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Microglial Infection by a Neurovirulent Murine Retrovirus Results in Defective Processing of Envelope Protein and Intracellular Budding of Virus Particles

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The observation of murine retrovirus infection of microglial cells in brain regions expressing spongiform neurodegenerative changes suggests that these cells may play an important role in pathogenesis. To evaluate this potential in vitro, murine microglial cells were infected in mixed glial cultures with the highly neurovirulent murine retrovirus, FrCas^E. The microglia were then isolated from the mixed cultures on the basis of their differential adherence and shown to be approximately 98% pure. The infected microglia expressed viral envelope protein at the plasma membrane, while viral budding was primarily intracellular. Evaluation of the viral envelope protein by immunoblotting indicated that the immunoreactive species produced was exclusively a 90-kDa precursor protein. Very little of the envelope protein was associated with particles released from these cells, and viral titers in the culture supernatant were low. Interestingly, these cells were still capable of infecting permissive target cells when seeded as infectious centers. This partially defective infection of microglial cells suggests a potential cellular means by which a neurovirulent retrovirus could disrupt normal microglia and in turn central nervous system motor system functioning.

Retroviral infections of the central nervous system (CNS) are capable of inducing severe clinical and histological changes in a wide variety of mammals, including humans. A common finding among a variety of type C murine retroviruses and lentiviruses is the infection of microglial cells in the CNS of affected individuals, and it is suspected that these cells may play an important part in the pathogenic process (2, 3, 17, 26, 31, 47, 49).

One such virus, called CasBrE, was first identified in populations of wild mice (13) and causes profound spongiform pathology which is primarily restricted to grey matter areas of the motor system (for reviews, see references 18, 34, and 51). Clinically, the disease is manifested by tremulous paralysis of the hind limbs with coincident wasting and ultimately death. The disease in the wild was originally shown to have a variable incidence and an extended time course, making the disease process difficult to study. However, rapid models of the disease with complete penetrance were ultimately developed (5, 35, 41). This progress was due in part to the molecular cloning of the causative viruses (19, 50) and identification of the neurovirulence determinants (35, 39, 52, 53). One of the fastest models resulted from the construction of a chimeric virus in which the envelope gene from a marginally neurovirulent molecular clone of CasBrE was placed into the background of a Friend murine leukemia virus (MuLV) which has rapid in vivo replication kinetics. Mice inoculated with this virus, called FrCas^E, exhibit clinical neurologic signs by 16 days after neonatal inoculation and reach the terminal stage of disease by day 25 (35).

Immunohistochemical and electron microscopic studies of this very rapid disease failed to show viral infection in the neurons which ultimately degenerate. What these studies did show, however, was an apparent colocalization of infected microglia and regions undergoing spongiform change (26). Similar studies with a neurovirulent temperature-sensitive mutant of Moloney MuLV, *ts*1, showed much the same picture, that is, no neuronal infection and significant microglial infection in regions of pathology (2, 3). Recent studies with one of the original CasBrE molecular clones have also shown this correlation (17). These observations suggest that the pathogenesis of spongiform change is indirectly mediated through microglial infection. Thus, in this report we have evaluated the interaction of the highly neurovirulent murine retrovirus, FrCas^E, with microglial cells in vitro, in an attempt to identify processes which could be involved in disease induction.

Microglial cell cultures were prepared by the shake-off method of Giulian and Baker, which takes advantage of the differential adherence of microglial cells in mixed glial culture (15). In these cultures, microglia tend to grow on top of a mixed glial monolayer composed primarily of astrocytes. Once released by shaking, microglial cells will attach to untreated plastic culture dishes more rapidly and tenaciously than other released cells (primarily oligodendroglia [24]). Our initial attempts to directly infect such enriched microglial cultures with the neurovirulent chimeric retrovirus FrCas^E failed to result in significant infection (less than 1 cell in 100; not shown). We suspected that this might be because our murine microglial cultures showed only a very limited amount of cell division, a prerequisite for most retroviral infections (46). This lack of cell division is in contrast to the initial report from Giulian and Baker, whose cultures lost the ability to divide only upon differentiation into ramified cells (15). To overcome this problem, we exposed the initial mixed glial cultures to the FrCas^E virus at a multiplicity of infection of approximately 1, 6 days after the initial plating (a time when the cells are 50 to 75% confluent). These cultures have been shown to contain microglial progenitor cells (40). Within 6 days, the mixed cultures were observed to be confluently infected when immu-

