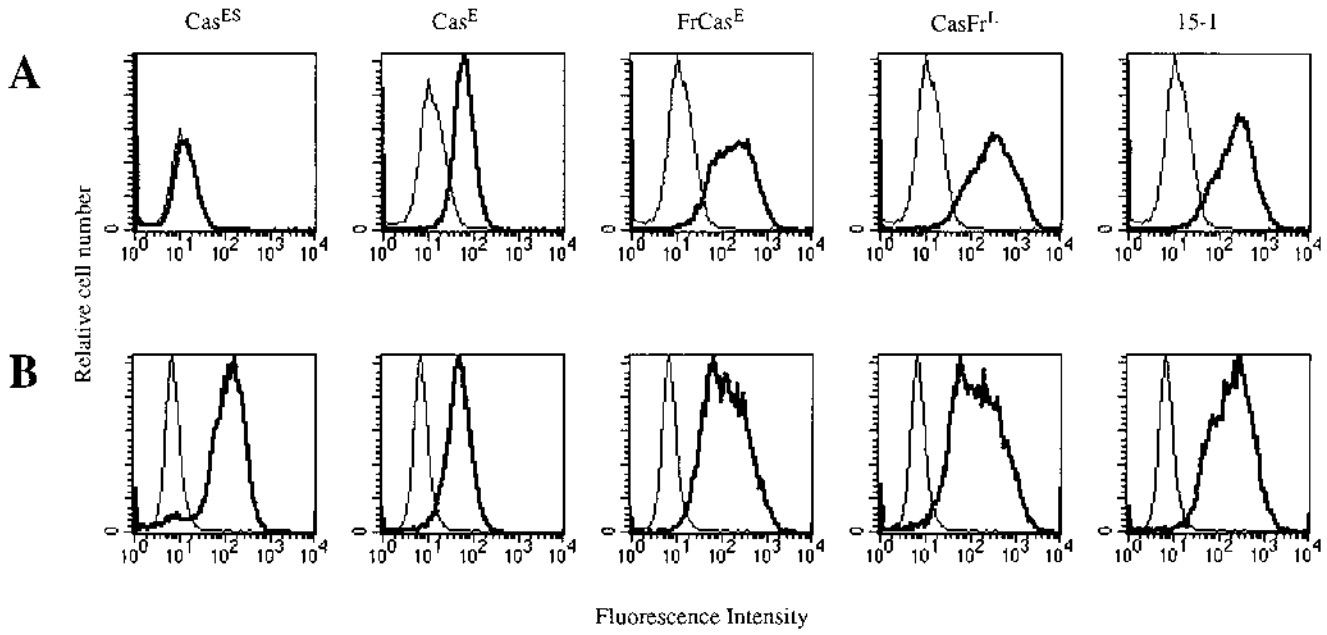

FIG. 2. CasBrE envelope protein expression in infected C17.2 cells by FACS. (A) C17.2 cell surface expression of CasBrE envelope protein with MAb 667 as the primary staining reagent; (B) intracellular staining on 0.1% Triton X-100-permeabilized cells with MAb 697. Primary antibody staining was followed by staining with fluorescein isothiocyanate–goat anti-mouse antibody prior to FACS analysis. The thin line in each graph indicates staining of uninfected C17.2 cells with both the primary and secondary antibodies, whereas the thick line represents staining of C17.2 cells infected with the virus listed above each column. Note a significant shift in the cellular expression of CasBrE *env* for all of the infected cells, except for surface expression on Cas^{ES}-C17.2 cells.

Lysates were monitored for the presence of virus by seeding into a focus assay with Dunni cells to detect total virus production (8). In some cases serial sections were evaluated by X-Gal histochemistry, immunohistochemistry, immunoblotting, and infectivity assays in order to identify all components associated with the transplant as well as to detect virus spread to host cells.

CasBrE envelope protein expression was determined by immunohistochemistry with fresh frozen or fixed frozen sections by using CasBrE-specific biotinylated MAb 667 or 83A25 as described previously (29). Alternatively, the CasBrE envelope protein was identified in paraffin sections by using the CasBrE-specific MAb 697, which recognizes denatured CasBrE envelope protein. Briefly, deparaffinized, rehydrated sections were incubated with MAb 697 supernatants for 1 h at 37°C, rinsed three times in PBS, incubated for 1 h with a 1:250 dilution of biotinylated horse anti-mouse IgG (rat adsorbed; Vector), rinsed three times in PBS, incubated for 30 min in Supersensitive peroxidase-conjugated streptavidin (Biogenex), rinsed three times in PBS, and developed with diaminobenzidine as described previously (29). The latter localization technique, while not as sensitive as that performed with frozen sections, allows for simultaneous localization of virus-expressing cells and histopathologic changes.

Immunohistochemical detection of F4/80, Mac-1, and GFAP was carried out as previously described (10, 29, 31).

Histopathology. Histologic assessment was carried out with brains which were immersion fixed in 4% paraformaldehyde–PBS for 4 h at room temperature, sectioned into 3- to 4-mm pieces, and fixed overnight in 4% paraformaldehyde–PBS prior to dehydration and embedding in paraffin. Four-micrometer sections were stained with hematoxylin and eosin (H&E) or rehydrated, immunostained, and then counterstained with hematoxylin alone. Sections were viewed by bright-field microscopy and photographed with Kodak Royal Gold 100 print film or Ektachrome 100 with a daylight filter.

RESULTS

C17.2 cells can express neurovirulent CasBrE virus and envelope protein at high levels in vitro. In order to evaluate the toxicity of the CasBrE envelope protein in vivo, two *env* expression constructs, Cas^{E} and Cas^{ES}, were generated to express membrane-bound (SU/TM) and secreted (SU) forms of this protein, respectively (Fig. 1). The initial transfection of these constructs into packaging cells resulted in the expression of envelope protein and incorporation of the Cas^{E} and Cas^{ES} vectors into virion particles (not shown). To evaluate the feasibility of using the multipotent neural progenitor cell line C17.2 (50) as a means to deliver CasBrE SU/TM and SU viral proteins to the CNS, C17.2 cells were infected with Cas^{E} and Cas^{ES} viruses. This infection was compared with infection with the replication-competent CasBrE molecular clone 15-1 and the Friend-CasBrE chimeric viruses $FrCas^{E}$ and $CasFr^{L}$. Immunostaining for surface and intracellular CasBrE envelope protein indicated readily detectable levels of expression of both replication-competent and defective virus infection (Fig. 2). For the Cas^{ES}-C17.2 cells, intracellular staining was present in the absence of cell surface staining, as might be predicted for a secreted protein product.

