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Age-Dependent Poliomyelitis of Mice: Expression of Endogenous Retrovirus Correlates with Cytocidal Replication of Lactate Dehydrogenase-Elevating Virus in Motor Neurons

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The widespread presence of endogenous retroviruses in the genomes of animals and humans has suggested that these viruses may be involved in both normal and abnormal developmental processes. Previous studies have indicated the involvement of endogenous ecotropic murine leukemia virus (MuLV) in the development of age-dependent poliomyelitis caused by infection of old C58 or AKR mice by lactate dehydrogenase-elevating virus (LDV). The only genetic components which segregate with susceptibility to LDV-induced paralytic disease are multiple proviral copies of ecotropic MuLV and the permissive allele, at the *Fv-1* locus, for N-tropic, ecotropic virus replication (*Fv-1*$^{n/n}$). Using in situ hybridization and Northern (RNA) blot hybridization, we have correlated the expression of the endogenous MuLV, both temporally and spatially, with LDV infection of anterior horn motor neurons and the development of paralysis. Our data indicate that treatment of 6- to 7-month-old C58/M mice with cyclophosphamide, which renders these mice susceptible to LDV-induced paralytic disease, results in transient increases in ecotropic MuLV RNA levels in motor neurons throughout the spinal cord. Peripheral inoculation of C58/M mice with LDV, at the time of elevated MuLV RNA levels, results in a rapid spread of LDV to some spinal cord motor neurons. LDV infections then spread slowly but progressively throughout the spinal cord, involving an increasing number of motor neurons. LDV replication is cytocidal and results in neuron destruction and paralysis of the infected animals 2 to 3 weeks postinfection. The slow replication of LDV in the spinal cord contrasts sharply with the rapid replication of LDV in macrophages, the normal host cells for LDV, during the acute phase of infection. The data indicate that the interaction between the endogenous MuLV with the generally nonpathogenic murine togavirus LDV occurs at the level of the motor neuron. We discuss potential mechanisms for the novel dual-virus etiology of age-dependent poliomyelitis of mice.

The genomes of all vertebrates, as far as it is known, carry large numbers of endogenous proviruses and other retrovirus-related sequences (17). The origins of these sequences and their potential function in either the normal or abnormal development and physiology of the host are not completely understood. However, pathogenic mechanisms involving endogenous retroviruses have emerged. For example, endogenous retroviruses have been linked to the development of tumors in certain inbred mouse strains (6, 7, 17, 37). In mice, four groups of proviruses have been distinguished: ecotropic, xenotropic, polytropic, and modified polytropic murine leukemia viruses (MuLV) (31). In highly leukemic mouse strains, such as AKR and C58, the development of T-cell lymphomas is dependent upon the replication of an endogenous ecotropic MuLV and recombination between this retrovirus and xenotropic-related and polytropic-related sequences, resulting in the formation of oncogenic recombinants (18, 31). Similarly, endogenous feline leukemia viruses have been shown to recombine with exogenous feline leukemia viruses to generate a variety of pathogenic variants which are associated with both lymphoproliferative and antiproliferative diseases of cats (25; J. I. Mullins and E. A. Hoover, *in* R. Gallo and F. Wong-Staal (ed.), *Retrovirus Biology: an Emerging Role in Human Diseases*, in press). Endogenous retroviruses of mice have also been shown to inactivate host genes by integrating within critical sequences resulting in genetic defects (32). In addition to the pathogenesis involving recombination among retroviruses and the inactivation of genes by integration, we describe a third pathogenic mechanism of an endogenous retrovirus. This mechanism involves the interaction between an endogenous ecotropic MuLV and an exogenous togavirus, lactate dehydrogenase-elevating virus (LDV), in causing a fatal, progressive neurological disease in C58/M mice and other highly ecotropic MuLV-expressing strains of mice.