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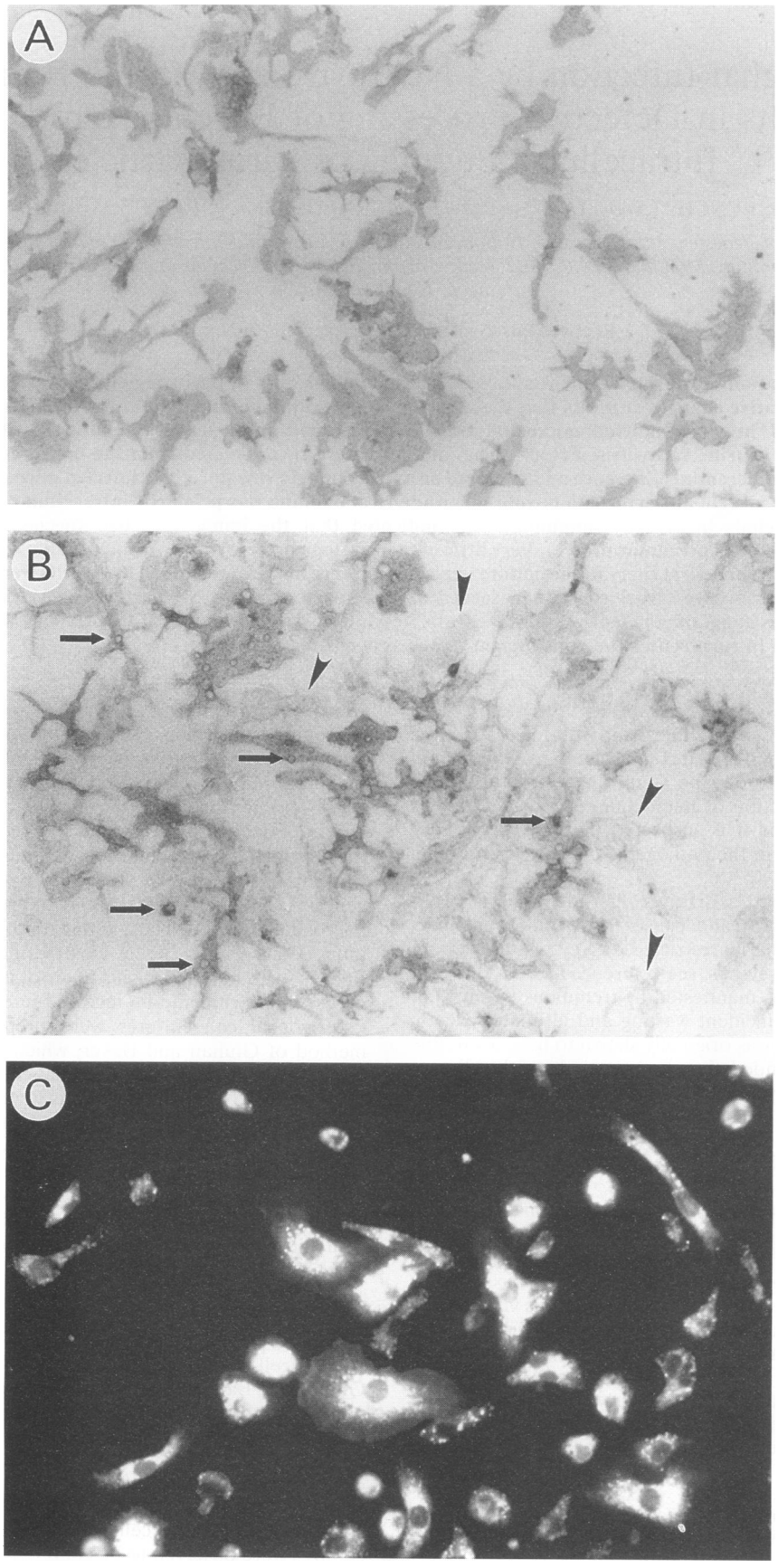
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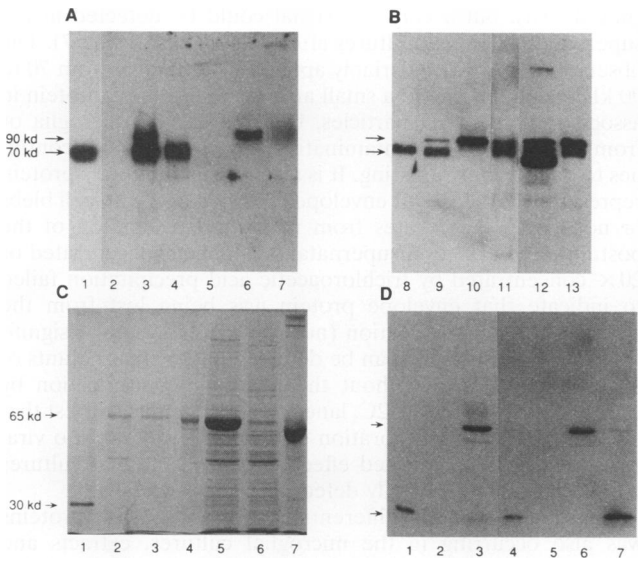


FIG. 2. Immunoblotting of FrCas^E-infected cultures indicates that microglia fail to process viral envelope protein. Identification of the envelope and *gag* viral proteins in cultures of microglia and mixed glial cells is shown in panels A and D, respectively. Envelope proteins were detected with monoclonal antibody 697 (29), and *gag* proteins were detected with monoclonal antibody R187 (7) as has been previously described (11, 27). A comparison between the envelope proteins observed in cultured cells with those seen in brain and spleen from mice exhibiting clinical neurological signs, using monoclonal 697, is shown in panel B. Panel C shows a Coomassie blue-stained gel and indicates the protein loads used to obtain the signals shown in panels A and D. Lanes: 1 and 8, FrCas^E virus; 2, uninfected mixed glial culture; 3 and 9, FrCas^E-infected mixed glial culture; 4, 7-day culture supernatant from FrCas^E-infected mixed glial culture; 5, uninfected microglia; 6 and 10, FrCas^E-infected microglia; 7, 50×-concentrated, 7-day culture supernatant from FrCas^E-infected microglial cells; 11, FrCas^E-infected spleen, 14 days postinfection (11); 12, FrCas^E-infected CNS, 14 days postinfection (11); 13, FrCas^E-infected NIH 3T3 cells. SDS-PAGE analyses was performed on 9% gels under reducing conditions.

nostained with FrCas^E envelope-specific monoclonal antibody 667, as has been described previously (26, 27, 29). Double staining with antibody markers for viral envelope protein and astrocytes (GFAP; Dako) (4), oligodendroglia (antigalactocerebroside; Accurate) (38), endothelia (anti-factor VIII; Biogenex) (30), microglia (Mac-1 [43, 44] and F4/80 [1]), or fibroblasts (antifibronectin; Biogenex) indicated that all these cell types were infected in the mixed cultures (not shown). These cultures were maintained for another 4 days, after which time the flasks were shaken at 180 rpm at 37°C for 4 h to release the microglia which were growing on top of the mixed glial monolayer. Cells released into the supernatant were removed, spun down at 2,000 rpm for 5 min, and resuspended in macrophage serum-free medium (GIBCO) containing granulocyte macrophage colony-stimulating factor (0.5 ng/ml; Peprotech) and colony-stimulating factor type 1 (5 U/ml; Sigma). Shake-off cells were plated at a density of 10^6 per well in an untreated TC-6 tissue culture plate (Linbro). After 1 h, the medium was removed and the plates were washed with Dulbecco's phosphate-buffered saline (DPBS) to remove cells which failed to attach during this period. Fresh medium was added, and the cells were grown overnight before further analysis. Immunohistochemical staining of the attached population for the macrophage marker, Mac-1 (Fig. 1A), indicated that greater than 98% of the isolated cells were microglia whether they came from infected or uninfected cultures. This enrichment was consistent with the original report for uninfected cells from Giulian and Baker (15). Microglial identity was also confirmed by immunostaining with monoclonal antibody F4/80 and the lectin ricin communis agglutinin I (45) (not shown). Contaminating cells included astrocytes, endothelial cells, fibroblasts, and oligodendroglia.

Analysis of the microglial cultures for FrCas^E viral infection was examined immunohistochemically by using monoclonal antibodies 667 and 697, both of which are specific for the FrCas^E envelope protein (29). The staining of nonpermeabilized, 10% formalin-fixed microglia cultures showed significant staining associated with the cell surface (Fig. 1B). Immunostaining after permeabilization by using 0.1% Triton X-100 or −20°C acetone showed additional immunoreactive protein with an intracellular location, consistent with the normal cellular trafficking of envelope protein (not shown). Greater than 95% of cells plated were positive for envelope expression, although the level of expression varied considerably from cell to cell (Fig. 1). Viral envelope protein could also be observed on cell blebs, both free and still associated with the microglial cells. These blebs were more numerous the longer the initial mixed glial cultures were maintained in vitro prior to isolation of the microglia. No staining was observed in uninfected cultures stained with the envelope-specific monoclonal antibodies or in infected cultures stained in the absence of the envelope-specific monoclonal antibodies (not shown).