Western blot analysis of cell-associated and supernatant fractions of infected C17.2 cells indicated that the Cas^{E} expression vector generated envelope precursor (pr85) and processed protein (gp70) with molecular mobilities indistinguishable from those resulting from productive virus infection (Fig. 3). Interestingly, a significant level of SU envelope protein was released into the tissue culture medium of Cas^{E}-expressing cells. While SU shedding from virions is a common feature of retroviruses, it is possible that some level of particle formation may have occurred with the Cas^{E} expression vector, since this vector is known to generate Gag proteins (44).

The production of CasBrE SU alone from Cas^{ES} showed a divergent phenotype. Envelope proteins exhibiting mobilities of ca. 75 and 60 kDa were associated with cell extracts, while a protein with a slightly slower mobility than virion gp70 (~75 kDa) was detected in the culture medium. Whether these differences arise as a result of altered translation termination, proteolysis, or differential glycosylation is not known.

Envelope protein or virus expression did not affect the viability, growth rate, or β-galactosidase expression of C17.2 cells. Infection of C17.2 cells with replication-competent virus yielded infectious virus at levels comparable to those for infected NIH 3T3 fibroblasts (Table 1). Analysis of C17.2 cells infected with the replication-defective Cas^{E} or Cas^{ES} virus did

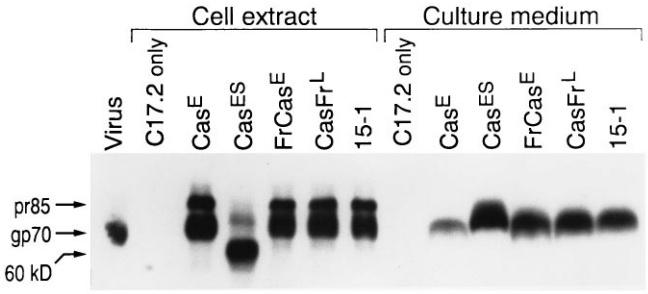

FIG. 3. CasBrE envelope protein expression and release from infected C17.2 cells in culture. Samples of postnuclear cell extracts or culture medium conditioned for 2 days from uninfected and infected C17.2 cells were resolved by SDS–9% PAGE and immunoblotted by using the CasBrE envelope-specific MAb 697 (28). Except for Cas^{ES}-infected cells, precusor (pr85) and processed (gp70) envelope proteins were indistinguishable in the cellular extracts. A significant amount of envelope protein is observed in the medium of Cas^{E}-C17.2 cells, indicating that gp70 is released from the cell surface. The expression of precursor (~75 kDa) and processed (~60 kDa) envelope proteins is observed in cellular extracts of Cas^{ES}-infected C17.2 cells. In contrast, protein released into the supernatant from Cas^{ES}-infected cells has a slightly slower mobility than protein expressed by CasE or the replication-competent viruses. The reasons for these mobility differences are not known, but the differences suggest that the p15E region is likely to be required for correct protein processing.

not show evidence of infectious virion release into culture supernatants, nor did they score in infectious-center assays.

Given the high levels of CasBrE envelope proteins that were expressed in Cas^{E}- and Cas^{ES}-C17.2 cells, we were interested in evaluating to what extent this protein could interact with the ecotropic receptor, MCAT-1. Therefore, *env*-expressing cells were assayed for their susceptibility to superinfection by a second ecotropic MuLV, Friend clone FB-29. It is well established that infection by one MuLV interferes with the subsequent infection by a second virus of the same tropism group by envelope protein occupation of available receptor sites (57). Cas^{E}-C17.2 cell SU/TM expression resulted in essentially complete superinfection interference ($>10^5$), whereas SU expression alone within Cas^{ES}-C17.2 cells reduced superinfection by approximately 2 log units (Table 1). These results indicated that while SU/TM interacts strongly with MCAT-1, the truncated envelope generated through expression of SU did not bind with the same affinity as the complete protein. These differences may reflect the differential mobilities noted in the Western blot analysis or a lack of oligomerization. The Cas^{ES} protein product was still reactive to MAbs 667 and 83A25 (12, 33), two envelope-specific MAbs which recognize conformational determinants. These latter results indicate that Env protein folding was at least partially intact.

To assess the ability of replication-competent-virus-infected C17.2 cells to regenerate the input virus, viral supernatants or virus-infected C17.2 cells (approximately 10^4 cells per animal) were inoculated into the peritoneal cavities of susceptible IRW neonates. For each individual infectious virus, i.e., $FrCas^{E}$, $CasFr^{L}$, and 15-1, the neurological disease induction kinetics were indistinguishable from those previously reported for the original virus stocks (41, 42). Both the $FrCas^{E}$ and $CasFr^{L}$ viruses induced disease with a rapid time course (time to clinical onset, 16 days). C17.2 15-1-inoculated mice failed to show clinical neurologic disease within 60 days of inoculation, although all animals were viremic (not shown). Peripheral inoculation of C17.2 cells infected with the defective Cas^{E} or Cas^{ES} virus showed no evidence of viremia or neurologic disease over a 2-month observation period.

Since the C17.2 cell line was originally generated by infection with v-Myc/neo- and β-Gal/neo-encoding defective retroviruses (46, 50), we evaluated the extent to which the cells infected with replication-competent and -incompetent viruses were incorporating β-galactosidase or neomycin transcripts into budding virus. X-Gal staining and neomycin selection of Dunni cells exposed to supernatants from C17.2 cells infected with replication-competent virus indicated that incorporation of β-galactosidase (Table 1) and neomycin (not shown) gene transcripts was occurring at a frequency of 2 to 3 log units less than that of the replication-competent virus itself. No detection of marker or selection gene incorporation into virions was noted for cells infected with the replication-defective viruses, Cas^{E} and Cas^{ES}.