To investigate whether specific microglial cell functional activity showed changes as a result of infection, cultures were evaluated for the ability to bind and endocytose acetylated low-density lipoprotein (AcLDL) and phagocytose 1.0-μm-diameter latex beads (15) 24 h after plating. As indicated in Fig. 1C and 3, endocytosis of AcLDL (fluorescently labeled with 1,1′-dioctodecyl-,3,3,3′,3′-tetramethyl indocarbocyaninate [DiI]) was unaffected as a result of virus infection. The microglial cells' ability to phagocytose latex beads (Polysciences) also showed no apparent differences from that of uninfected cells (not shown). These results suggest, at least at a superficial level, that viral infection was not disruptive to the

FIG. 1. Infection of microglial cells by the neurovirulent virus FrCas^E in vitro. Microglia were enriched from cultures of mixed glial cultures after infection with FrCas^E and evaluated immunohistochemically for microglial markers Mac-1 (A; magnification, ×340), F4/80, and RCA-1 (not shown). By these criteria, cultures were greater than 98% pure microglia. Immunohistochemical staining of nonpermeabilized microglial cells for FrCas^E envelope protein, using monoclonal antibody 667, is shown in panel B (magnification, ×340). Greater than 95% of the microglial cells were positive for envelope protein, although the level of surface expression was quite variable from cell to cell. Low-level microglial staining (indicated by arrowheads) was easily distinguished from unstained cells in the same culture. Unstained cells were difficult to demonstrate photographically and thus are not shown. Staining of infected cultures without the primary antibody was essentially nonexistent. In addition, staining of microglial cells derived from uninfected cultures failed to show any immunostaining with virus-specific antibodies 667 and 697 (not shown). Blebbing from microglia can be seen on a number of cells (arrows in panel B). In addition to the expression of microglial markers, the infected cells were also tested for the ability to bind and phagocytose DiI-AcLDL as described by Giulian and Baker (15). Panel C shows the presence of the fluorescent dye in intracytoplasmic vesicles of all the infected microglial cells regardless of their level of *env* expression (magnification, ×865).

endocytic or phagocytic activity specific to these cells. In addition, no cytotoxicity of virally infected mixed glial or microglial cultures was observed, as cultures could be maintained for at least 3 months without significant cell loss.

As previously mentioned, the microglia used in these experiments (either infected or uninfected) showed only limited potential for cell division once isolated from the mixed glial culture. Contaminating astrocytes, fibroblasts, and endothelial cells continued to divide (albeit quite slowly in the macrophage serum-free medium) and could represent a substantial contaminant (up to 5% of the total cells) after 2 to 3 weeks of culturing the shake-off population. Therefore, biochemical and infectivity assays were carried out within the first 7 days after microglial shake-off and plating to minimize the potential contribution of contaminating nonmicroglial cells.

A variety of viral envelope-immunoreactive species have been observed in vivo for the $FrCas^E$ virus (11, 27). These varied envelope proteins might arise from defective retroviral envelope protein processing such as has been reported in vitro for astrocytes (42) and thymus- or bone marrow-derived TB cells (50) infected with *ts*1 Moloney MuLV and for fibroblasts and lymphocytes infected with the feline leukemia virus FeLV-FAIDS (37). Alternatively, some of these species may be due to cell-type-specific glycosylation such as that reported for feline immunodeficiency virus (36). Therefore, we evaluated the expression of $FrCas^E$ viral envelope protein in both mixed glial cultures and the enriched microglial cell cultures by Western blotting (immunoblotting) using monoclonal antibody 697 (Fig. 2A and B) (11, 27). Total microglial cell extracts showed only a 90-kDa protein (lanes 6 and 10), whereas mixed CNS cultures expressed both 90- and 70-kDa envelope proteins (lanes 3 and 9). Given that the analysis was done under reducing conditions, these results suggest that the viral envelope precursor protein was not proteolytically cleaved after synthesis in the endoplasmic reticulum. To confirm this possibility, parallel samples were evaluated with polyclonal antibody specific for p15E by Western blotting (7). The results indicated that the 90-kDa band was immunoreactive for the transmembrane p15E protein and thus represents an uncleaved precursor protein (not shown). Whether this protein also fails to undergo normal glycosylation has yet to be evaluated. Since only the 90-kDa envelope precursor protein was observed in microglia when analyzed by immunoblotting, it is assumed that it is the same protein expressed on the cell surface of the infected microglial cells. It is of interest that the $FrCas^E$ envelope protein did not appear to accumulate in an intracellular compartment as was observed in astrocytes by Shikova et al. for the *ts*1 virus (42). Reports on other MuLVs have suggested that proteolytic cleavage, glycosylation, and disulfide bond formation may be necessary for transit to the cell surface and release in infectious virions (16, 21). However, observations of mutants of Rous sarcoma virus have shown that uncleaved envelope precursor protein is expressed on the plasma membrane (12, 25), and depending on the nature of the mutation, the precursor protein can be efficiently incorporated into viral particles (12).

To examine whether a mature gp70 or 90-kDa precursor protein was being made in the infected microglial cells and released from the cell surface in association with viral particles, filtered (0.22-μm-pore-size filter) supernatants from 1-, 4-, and 7-day cultures were subjected to ultracentrifugation at 300,000 $\times$ *g* for 45 min and then resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (effective concentration, 50$\times$). Western blotting analysis of these samples indicated no detectable envelope-immunoreactive protein in the supernatants after 1 and 4 days (not shown), but a very low signal could be detected in the supernatants of a few cultures after 7 days (Fig. 2, lane 7). The observed signal which variably appears as a smudge from 70 to 90 kDa could be due to a small amount of envelope protein in association with viral particles, from either the microglia or from a nonmicroglial contaminating cell fraction which continues to divide after replating. It is also possible that this protein represents the release of envelope in association with cell blebs or nonspecific aggregates from microglia. Evaluation of the postultracentrifugation supernatants either unconcentrated or 20$\times$ concentrated by trichloroacetic acid precipitation failed to indicate that envelope protein was being lost from the virions during centrifugation (not shown). However, a significant Env protein signal can be detected in the supernatants of mixed glial cultures without the need for concentration by ultracentrifugation (Fig. 2C, lane 4). These results suggest that the cleavage and incorporation of envelope protein into viral particles was accomplished effectively in mixed glial cultures but was partially or wholly defective in microglial cells.

To evaluate whether differential processing of Gag proteins was also occurring in the microglial cultures, extracts and supernatants were evaluated by Western blotting using a Gag-specific monoclonal antibody, R187 (7, 11). Protein equivalents were loaded as shown for envelope protein evaluation (Fig. 2A and C), and Gag immunoreactivity was observed in both the microglial culture and the supernatant, with pr65 being the predominant cell-associated form and p30 being predominant in the supernatants. This is consistent with the patterns expressed in mixed glial cultures (Fig. 2D, lanes 2 and 3). Given the difficulty of detecting envelope protein in the microglial culture supernatants (compared with infected mixed glial cultures) it was surprising that there was a significant level of $p30^{gag}$ protein. This finding suggests that microglia are capable of budding viral particles, but particles that are devoid of the normal gp70 protein, and perhaps contain no envelope glycoprotein at all. These data most closely parallel the observations of Linial et al., who found that a mutant of Rous sarcoma virus expressed envelope precursor on the cell surface but failed to incorporate it into virions (25).