CNS engraftment of C17.2 cells and expression of CasBrE envelope proteins. To assess the ability of C17.2 cells to deliver the envelope protein to the brain, neonatal IRW mice were inoculated within the lateral cerebral ventricles with either Cas^{E}-C17.2 cells, Cas^{ES}-C17.2 cells, or noninfected C17.2 cells. Mice were evaluated at 2, 3, and 4 weeks postimplantation to assess the extent of cellular engraftment. All cell lines were observed to engraft with high efficiency into the brains of neonatal IRW mice, as demonstrated by X-Gal staining for β-galactosidase in the transplanted cells (Fig. 4B; see Fig. 5A and D). All mice examined had significant levels of engrafted cells, although the amount and specific localization of engrafted cells varied from individual to individual. There were no apparent engraftment differences between noninfected and envelope protein-expressing C17.2 cells. Engraftment was most consistently found in the cerebral cortex, striatum, and septal regions. Therefore, these areas were examined in all experimental regimens. In addition, significant engraftment was also frequently observed in the hippocampus, thalamus, brain stem, colliculus, olfactory bulb, and cerebellum, as pre-

TABLE 1. Virus production, interference, and pathogenic properties of CasBrE *env*-expressing C17-2 cells

Construct	Virus production (FFU/ml)		Superinfection interference (FFU/ml)		No. with CNS disease[a]/total no.	
	CasBrE	β-Galactosidase	FB29 titer	Fold reduction	IRW mice	FVB mice
None	<10	<10	6.4×10^6	NA[b]	NB[c]; 0/8	ND
Cas^{E}	<10	<10	<10	$>10^5$	0/4; 0/4	ND
Cas^{ES}	<10	<10	1.0×10^5	64	0/3; 0/4	ND
15-1	1.4×10^6	1.7×10^4	ND	ND	0/4; 0/3	ND
$FrCas^{E}$	1.8×10^6	3.0×10^3	<10	$>10^5$	4/4; 3/3	4/4; 5/5
$CasFr^{L}$	2.4×10^6	5.1×10^3	<10	$>10^5$	5/5; 5/5	0/8; 0/7

[a] CNS disease was evaluated after i.p. inoculation of either tissue culture supernatants (first set of numbers) or C17.2 cells themselves expressing envelope protein-virus constructs (second set of numbers). Mice were evaluated for clinical disease for at least 60 days.
[b] NA, not applicable.
[c] ND, not done.