To evaluate whether the defective processing of CasBrE viral envelope protein was influenced by the Friend MuLV background into which the envelope gene was cloned (35) or was a unique property of the envelope sequence alone, an envelope gene expression vector was generated to determine whether defective envelope expression could be observed in the absence of *gag* and the 5′ *pol* genes. This was accomplished by deleting the *gag-pol* region of $FrCas^E$ viral genome from *Pst*I to *Hin*dIII as has been previously described for CasBrE (20). This results in an envelope gene that is driven by the Friend MuLV long terminal repeat (LTR). Transfection of this ecotropic *env* expression plasmid into PA317 psi amphoteric packaging cell cultures (32) resulted in spread of the LTR *env* vector throughout the culture in a fashion analogous to the ping-pong experiments described by Kozak and Kabat (23). These cultures produced packaged LTR-*env* virus at titers of 10^4 to 10^5 focus-forming units (FFU)/ml without the generation of any detectable helper virus. Infection of mixed glial cultures with LTR-*env*/PA317 viral supernatants (as described above) resulted in infection and expression of the envelope protein. Isolation of microglial cells from these cultures indicated that approximately 20% of these cells were expressing surface envelope protein by immunohistochemistry using monoclonal antibody 667 (not shown). Analysis by immunoblotting using monoclonal antibody 697 indicated that the envelope protein expression by mixed glia and microglia was indistinguishable from that observed from $FrCas^E$ virus-in-

TABLE 1. Infectivity associated with FrCas^E-infected microglia

Prepn	Virus titer[a]		Infectious centers[b]		In vivo infection, DiI-AcLDL sorted[c]
	24 h	96 h	DiI sorted	Trypsin treated	
Microglia	6.2×10^1	2.1×10^2	5.6×10^5	5.1×10^5	5/5
Primary CNS	6.1×10^4	1.0×10^5	ND[d]	ND	4/4

[a] Average of four different cultures, expressed as focus-forming units per milliliter generated by 10^6 microglial cells over the given incubation periods (9).
[b] Per 10^6 microglial cells seeded (8).
[c] 10^3 cells were inoculated intraperitoneally into neonatal IRW mice. Post shake-off, DiI-AcLDL-treated primary CNS cultures were used as the control for in vivo infectivity. Values represent numbers of mice expressing clinical neurodegenerative disease within 20 days of inoculation.
[d] ND, not determined.

fected cells, i.e., 90-kDa precursor expression in microglia and processing to gp70 in mixed glia (not shown). Thus, the altered processing of the envelope protein was likely attributable to the envelope gene alone.

The envelope protein profiles observed for infected microglia and mixed glial cultures were also compared with the immunoreactivity seen in extracts from the infected brain and spleen of clinically sick animals and infected NIH 3T3 cells (Fig. 2B). The comparison of the infected brain (lane 12; see also references 11 and 27) with mixed glial and microglia culture (lanes 9 and 10, respectively) indicates an apparent reduction in molecular complexity that can result from isolation of individual CNS components. While the presence of abundant 90- and 70-kDa proteins in the CNS sample are consistent with the observation of microglial and endothelial cell infection in vivo (2, 3, 26), the predominant 65-kDa envelope protein has no counterpart in the mixed glial or microglial cultures. Preliminary results from our laboratory indicate that this 65-kDa protein is likely to be contributed by infected neurons which fail to normally glycosylate the Env protein (28). Neurons are clearly absent from the cultures analyzed in this report. However, caution must be exercised in suggesting that specific envelope protein species may be diagnostic for specific-cell-type infection. As observed here for FrCas^E-infected NIH 3T3 fibroblasts (lane 13) and spleen, and as noted for *ts*1 Moloney MuLV-infected endothelial cells and astrocytes (42), it is clear that other tissues and cell types can express significant levels of the precursor protein. To further complicate matters, it is possible that the ability of cells to express and process protein in vivo is quite different from that in vitro. Therefore, it should prove instructive to assess whether the processing defect observed in vitro also occurs in microglia in vivo.

To test whether the infected cultures were capable of making infectious virus, virus titers in the supernatants from the infected mixed glial and microglial cultures were determined after 1 and 4 days, using a focal immunoassay (9). The results, shown in Table 1, indicate an inability of microglia to produce or release infectious virions into the supernatant. The low virus titers found may be explained by the nature and paucity of envelope protein observed in the Western blotting experiment.

Given the relative inability of infected microglia to produce infectious particles, we also investigated the potential of these cells to infect permissive target cells as infectious centers. FrCas^E-infected mixed glial cultures were labeled with 2 μg of DiI-AcLDL (Molecular Probes) per ml for 5 h. Shake-off cells obtained from these cultures were washed three times and then selected for DiI fluorescence by flow cytometry using a Becton Dickinson FACStar. The sorting gates (see Fig. 3) selected the brightest 63% of the shake-off population to minimize contamination with nonmicroglial cell populations