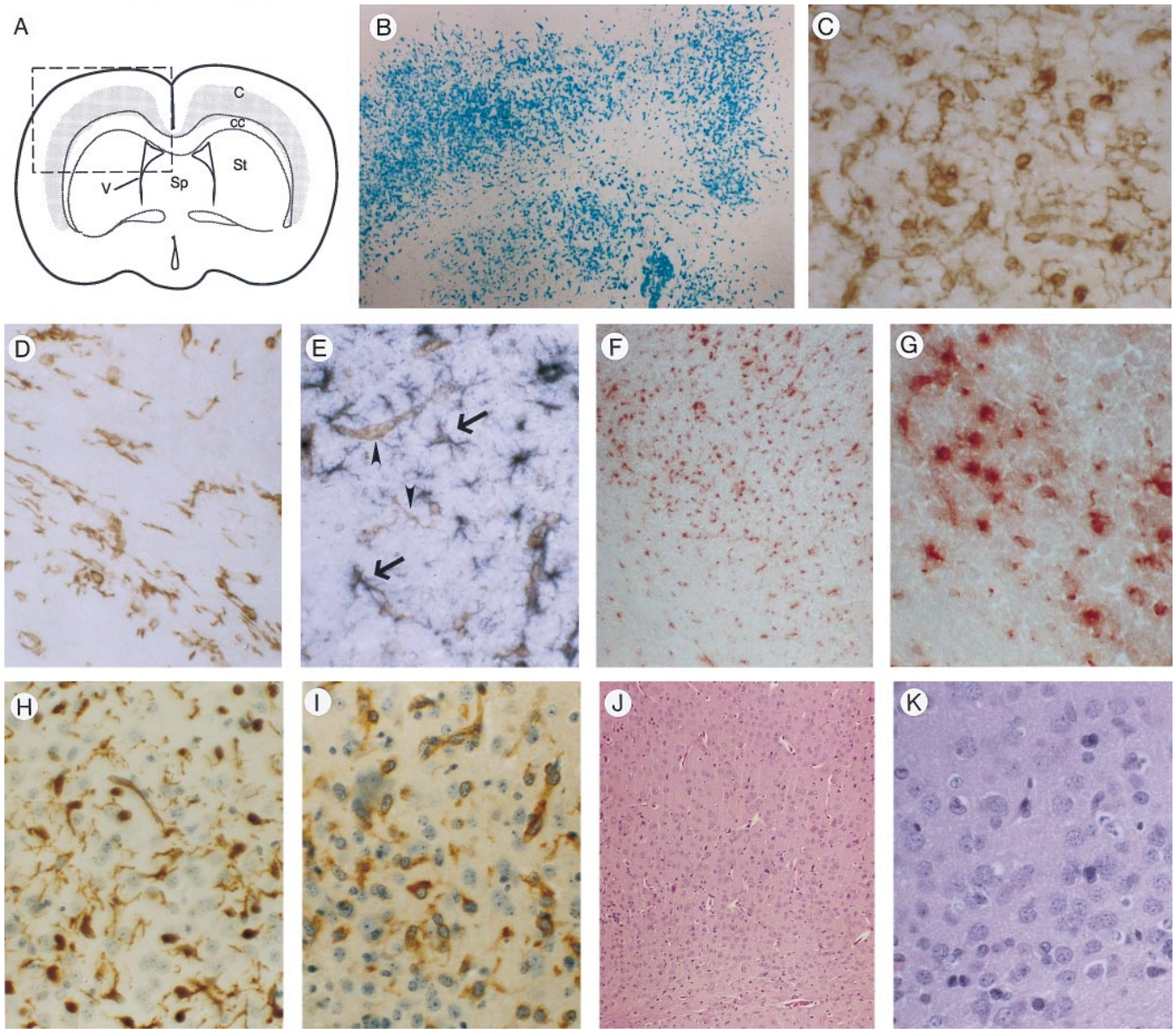

FIG. 4. Engraftment of CasBrE envelope protein-expressing C17.2 cells in the CNS. (A) Diagram of a coronal section through the forebrain of a mouse, with the cortex (C), corpus callosum (cc), septum (Sp), and striatum (St) indicated. This region was evaluated in all transplanted animals because of the high levels of cellular engraftment which were consistently seen in this segment of the CNS. The shaded region indicates where pathology has been observed to arise with rapid kinetics either upon neonatal infection with the highly neurovirulent chimeric CasBrE virus FrCas^E (10, 29, 30) or upon transplantation of FrCas^E-infected microglia at P10 (31). (B) Example of X-Gal staining in a 20-μm section from a brain engrafted with Cas^E-C17.2 cells 21 days after intraventricular inoculation and prepared by snap freezing. This region corresponds to the boxed area in panel A. Similar results were observed for C17.2 cells alone and Cas^{ES}-C17.2 cells. (C and D) Examples of CasBrE envelope protein expression (brown; diaminobenzidine reaction product) observed in the cortex (C) and the corpus callosum (D) of Cas^E-C17.2-transplanted mice after immunostaining of 10-μm fixed cryopreserved frozen sections with biotinylated MAb 667 (see Materials and Methods). (E) Cas^E-C17.2 cell-engrafted brain double immunolabeled with antibody specific for GFAP to identify astrocytes (dark blue) and with MAb 667-biotin to identify CasBrE envelope protein-expressing cells (brown). Note that in some cells (arrows) these antigens were colocalized, while in other cells (arrowheads) envelope alone was expressed. This result indicated that donor cells differentiated into astrocytes (arrows) as well as other lineages (arrowheads). (F) Low-power field of an IRW brain engrafted with Cas^{ES}-C17.2 cells which has been immunostained for the CasBrE envelope protein (red) by using MAb 697 and AEC as the chromagen. (G) Higher-magnification view of Cas^{ES}-C17.2 expression of SU in the deep layers of the cortex. Staining is noted in the cell bodies as well as diffusely in the surrounding parenchyma, giving the impression that protein was being secreted from these cells. (H and I) Immunostaining of 4-μm paraffin sections from the cortex of a CasE-C17.2 cell-engrafted brain 4 weeks postinoculation by using antibodies specific for β-galactosidase and CasBrE envelope protein (MAb 697), respectively. These sections were counterstained with hematoxylin to reveal the tissue morphology. Despite high levels of cellular engraftment and envelope expression, no evidence of spongiform neurodegeneration was noted. This was confirmed by H&E staining of adjacent paraffin sections (J) and at higher power (K). Similar observations were made for Cas^{ES}-C17.2 cell-engrafted brains (not shown). Magnifications: panel B, ×17; panels C, D, E, G, H, I, and K, ×170; panels F and J, ×43.

viously reported (49, 50, 52, 54). No engraftment was noted in the caudal spinal cords of any mice examined by using this administration technique (n = 14).

Brain regions containing engrafted cells were immunohistochemically examined for the expression of CasBrE envelope protein by using highly specific MAb probes (29, 33). CasBrE envelope protein expression was readily apparent in all areas containing engrafted cells (Fig. 4C, D, F, G, and I). Expression levels were comparable to those observed in virus-infected brains. Colocalization of envelope protein with glial fibrillary

TABLE 2. Features associated with CNS transplantation of CasBrE envelope- and virus-expressing C17.2 cells

Transplanted C17.2 cells	No. affected/total no.				Infectivity[a]	
	Clinical disease		Spongiosis			
	IRW mice	FVB mice	IRW mice	FVB mice	IRW mice	FVB mice
No virus	0/18	0/23	0/12	0/9	−	ND[b]
Cas^{E}	0/31	0/17	0/15	0/12	−	ND
Cas^{ES}	0/24	0/12	0/9	0/8	−	ND
$CasFr^{L}$	8/8	0/19	4/4	0/12	+	+
15-1	12/13[c]	ND	7/8[d]	ND	+	ND
$FrCas^{E}$	8/8	13/13	4/4	9/9	+	+

[a] This parameter measured whether infectious virus could be detected by a focus assay when extracts from frozen sections were prepared in parallel with X-Gal-stained sections. Focus assays with adjacent sections often gave results which varied quantitatively despite similar X-Gal staining. This was likely due to incomplete tissue homogenization. +, foci detected in all sections examined; −, foci never detected.

[b] ND, not done.

[c] Clinical neurological disease in these animals was not characterized by any abnormalities in the hind limbs. Tremors involving the head were noted, with some kyphosis, decreased activity, and ungroomed fur as notable signs. See footnote *d* regarding the one asymtomatic animal.

[d] The one animal without spongiform changes did not demonstrate β-galactosidase-positive engrafted cells or peripheral viremia and corresponded to the animal failing to exhibit clinical signs. This animal was not tested for CNS virus or envelope expression.

acidic protein (GFAP) by double-label immunohistochemistry indicated that the envelope protein-expressing C17.2 cells were capable of differentiating into astrocytes (Fig. 4E). The observation that not all envelope-positive donor-derived cells were labeled with GFAP suggested that the transplanted C17.2 cells differentiate towards other lineages as well. Morphological examination of β-galactosidase-positive cells indicated that in addition to astrocyte morphologies, many of the transplanted cells exhibited neuronal and oligodendroglial phenotypes. Although not extensively explored for the purposes of this study, this interpretation is consistent with previous reports showing that these multipotent neural progenitors can differentiate into neurons, astrocytes, and oligodendrocytes in vivo (49–54).

Homogenates of Cas^{E}- and Cas^{ES}-C17.2-engrafted brain regions were evaluated for the presence of replication-competent virus by using a focus assay (8) (Table 2). No infectious virus was detected in brains transplanted with C17.2 cells expressing Cas^{E} or Cas^{ES}, suggesting that expression vector recombination with endogenous viral sequences did not occur.

Envelope protein expression does not result in acute spongiform neurodegeneration. To evaluate whether the CasBrE envelope protein expression from C17.2 cells was capable of inducing pathologic changes, Cas^{E}-C17.2-engrafted mice were analyzed histologically through 4 weeks after intraventricular inoculation. Because engraftment appeared to be occurring most reproducibly in the neocortex, striatum, and septum, paraffin sections including these regions (Fig. 4A) were subjected to detailed histochemical analysis. The deep layers of the cerebral cortex have been previously shown to be highly susceptible to the degenerative effects of CasBrE infection (Fig. 4A) (10, 29, 31, 40). Sites of cellular engraftment were demonstrated immunohistochemically in paraffin sections by using antibodies specific for β-galactosidase (Fig. 4H) or envelope protein (Fig. 4I). Counterstaining of these sections with hematoxylin failed to reveal any evidence of spongiform neurodegeneration. Adjacent sections were stained with H&E and also did not shown evidence of spongiosis in the deep layers of the cortex (Fig. 4J and K) or in the stiatum, septum, or brain stem (not shown).

Evaluation of engrafted brains for induction of pathology was limited to 4 weeks after ventricular inoculation because of concern about rejection of CasBrE-expressing C17.2 cells. CNS changes suggesting that rejection might be occurring included infiltration of inflammatory cells with associated perivascular cuffing. The infiltrates stained positive with antibodies specific for T cells, including anti-CD3, -CD8, and -CD4 (not shown). Since spongiform pathology can arise very quickly once brain development passes the first postnatal week (10, 30, 31), the 4-week window is sufficient for evaluating the acute effects of CasBrE envelope protein expression. Evaluation of the chronic effects of *env* expression in the CNS may require better understanding of the nature of the CasBrE-C17.2-associated inflammation.

Because previous experiments from our laboratories have suggested that glial activation may prevent the induction of spongiform neurodegeneration (31), we evaluated whether glial activation was occurring upon transplantation of C17.2 cells. Sections from transplanted brains were stained for activated astrocytes by using anti-GFAP and for activated microglial cells by using the MAbs Mac-1 and F4/80. GFAP staining was elevated over that of uninjected controls in 7 of 10 mice examined at 3 weeks postinoculation (Fig. 4E). This elevation occurred whether mice were given C17.2 cells alone (three of five mice) or Cas^{E}-C17.2 cells (four of five mice) and was not restricted to the sites of cellular engraftment. In contrast, no activation of microglial cells was seen in any of the engrafted mice examined ($n = 12$) prior to the fifth postnatal week.

To examine whether astrocyte activation associated with the transplantation procedure could prevent spongiform pathologic changes from occurring, normal C17.2 cells were transplanted into the brains of mice along with simultaneous intraperitoneal (i.p.) inoculation of the $FrCas^{E}$ virus. Spongiform neurodegeneration appeared in these animals despite the presence of transplanted cells and similar levels of astroglial activation (not shown). In a similar fashion, transplantation of 15-1-, $CasFr^{L}$- or $FrCas^{E}$-expressing C17.2 cells in the brains of IRW animals resulted in the appearance of spongiform changes coincident with increased GFAP expression. Microglial activation, as determined by elevations in F4/80 and Mac-1 expression levels, was not noted under any transplant or control conditions. Therefore, these data indicate that astroglial activation is not sufficient to prevent the appearance of spongiform neurodegenerative changes.

Engraftment of N- and NB-tropic-virus-producing C17.2 cells in *Fv-1*^{b} mice. Since envelope protein expression from neural progenitors did not appear to be sufficient for inducing acute neurodegeneration, we pursued a strategy to identify the virus replication cycle events that were necessary. Therefore, we next evaluated the effect of virus expression in a host in which early virus replication events are permitted but productive infection is not. This was accomplished by introducing the N-tropic virus $CasFr^{L}$ into C17.2 progenitor cells and then transplanting them into FVB mice (homozygous for the *Fv-1*^{b} allele). Mice were examined through 4 weeks postinoculation for the presence of engrafted cells, virus expression, and spongiform neurodegeneration. Significant levels of cellular engraftment and CasBrE envelope expression were noted in areas susceptible to the neurodegenerative effects of the virus; however, no evidence of coincident spongiform changes was observed through the 4-week observation period (Fig. 5A to F). To evaluate whether $CasFr^{L}$-C17.2 cells continued to express replication-competent virus in the *Fv-1*-restricted FVB mouse brains, homogenates from engrafted regions were as-