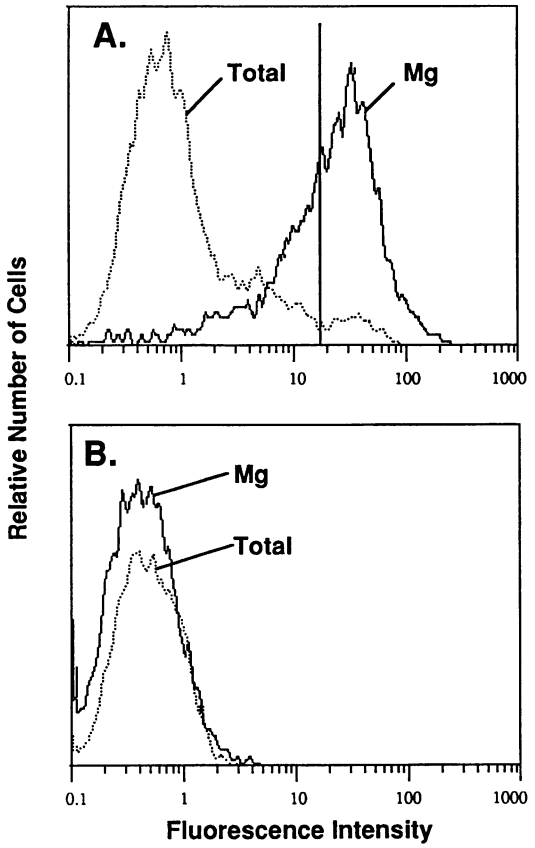
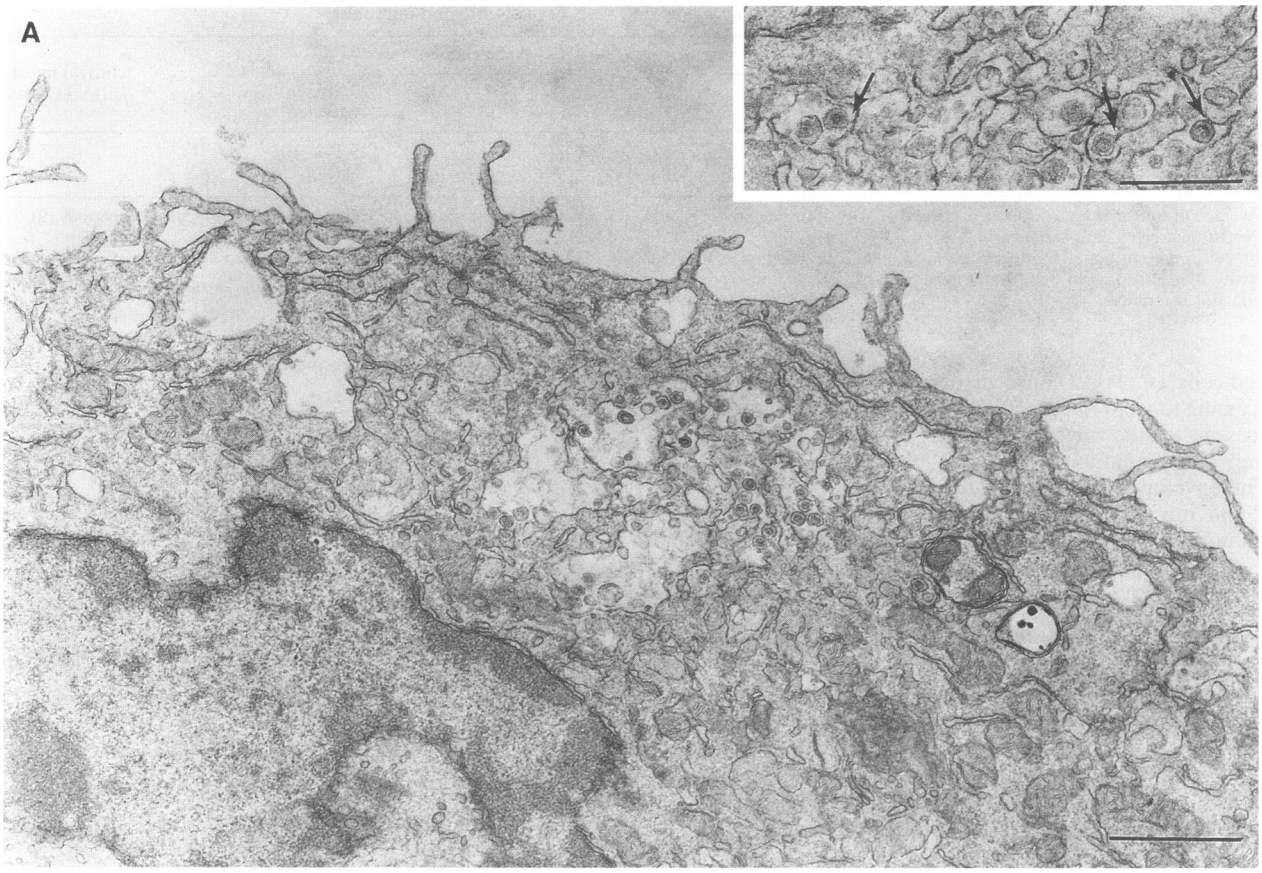


FIG. 3. Sorting of DiI-AcLDL-labeled FrCas^E-infected microglia for infectious center assay. Mixed glial cultures were labeled with 2 μg of DiI-AcLDL per ml of culture medium (A) or not labeled (B) and incubated for 5 h at 37°C. Cultures were then shaken for 4 h, and the released cells (Mg) were washed three times in DPBS containing 2% fetal calf serum prior to sorting. A nonshaken parallel culture (Total) was released by trypsinization and then washed. All cells were resuspended in DPBS–2% fetal calf serum. The microglial population is clearly much brighter than the majority of cells in the total population treated with DiI-AcLDL. Brightly labeled cells represented approximately 7% of the total mixed glial population. Staining of nonreleased postshake cultures with the microglial cell-specific monoclonal antibodies Mac-1 and F4/80 indicated that many microglia are not released by the shake-off (not shown), as has been previously reported (15). The shake-off population was sorted, using the gate shown in panel A (heavy black line), to minimize the contamination due to shake-off released, nonmicroglial cells. Forward scatter gates were also used to eliminate cell aggregates and cellular debris. The gates selected 63% of the total shake-off population, and these cells were used for the infectious center assay (Table 1).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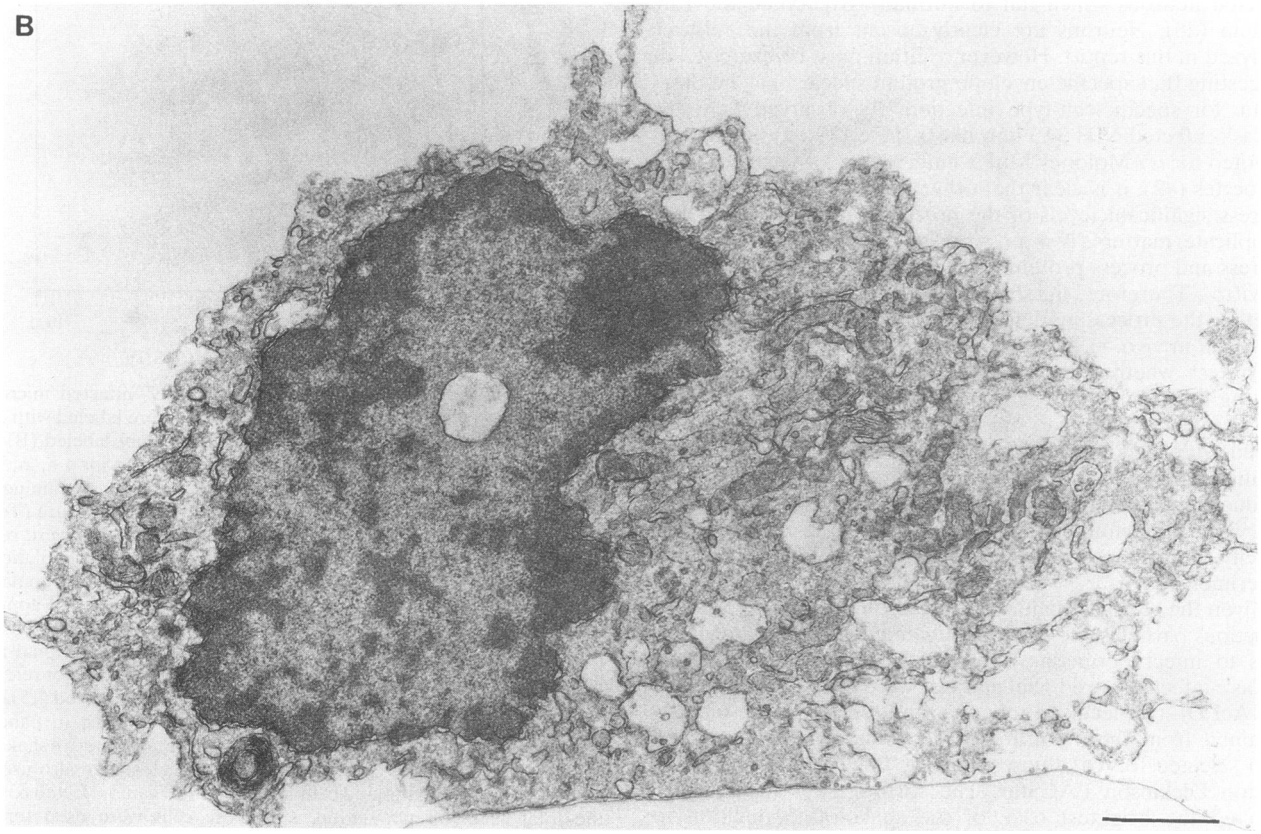
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present at low levels in the shake-off. For the infectious center assay, sorted cells were washed in DPBS, seeded at densities of between 2,000 and 20 cells in individual wells of TC-6 tissue culture plates, and allowed to attach overnight; 10^6 NIH 3T3 cells were then overseeded, and foci were scored 3 days later (8). The results (Table 1) indicated that over half of the cells highly positive for AcLDL binding and uptake scored as infectious centers, a value over 3 orders of magnitude greater than would have been expected on the basis of viral titers in the supernatants. This number of infectious centers was similar to that observed for nonsorted shake-off cells that were treated with 0.125% trypsin for 10 min at room temperature (to inactivate any virus adsorbed to the microglial cell surface; Table 1). Examination of the foci identified in the infectious center assay of DiI-AcLDL-labeled cells indicated that approximately 80% of the foci showed the presence of a fluorescent (DiI-labeled) microglial cell.

Analysis of the infectivity of the sorted microglial cells in vivo was carried out by intraperitoneal inoculation into five neonatal IRW mice of approximately 10^3 DiI^+ cells per animal. This resulted in a 100% incidence of clinical neurological disease within 17 days of inoculation. It has been demonstrated by Czub et al. (10) that injections of IRW mice with $FrCas^E$ at levels of 250 FFU per animal or less results in a lengthening of the incubation period. The injection of 10^3 microglial cells would be expected to result in the production of less than 1 FFU/day. If in vivo infection were to occur by the production of cell-free virus, we would have expected to see a significant increase in the incubation period. Since we observed very rapid disease, the results suggest that the microglia infect susceptible target cells both in vitro and in vivo by a means other than the normal production of cell-free virus.

It has been well documented that monocyte/macrophage cells infected with human immunodeficiency virus bud virus into intracellular vacuolar compartments (14, 33); however, human immunodeficiency virus-infected microglial cells were shown by Watkins et al. to bud virus from the cell surface (48). Therefore, we examined our enriched microglial cultures by transmission electron microscopy for the presence of viral particles. Figure 4A clearly shows the presence of C-type particles in membrane-bound intracellular compartments. The exact nature of this compartment is not clear but could represent endosomes or endoplasmic reticulum. This observation may be unique to cell culture, as intracellular budding into microglia in vivo has yet to be observed in animals infected with either $FrCas^E$ (26) or *ts*1 Moloney MuLV (2). In addition to the intracellular particles, virions could also be seen associated with the cell surface of some cells, indicating that particles may be released by exocytosis (not shown). We were unable to find any examples of virus budding directly from the cell surface. The release of intracellular particles would support the observation of pelletable Gag protein in the supernatants of infected microglial cells (Fig. 2D, lane 7). No other pathological anomalies were noted when infected and uninfected cells were compared at the ultrastructural level (e.g., Fig. 4).

These findings on microglial infection are quite different from what was observed in astrocytes infected by *ts*1 Moloney MuLV. In astrocytes, *ts*1 infection is cytopathic, is inefficient in transport and processing of envelope precursor, and shows intracellular budding of virus particles, yet these cells still express infectious virions at high levels (42). Whether intracellular budding of virions is a common feature in our mixed glial cultures has yet to be examined; however, no cytopathology has yet been observed at the light microscopic level over extended periods.

If the viral particles from microglial culture have little or no envelope protein and are noninfectious, how then do they transmit virus in the infectious center assay? Chesebro et al. reported that a MuLV-infected cell's ability to score as an infectious center is dependent on the rate of virus production by that cell, which requires the secretion of 1 FFU/40 h (8). It is that clear infected microglia do not satisfy this requirement, as they produce 2×10^{-4} FFU/40 h. To account for the observed high infectious center titer by the production of free virus, microglia would need to produce infectious virus at a rate approaching that reported above (1 FFU/40 h), although the virus would have to have a very short half-life to account for the observed low virus titer. Comparison of the viral titers from the 24- and 96-h time points would suggest that this may not be the case, since viral titer increases almost linearly with increasing incubation period. However, the observed virus titer might be totally accounted for by contamination of the microglial cultures with other cell types with productive viral infection.

While we have yet to find evidence that virus containing gp70 is made in microglial cells, cell-to-cell contact may allow the envelope precursor to facilitate virus budding directly into the target cell with high efficiency. Cell-to-cell contact may alternatively allow for the release of viral particles contained within intracellular compartments. Whether this intracellular virus is somehow qualitatively different has yet to be determined. Another, perhaps more likely possibility is that the target cell provides the proteolytic enzyme necessary for cleavage of the 90-kDa envelope precursor protein expressed on the cell surface. Once cleaved, it may be readily incorporated into infectious virions or allow direct budding into the target cell. Dong et al. have demonstrated that Rous sarcoma virus virions containing the envelope precursor are not infectious but can be made infectious with exposure to low levels of trypsin (12). It will be of interest to determine whether the release of infectious virions can be induced with mild trypsin treatment in this system.

While the exclusive production of 90-kDa envelope protein by microglial cells may explain their differential infectivity behavior, the question remains as to why $FrCas^E$-infected microglia fail to process the envelope precursor to gp70 and p15E. While the lack of the appropriate protease in the endoplasmic reticulum may be one possibility, others also exist. Mutagenesis studies on N-linked glycosylation sites of Friend MuLV envelope protein indicate that alteration of the highly conserved gs4 glycosylation site prevents subsequent processing of 90-kDa to gp70 and p15E and is associated with a loss of viral infectivity (21). Thus, the absence of the appropriate glycosylation machinery which can recognize the gs4 site in the

FIG. 4. $FrCas^E$-infected microglia bud virus intracellularly. Infected (A) and uninfected (B) microglial cells were examined by electron microscopy (6) 5 days after shake-off to minimize the potential observation of viral particles phagocytosed while the microglia were still in mixed glial cell culture (bar = 1 μm). Viral particles were readily apparent in membrane-bound intracellular compartments resembling vacuoles and endoplasmic reticulum and could occasionally be seen still in the process of budding (inset, panel A; bar = 0.5 μm). Comparison with the uninfected microglia shows that the translucent compartments (a site of apparent virus accumulation) are not restricted to infected cells and were therefore not induced by virus infection and intracellular budding.