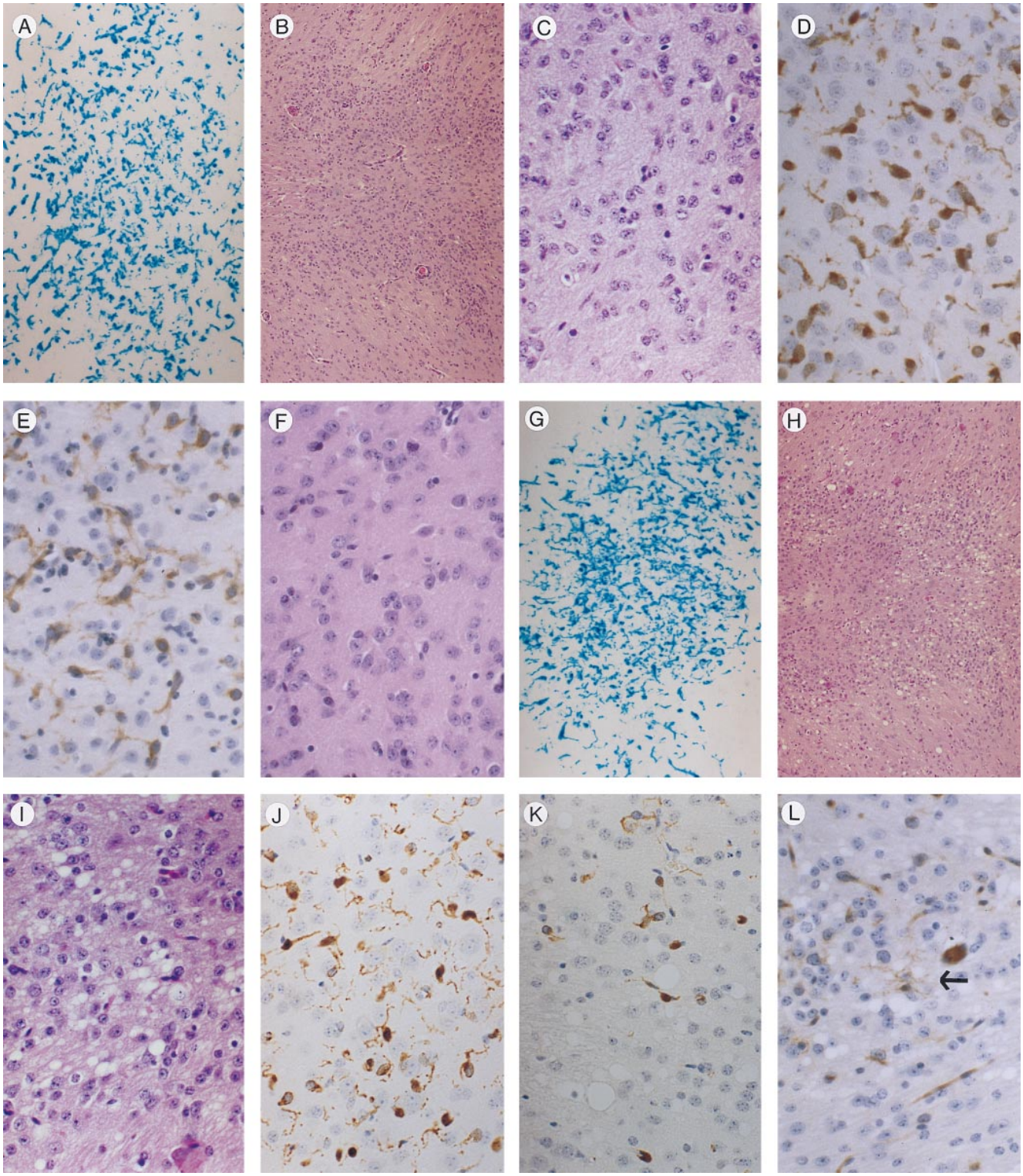

FIG. 5. Engraftment of N- and NB-tropic CasBrE virus-expressing C17.2 cells into the brains of FVB mice (*Fv-1*^{b}). (A to F) Engraftment, histology, and envelope protein expression in N-tropic CasFr^{L}-C17.2 cell-engrafted FVB mouse brains. (A) Significant engraftment of CasFr^{L}-C17.2 cells in the septum as revealed by X-Gal staining (blue). Similar X-Gal staining was noted in the striatum, corpus callosum, and cortex (not shown). (B and C) Low- and high-power fields, respectively, of corresponding paraffin sections from an engrafted septum stained with H&E. No evidence of spongiform change is associated with engraftment. (D and E) Immunostaining (brown) for β-galactosidase and CasBrE envelope, respectively, in paraffin sections from the cortex. Again, the hematoxylin counterstaining does not reveal evidence of associated spongiform pathology. A parallel section stained with H&E (F) supports this observation. (G to L) Engraftment and histology resulting from transplanting NB-tropic FrCas^{E}-C17.2 cells in FVB mouse brains, for comparative purposes. (G) X-Gal staining (blue) in the septum, a brain area where pathology has not been previously observed (10) (Fig. 4A). (H and I) Low- and high-power fields, respectively, showing the corresponding spongiform changes that are associated with septal engraftment (H&E). (J and K) Immunostaining (brown) for β-galactosidase in the cortex (corresponding to the shaded regions in Fig. 4A). Note that in panel J abundant engraftment of FrCas^{E}-C17.2 cells is not associated with spongiosis. In panel K abundant spongiosis is apparent but without high levels of engrafted cells. (L) Envelope protein immunostaining (brown) in cells characteristic of ramified microglia (arrow). The appearance of envelope protein expression in this cell type directly correlated with spongiform pathology, which is clearly observable in the background. No such staining of these cells was noted in CasFr^{L}-C17.2 cell-engrafted brains through 4 weeks (not shown). All panels correspond to brains taken from mice 4 weeks postinoculation. Panels A and G were fresh frozen 20-μm sections. All remaining panels were 4-μm paraffin sections. Magnifications: panels A, B, G, and H, ×43; panels C to F and I to L, ×170.

sayed for the presence of virus. Virus production was observed at 2, 3, and 4 weeks postengraftment despite the lack of spongiform neurodegeneration (Table 2).

As a control, C17.2 cells infected with the nonrestricted NB-tropic virus FrCas^E were inoculated into the ventricles of FVB mice. Examination of these mice showed cellular engraftment (Fig. 5G, J, and K), envelope expression (Fig. 5L), and spongiform neurodegeneration (Fig. 5H, I, K, and L) by 2 weeks of age, and all FrCas^E-C17.2 mice had clinical neurologic disease by 3 weeks posttransplantation. To evaluate whether there was a direct correlation between the engrafted FrCas^E-C17.2 cells and spongiform pathology, paraffin sections were immunostained for β-galactosidase and counterstained with hematoxylin. Donor-derived cells localized to CNS areas where pathology typically arises; however, as shown in Fig. 5J and K, FrCas^E-expressing, β-galactosidase-positive engrafted cells were not always visibly colocalized with spongiosis. Therefore, we examined the degenerating areas for evidence of virus spread to host cells by staining for envelope protein, since the NB-tropic virus, FrCas^E, is not restricted by the *Fv-1* gene in FVB mice. Pathology most closely correlated with cellular staining characteristic of ramified microglial cells. An example of this type of staining is shown in Fig. 5L. In the absence of this characteristic infection, no pathologic changes were observed.

When the brains of FVB mice transplanted with FrCas^E-C17.2 cells were surveyed for the appearance of spongiform degeneration, novel areas of involvement were noted, most particularly in the striatum and septum (Fig. 4A and 5H and I), areas showing frequent cellular engraftment. Since pathology in this area has not been reported for the FrCas^E virus, these results suggest that pathology arose as a result of the transplanted FrCas^E-expressing C17.2 cells rather than from virus entering this region from the circulation. To address directly the latter possibility, IRW mice were transplanted with C17.2 cells infected with the 15-1 virus. Peripheral i.p. inoculation of neonatal mice with the 15-1 virus induces clinical neurologic disease only at a very low incidence (<10%) and only after an extended time course (>6 months) (41) and does not result in acute CNS infection or pathology (8, 9, 30). Transplantation of 15-1 C17.2 cells in IRW mice resulted in extensive virus expression, both in the transplanted C17.2 donor cells and in endogenous microglial cells, and extensive associated spongiosis at 2 and 3 weeks posttransplantation. In regions where no cellular engraftment occurred, no microglial infection or spongiosis was detected. These areas were specific for each animal, depending on the distribution of the engrafted cells. It was of note that no pathology was observed in the caudal spinal cords of any of the mice examined. This was consistent with the lack of hind limb paralysis or abnormal adduction reflex in these mice. Thus, the correlation of transplants with pathology and with the presence or absence of typical clinical neurologic signs indicate that the release of virus from the C17.2 cells appears to be directly responsible for the infection of endogenous CNS microglial cells and the induction of spongiform neurodegeneration. A summary of the engraftment, virus production, histopathology, and clinical disease resulting from the different inoculation regimens is shown in Table 2.