CasBrE envelope may also be a site of disruption. Since cleavage of the murine retroviral 90-kDa protein into gp70 and p15E is reported to take place in the endoplasmic reticulum, envelope protein interaction with another protein upon its initial secretion into this compartment could interfere with either cleavage or glycosylation. A related observation was recently reported for the cationic amino acid transporter, which acts as an ecotropic retrovirus receptor. These studies showed altered glycosylation of the transporter in virally infected cells (22). In addition, a 50% reduction in arginine uptake as a result of this infection was found. If the altered processing of the envelope protein in microglia is due to interaction with a similar cellular protein, this might explain both the altered envelope expression and the potential to disrupt microglial cellular physiology followed by induction of spongiform change in the CNS. It is also possible that production of the 90-kDa protein is itself toxic to other cells in the CNS, whereas the gp70 protein is innocuous.

While alteration of microglial cells constitutes one site for disruption, we have also observed altered glycosylation of the $FrCas^E$ envelope protein in cerebellar neurons in vivo and in vitro (28). This altered glycosylation results in expression of a 65-kDa envelope protein, as shown in Fig. 2B for infected CNS. Thus, it is of considerable interest to evaluate viral envelope protein-endoplasmic reticulum/receptor protein interactions in a variety of cell types with both neurovirulent and nonneurovirulent murine retroviruses. Whether such alterations of microglial and/or neuronal physiology occur in vivo remains to be determined.

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REFERENCES

1. **Austyn, J. M., and S. Gordon.** 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. **11**:805–815.
2. **Baszler, T. V., and J. F. Zachary.** 1990. Murine retroviral-induced spongiform neuronal degeneration parallels resident microglial cell infection: ultrastructural findings. Lab. Invest. **63**:612–623.
3. **Baszler, T. V., and J. F. Zachary.** 1991. Murine retroviral neurovirulence correlates with an enhanced ability of virus to infect selectively, replicate in, and activate resident microglial cells. Am. J. Pathol. **138**:655–671. (Erratum, **138**:1058.)
4. **Bignami, A., L. F. Eng, D. Dahl, and C. T. Uyeda.** 1972. Localization of glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res. **43**:429–435.
5. **Brooks, B. R., J. R. Swarz, O. Narayan, and R. T. Johnson.** 1979. Murine neurotropic retrovirus spongiform polioencephalomyelopathy: acceleration of disease by virus inoculum concentration. Infect. Immun. **23**:540–544.
6. **Brown, W. J., and M. G. Farquhar.** 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. Methods Cell Biol. **31**:553–569.
7. **Chesebro, B., W. Britt, L. Evans, K. Wehrly, J. Nishio, and M. Cloyd.** 1983. Characterization of monoclonal antibodies reactive with murine leukemia viruses: use in analysis of strains of Friend MCF and Friend ecotropic murine leukemia virus. Virology **127**:134–148.
8. **Chesebro, B., K. Wehrly, K. Watson, and K. Chesebro.** 1978. Murine leukemia virus infectious centers are dependent on the rate of virus production by infected cells. Virology **84**:222–226.
9. **Czub, M., S. Czub, F. McAtee, and J. Portis.** 1991. Age-dependent resistance to murine retrovirus-induced spongiform neurodegeneration results from central nervous system-specific restriction of virus replication. J. Virol. **65**:2539–2544.
10. **Czub, M., F. J. McAtee, and J. L. Portis.** 1992. Murine retrovirus-induced spongiform encephalomyelopathy: host and viral factors which determine the length of the incubation period. J. Virol. **66**:3298–3305.
11. **Czub, S., W. P. Lynch, M. Czub, and J. L. Portis.** Kinetic analysis of the spongiform neurodegenerative disease induced by a highly virulent murine retrovirus. Lab. Invest., in press.
12. **Dong, J. Y., J. W. Dubay, L. G. Perez, and E. Hunter.** 1992. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. J. Virol. **66**:865–874.
13. **Gardner, M. B., B. E. Henderson, J. E. Officer, R. W. Rongey, J. C. Parker, C. Oliver, J. D. Estes, and R. J. Huebner.** 1973. A spontaneous lower motor neuron disease apparently caused by indigenous type-C RNA virus in wild mice. J. Natl. Cancer Inst. **51**:1243–1254.
14. **Gendelman, H. E., et al.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J. Exp. Med. **167**:1428–1441.
15. **Giulian, D., and T. J. Baker.** 1986. Characterization of ameboid microglia isolated from developing mammalian brain. J. Neurosci. **6**:2163–2178.
16. **Gliniak, B. C., S. L. Kozak, R. T. Jones, and D. Kabat.** 1991. Disulfide bonding controls the processing of retroviral envelope glycoproteins. J. Biol. Chem. **266**:22991–22997.
17. **Gravel, C., D. G. Kay, and P. Jolicoeur.** 1993. Identification of the infected target cell type in spongiform myeloencephalopathy induced by the neurotropic Cas-Br-E murine leukemia virus. J. Virol. **67**:6648–6658.
18. **Jolicoeur, P., C. Gravel, and D. G. Kay.** 1992. Pathogenesis of murine spongiform myeloencephalopathy induced by a murine retrovirus, p. 199–224. *In* R. P. Roos (ed.), Molecular neurovirology. Humana Press Inc., Totowa, N.J.
19. **Jolicoeur, P., N. Nicolaiew, L. DesGroseillers, and E. Rassart.** 1983. Molecular cloning of infectious DNA from ecotropic neurotropic wild mouse retrovirus. J. Virol. **45**:1159–1163.
20. **Kay, D. G., C. Gravel, F. Pothier, A. Laperriere, Y. Robitalle, and P. Jolicoeur.** 1993. Neurological disease induced in transgenic mice expressing the env gene of the Cas-Br-E murine retrovirus. Proc. Natl. Acad. Sci. USA **90**:4538–4542.
21. **Kayman, S. C., R. Kopelman, S. Projan, D. M. Kinney, and A. Pinter.** 1991. Mutational analysis of N-linked glycosylation sites of Friend murine leukemia virus envelope protein. J. Virol. **65**:5323–5332.
22. **Kim, J. W., and J. M. Cunningham.** 1993. N-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells. J. Biol. Chem. **268**:16316–16320.
23. **Kozak, S. L., and D. Kabat.** 1990. Ping-pong amplification of a retroviral vector achieves high-level gene expression: human growth hormone production. J. Virol. **64**:3500–3508.
24. **Levinson, S. W., and K. D. McCarthy.** 1991. Astroglia in culture, p. 309–336. *In* G. Banker and K. Goslin (ed.), Culturing nerve cells. The MIT Press, Cambridge, Mass.
25. **Linial, M., J. Fenno, W. N. Burnette, and L. Rohrschneider.** 1980. Synthesis and processing of viral glycoproteins in two nonconditional mutants of Rous sarcoma virus. J. Virol. **36**:280–290.
26. **Lynch, W. P., S. Czub, F. J. McAtee, S. F. Hayes, and J. L. Portis.** 1991. Murine retrovirus-induced spongiform encephalopathy: productive infection of microglia and cerebellar neurons in accelerated CNS disease. Neuron **7**:365–379.
27. **Lynch, W. P., and J. L. Portis.** 1993. Murine retrovirus-induced spongiform encephalopathy: disease expression is dependent on postnatal development of the central nervous system. J. Virol. **67**:2601–2610.
28. **Lynch, W. P., and J. L. Portis.** Unpublished observations.
29. **McAtee, F. J., and J. L. Portis.** 1985. Monoclonal antibodies specific for wild mouse neurotropic retrovirus: detection of comparable levels of virus replication in mouse strains susceptible and resistant to paralytic disease. J. Virol. **56**:1010–1022.
30. **McComb, R. D., T. R. Jones, S. V. Pizzo, and D. D. Bigner.** 1982. Specificity and sensitivity of immunohistochemical detection of factor VIII/V on Willebrand factor antigen in formalin-fixed parafin-imbedded tissue. J. Histochem. Cytochem. **30**:371–377.
31. **Michaels, J., R. W. Price, and M. K. Rosenblum.** 1988. Microglia

in the giant cell encephalitis of acquired immune deficiency syndrome: proliferation, infection and fusion. Acta Neuropathol. (Berlin) **76:**373–379.

32. **Miller, A. D., M. Law, and I. M. Verma.** 1985. Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. Mol. Cell. Biol. **5:**431–437.
33. **Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman.** 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. J. Virol. **62:**2578–2586.
34. **Portis, J. L.** 1990. Wild mouse retrovirus: pathogenesis. Curr. Top. Microbiol. Immunol. **160:**11–27.
35. **Portis, J. L., S. Czub, C. F. Garon, and F. J. McAtee.** 1990. Neurodegenerative disease induced by the wild mouse ecotropic retrovirus is markedly accelerated by long terminal repeat and *gag-pol* sequences from nondefective Friend murine leukemia virus. J. Virol. **64:**1648–1656.
36. **Poss, M. L., S. W. Dow, and E. A. Hoover.** 1992. Cell-specific envelope glycosylation distinguishes FIV glycoproteins produced in cytopathically and noncytopathically infected cells. Virology **188:**25–32.
37. **Poss, M. L., S. L. Quackenbush, J. I. Mullins, and E. A. Hoover.** 1990. Characterization and significance of delayed processing of the feline leukemia virus FeLV-FAIDS envelope glycoprotein. J. Virol. **64:**4338–4345.
38. **Raff, M. C., R. Mirsky, K. L. Fields, R. P. Lisak, S. H. Dorfman, D. H. Silberberg, N. A. Gregson, S. Leibowitz, and M. C. Kenedy.** 1978. Galactocerebroside is a specific cell surface marker for oligodendrocytes in culture. Nature (London) **274:**813–816.
39. **Rassart, E., L. Nelbach, and P. Jolicoeur.** 1986. Cas-Br-E murine leukemia virus: sequencing of the paralytogenic region of its genome and derivation of specific probes to study its origin and the structure of its recombinant genomes in leukemia tissues. J. Virol. **60:**910–919.
40. **Richardson, A., C. Hao, and S. Fedoroff.** 1993. Microglia progenitor cells: a subpopulation in cultures of mouse neopallial astroglia. Glia **7:**25–33.
41. **Sharpe, A. H., R. Jaenisch, and R. M. Ruprecht.** 1987. Retroviruses and mouse embryos: a rapid model for neurovirulence and transplacental antiviral therapy. Science **236:**1671–1674.
42. **Shikova, E., Y. C. Lin, K. Saha, B. R. Brooks, and P. K. Wong.** 1993. Correlation of specific virus-astrocyte interactions and cytopathic effects induced by *ts*1, a neurovirulent mutant of Moloney murine leukemia virus. J. Virol. **67:**1137–1147.
43. **Springer, T., G. Galfre, D. S. Secher, and C. Milstein.** 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. **9:**301–306.
44. **Springer, T., G. Galfre, D. S. Secher, and C. Milstein.** 1978. Monoclonal xenogenic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. Eur. J. Immunol. **8:**539–551.
45. **Streit, W. J., and G. W. Kreutzberg.** 1987. Lectin binding by resting and reactive microglia. J. Neurocytol. **16:**249–260.
46. **Varmus, H., and R. Swandstrom.** 1982. Replication of retroviruses, p. 467–469. *In* R. Weiss et al. (ed.), RNA tumor viruses: molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Vazeux, R., et al.** 1987. AIDS subacute encephalitis. Identification of HIV-infected cells. Am. J. Pathol. **126:**403–410.
48. **Watkins, B. A., H. H. Dorn, W. B. Kelly, R. C. Armstrong, B. J. Potts, F. Michaels, C. V. Kufta, and D. M. Dubois.** 1990. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. Science **249:**549–553.
49. **Wiley, C. A., R. D. Schrier, J. A. Nelson, P. W. Lampert, and M. B. Oldstone.** 1986. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proc. Natl. Acad. Sci. USA **83:**7089–7093.
50. **Wong, P. K. Y., M. M. Soong, R. MacLeod, G. E. Gallick, and P. H. Yuen.** 1983. A group of temperature-sensitive mutants of Moloney leukemia virus which is defective in cleavage of *env* precursor polypeptide in infected cells also induces hind-limb paralysis in newborn CFW/D mice. Virology **125:**513–518.
51. **Wong, P. K. Y., and P. H. Yuen.** 1992. Molecular basis of neurologic disorders induced by a mutant ts1, of Moloney murine leukemia virus, p. 161–197. *In* R. Roos (ed.), Molecular neurovirology: pathogenesis of viral CNS infections. Humana Press, Totowa, N.J.
52. **Yuen, P. H., D. Malehorn, C. Knupp, and P. K. Y. Wong.** 1985. A 1.6-kilobase-pair fragment in the genome of the *ts*1 mutant of Moloney murine leukemia virus TB that is associated with temperature sensitivity, nonprocessing of $Pr80^{env}$, and paralytogenesis. J. Virol. **54:**364–373.
53. **Yuen, P. H., E. Tzeng, C. Knupp, and P. K. Y. Wong.** 1986. The neurovirulent determinants of *ts*1, a paralytogenic mutant of Moloney murine leukemia virus TB, are localized in at least two functionally distinct regions of the genome. J. Virol. **59:**59–65.