Quantitative and qualitative features of CasBrE envelope expression in the CNS. In order to evaluate whether pathologic changes may be dependent on quantitative or qualitative features of CasBrE envelope expression in the CNS, envelope protein expression in transplanted brains was evaluated by immunoblotting of forebrain sections (Fig. 6A). By using β-galactosidase protein expression as a means for evaluating the relative level of transplantation, the levels of envelope protein

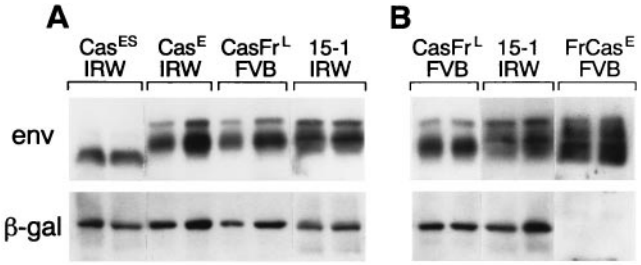

FIG. 6. CNS expression of envelope protein after transplantation of C17.2 cells. Extracts from forebrain sections (Fig. 4A) of CasBrE *env*- or virus-expressing C17.2 cell transplanted mice were evaluated for expression of envelope proteins (env) along with the corresponding levels of β-galactosidase (β-gal) by immunoblotting. Lanes are marked with the type of C17.2 cells which were transplanted (e.g., Cas^{ES}) and the strain of mouse in which the inoculations took place (*Fv-1^n* IRW or *Fv-1^b* FVB). Examples from two different brains for each transplantation regimen are shown and correspond to 3 weeks posttransplantation. Equivalent levels of brain extracts from each inoculation regimen were used. (A) Significant levels of envelope protein were made with comparable levels of β-galactosidase for brains transplanted with C17.2 cells expressing CasBrE virus or envelope protein only. This suggests that envelope protein expression levels are not responsible for the differences noted in the appearance of spongiosis. Qualitatively, the proteins made in the brains of these animals are generally reflective of the protein made in the cell extracts in vitro (Fig. 3). However, in the case in which virus expression from transplanted cells resulted in host infection (e.g., lanes 15-1 IRW), additional envelope protein heterogeneity is apparent in the region of gp70 when compared with mice transplanted with cells expressing *Fv-1*-restricted virus (CasFr^L FVB). This is more clearly illustrated in panel B, in which the samples were further resolved by SDS-PAGE. Comparison with a sample taken from a mouse inoculated i.p. with a nonrestricted virus (FrCas^E-FVB) also shows a high level of envelope protein complexity. This pattern is similar, but not identical to that seen after transplantation with cells expressing nonrestricted FrCas^E- and CasFr^L-C17.2 cells transplanted into IRW mice. FrCas^E-C17.2 cells transplanted into FVB mice (not shown) gave patterns similar those shown for 15-1 C17.2 cells inoculated into IRW mice.

expressed were compared. No significant differences in *env* expression between mice which exhibited pathologic changes and those which did not were observed. In contrast, qualitative examination of the envelope protein profiles indicated that the expressed protein from transplanted mice fell into two categories. Samples from transplants failing to induce pathology showed a simple pattern of envelope expression consisting of a pr85 precursor band and a processed gp70 protein (e.g., CasFr^L-C17.2/FVB). This pattern was akin to that associated with the cellular fraction of cultured C17.2 cells (Fig. 3). Samples derived from transplants expressing pathology, i.e., FrCas^E-C17.2/FVB, CasFr^L C17.2/IRW, and 15-1 C17.2/IRW, showed additional envelope heterogeneity in the processed protein (15-1 IRW in Fig. 6B). A comparison of the envelope protein profiles from transplanted animals with FrCas^E inoculated i.p. showed that 15-1 C17.2-transplanted mice express an envelope protein profile intermediate between those for CasFr^L and i.p. inoculated FrCas^E. Whether the envelope protein heterogeneity noted in diseased animals could be a primary cause or a secondary manifestation of the pathogenic changes is not known, but since infection of endogenous host cells occurred with the viruses which induced pathology, the additional protein species observed can likely be attributed to the appearance of this infection.

DISCUSSION

The means by which retroviruses induce neuropathologic changes in the CNS remains a challenging experimental problem. In this study we have explored this problem by in vivo reconstitution of defined stages of CNS infection by the neurovirulent virus CasBrE. These studies sought to address two

basic questions. First, is CNS expression of the *env* gene sufficient to induce spongiform neurodegeneration? Second, is the ability of CasBrE to cause pathogenic changes cell type dependent?

The first question is predicated on prior genetic mapping studies showing that the primary murine retroviral neurovirulence determinants reside within the *env* gene (11, 40, 41, 61, 62). The envelope protein is involved in binding and fusion of the virus to the cell surface, which for ecotropic viruses is mediated through interaction with the cellular protein MCAT-1, a basic amino acid transporter (1). Thus, it might be predicted that the CasBrE envelope protein could directly cause spongiform neurodegeneration by binding to MCAT-1 on the surfaces of neurons and glia, thus blocking basic amino acid transport. Alternatively, the CasBrE envelope could interact with other, yet-to-be identified, CNS receptors, which results in activation or inhibition of normal CNS function.

To test these hypotheses, we used transplanted multipotent neural progenitor cells as a vehicle for delivering CasBrE envelope proteins to a susceptible CNS. These engineered cells were capable of robust engraftment into the developing CNS as integral cytoarchitectural components. Our results indicated that although this expression system effectively delivered high levels of CasBrE envelope protein to susceptible CNS areas, the expression was not in and of itself neurotoxic. Since the transplanted cells expressing the envelope protein were observed to express differentiated phenotypes, it was suggested that the envelope protein interactions within these cell types were unlikely to be a primary factor in neurodegeneration. The most straightforward interpretation of these findings is that in order for the envelope protein to mediate neurologic changes, this protein must be eliciting its effects at a stage of the virus replication cycle after receptor binding, in cells other than those which are neuroectodermally derived. This explanation is consistent with prior observations of neuronal infection without degeneration in mice inoculated with the highly neurovirulent chimeric retrovirus FrCas^E (29, 30) and CasBrE (23).

In order to explore whether early or late virus replication events were necessary for inducing spongiform neurodegeneration, we sought to develop a system whereby neurovirulent virus replication would be specifically inhibited at an intermediate stage. It is known that various mouse strains differ widely in their susceptibilities to infection and disease induction by the MuLVs. One specific locus which has been shown to restrict Friend virus-induced leukemia (26) and neurodegenerative disease induced by CasBrE is *Fv-1* (13, 14, 18, 38, 39). The *Fv-1* locus contains two alleles designated *n* and *b* to represent the ability of virus to infect NIH Swiss or BALB/c mice, respectively. N-tropic viruses will infect mice homozygous for the *n* allele and are unable to infect mice containing the *b* allele, and vice versa. NB-tropic viruses are not restricted by *Fv-1*. The viral sequences responsible for *Fv-1* restriction have been mapped to a limited region of the viral *gag* gene. The *gag* gene sequences, which are different for N-, B-, and NB-tropic viruses, affect the structure of the p30 Gag protein, which is a major structural protein of the viral capsid. The *Fv-1* gene product interacts with p30 Gag and inhibits viral replication at the level of proviral integration (20, 21; reviewed in references 18 and 57).

In this study, we used the *Fv-1* restriction in concert with constitutive virus expression from engrafted neural progenitor cells to address whether virus replication cycle events in host cells, prior to proviral integration, are responsible for inducing spongiform lesions. In this model system, virus-expressing cells did not result in coincident spongiform changes, indicating that virus penetration events through reverse transcription in host cells are not sufficient to induce disease. In contrast, transplantation of neural progenitor cells expressing nonrestricted virus resulted in the appearance of spongiform neurodegeneration. However, it was the expression of CasBrE envelope protein in host microglial cells rather than in the engrafted neural progenitor cells which correlated with pathologic changes. In this model the neural progenitor cells were necessary for delivering replication-competent virus to the CNS, but the formative events in neurodegeneration are occurring in infected microglia. More specifically, these results suggest that late rather than early virus replication events in microglia are necessary for retrovirus-induced disease. Whether this involves envelope protein synthesis, processing, and/or release in budding virus particles remains to be determined.

The interpretation that infection or *env* expression in cells of neural origin is not associated with the induction of neurodegeneration is consistent with previous reports by us (28, 29, 31) and others (2, 3, 17) indicating that the infection of microglia most closely correlates with pathologic changes in the CNS. Similar speculations have been made for human immunodeficiency virus, given that the infection of monocyte/macrophage cells is necessary for neurotoxicity in vitro (15, 16, 27) and is closely associated with neurodegenerative changes in vivo (34, 55, 59). The supposition that microglial infection is central to retroviral neuropathogenesis is supported by our previous transplantation experiments using FrCas^E-infected microglia (31). In these experiments, infected microglia were introduced into postnatal-day-10 mice, which are developmentally resistant to CNS infection. Transplantation resulted in the focal appearance of pathology coincident with infected microglia. The neural progenitor cell transplant experiments presented here also support the idea that virus infection of microglial cells is directly responsible for inducing spongiform neurodegeneration. Evaluation of how CasBrE infection, or *env* expression, in this cell type causes disease will require CNS reconstitution of the microglial compartment, wherein virus replication is restricted to and within microglia and virus spread is limited within the host.

We suggest two possible explanations for how the CasBrE envelope protein could be inducing pathology by interactions within microglia. First, infection of microglia could result in MCAT-1 interactions that block basic amino acid transport. It was clear from our present study that CasBrE envelope–MCAT-1 interactions in C17.2 cells were sufficiently high to completely inhibit FB29 superinfection. However, whether the CasBrE envelope protein can inhibit MCAT-1 amino acid transport is not known. Studies by others have demonstrated that ecotropic envelope–MCAT-1 interactions result in a 50 to 70% decrease in arginine transport activity after either virus infection of MCAT-1 containing cells (56) or coexpression of envelope protein and MCAT-1 in oocytes (24). However, the ability of ecotropic envelope protein to inhibit MCAT-1-mediated arginine transport is only about 25% when cells are exposed to saturating concentrations of extracellular gp70 (56). Thus, MCAT-1–envelope interactions upon synthesis within an intracellular compartment may result either in restricted MCAT-1 trafficking to the plasma membrane or in higher-affinity envelope-receptor binding. Either of these events could result in the greater transport inhibition observed upon MCAT-1–envelope coexpression.

Following this line of reasoning, infection of microglia, rather than extracellular exposure to envelope protein or virus, might completely block amino acid transport by increased binding or altered MCAT-1 trafficking. Such MCAT-1 inhibition could deny microglial cells sufficient arginine to carry out their normal scavenger and degradative functions in the

CNS. It has been well documented that arginine serves as the precursor for nitric oxide (NO), a molecule which has been implicated in a wide variety of physiologic functions in macrophages and in the CNS (6). Disruption of normal NO homeostasis in microglia might be sufficient to precipitate pathologic changes. While there are currently no data addressing this hypothesis, we have begun to test whether CasBrE infection of microglia in vitro alters NO homeostasis.

An alternative proposal for CasBrE envelope protein-mediated pathogenesis would require that microglial infection results in the production of a novel CasBrE envelope protein which has neurotoxic properties. Previously, and in this report, we have identified novel envelope protein expression patterns in the brains of mice infected with CasBrE viruses (10, 28, 30). Our current experiments showed that the envelope expression patterns were very simple when virus spread to the host was restricted. However, when microglial cells were infected and neurodegenerative changes were noted, additional envelope protein complexity became apparent. Whether the appearance of additional envelope proteins is specifically due to microglial infection and represented the appearance of "Env neurotoxins" or is simply a consequence of neurodegeneration is not known. Interestingly, in vitro infection of microglia by CasBrE results in defective envelope processing. Specifically, envelope precursor protein is not proteolytically cleaved from the pr85 precursor to gp70 and p15E. This processing defect correlates with an inability of infected cells to bud virions from the cell surface (28). Although it has been reported that macrophages produce neurotoxic human immunodeficiency virus gp120 in vitro (27), our initial attempts to show CasBrE infection-specific microglial neurotoxicity in spinal cord slices in vitro have not revealed evidence in support of this proposal (4). It may be necessary, therefore, to have a mature, active, functioning CNS in order to observe neurologic changes. Thus, in vivo reconstitution experiments may be the only reliable means for testing these hypotheses.

Particular note should be made of the strategy by which the questions in this study were approached. Multipotent neural progenitor cells, which can be easily genetically manipulated ex vivo prior to transplantation and which can integrate throughout the CNS in a cytoarchitecturally appropriate manner without altering other neurobiologic functions, can serve as cellular vehicles for gene transfer (49, 50). These cells have received attention of late for their ability to transfer therapeutic gene products to the CNS (25, 51–53). However, here we demonstrate that these cells also can be used to generate models of pathology via the transfer of potentially toxic molecules or viral particles. This approach provides a novel means to sequentially dissect the role that particular factors play in complex pathologic processes. Therefore, we hope that this study might serve as a paradigm for testing other molecules which are candidates for inducing pathology in other types of neurodegenerative conditions, including those which are not virally related. This strategy may prove more expeditious and efficient for certain CNS diseases than creating transgenic or knockout mouse models. Furthermore, once such neural progenitor-mediated models of pathology have been generated, these animals may then serve as testing grounds for various therapeutic interventions.

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