Although LDV generally establishes only an asymptomatic, lifelong, persistent infection in all strains of mice, old C58/M or AKR mice may develop a progressive, paralytic disease, age-dependent poliomyelitis (ADPM), 2 to 3 weeks postinfection (p.i.) with a neuropathogenic strain of LDV (24). Earlier work had shown that the susceptibility of mice to ADPM is genetically linked to the presence of multiple proviral copies of N-tropic, ecotropic MuLV and homozygosity of the allele at the locus that permits the replication of N-tropic MuLV (*Fv-1*$^{n/n}$) (23, 24, 26). The susceptibility of C58/M mice to ADPM also increases with increasing age of the mice and is greatly enhanced by treatment with cyclophosphamide or X-irradiation (23, 24, 26). C58/M mice 12 months of age or older have been reported to be 50 to 60% susceptible to ADPM (24). We find that less than 10% of 5- to 10-month-old C58/M mice raised in our colony develop paralytic disease after LDV infection but that the incidence of ADPM is increased to nearly 100% if these mice are X-irradiated or treated with cyclophosphamide 1 to 2 days prior to LDV infection (see below). Two-month-old C58/M mice remain asymptomatic after LDV infection regardless of whether or not they have been X-irradiated or treated with cyclophosphamide (23, 24).

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LDV replication in mice occurs primarily in a subset of macrophages and is cytocidal (13, 34, 35). Systemic LDV infections persist by continuous passage of LDV to newly generated macrophages derived from nonpermissive precursor cells (34). Although LDV infects the same subpopulation of macrophages in C58/M mice as in other mouse strains, we have recently shown that the expression of endogenous, ecotropic MuLV extends its host cell range to include ventral horn motor neurons (10). Northern (RNA) blot hybridization analyses demonstrated 20- to 50-fold-increased accumulations of 3- and 8.2-kilobase (kb) RNA specific for the endogenous ecotropic MuLV in the spinal cords of C58/M mice with increasing age and after treatment with cyclophosphamide (10); these RNA accumulations occur concomitantly with the increased susceptibility of the mice to ADPM. An earlier study had demonstrated that the development of paralysis in the susceptible C58/M mice after LDV infection correlates with the appearance of LDV RNA and antigens in motor neurons, thus indicating LDV replication in these neurons (9). Paralysis results from extensive motor neuron destruction, which is generally, but not always, associated with an influx of inflammatory cells (33).

The observed increases in ecotropic MuLV RNA levels in spinal cord neurons with increasing age of the mice and after cyclophosphamide treatment were restricted to the spinal cord and were associated specifically with motor neurons (10). After cyclophosphamide treatment, no ecotropic retrovirus RNA was detected in the brain and no changes in ecotropic MuLV RNA levels were detected in any tissue other than the spinal cord, including thymus and spleen. In addition, cyclophosphamide treatment did not cause an increase in ecotropic viral RNA levels in any tissue of young C58/M mice that are not susceptible to ADPM (10). Northern hybridization of probes specific for polytropic and xenotropic sequences indicated no change in the expression of these sequences in any tissue in relation to susceptibility to ADPM (10).

The results of these previous studies (9, 10) and those of the present report suggest that increased expression of the ecotropic retrovirus in the ventral horn motor neurons renders these neurons susceptible to cytocidal infection by LDV, thereby explaining the genetic linkage between susceptibility of mice to LDV-triggered ADPM and the presence of an ecotropic provirus(es). The enhanced susceptibility of C58/M mice to ADPM with increasing age and after X irradiation or cyclophosphamide treatment seems to be mediated through an increased expression of the ecotropic MuLV in spinal cord motor neurons. This concept of an interaction between two generally nonpathogenic, unrelated viruses, the ecotropic provirus and LDV, in causing neurological disease represents a novel mechanism of viral pathogenesis. In its dependence on multiple predisposing factors, ADPM resembles several human neurological disorders in which age-related, genetic, immunological, environmental, and viral factors may play an etiological role.

MATERIALS AND METHODS

Mice and virus. C58/M mice were bred in our animal facility at the University of Minnesota. The breeding nucleus was originally obtained in 1987 from W. Murphy (University of Michigan). C58/M mice were injected intraperitoneally with 200 mg of cyclophosphamide per kg (Sigma Chemical Co., St. Louis, Mo.) 48 h prior to the removal of tissues or infection of the mice by intraperitoneal inoculation of 10^6 50% infectious doses (ID_{50}) of LDV_v, a neurovirulent strain of LDV. LDV_v was obtained from a C58/M mouse with severe paralysis 10 days p.i. with LDV_M (obtained from W. Murphy). The plasma from this mouse was injected into 20 C58/M mice, and their plasma was harvested 24 h p.i. LDV titers were estimated by an endpoint dilution assay in mice (4, 27).

RNA extraction, Northern blot analysis, and nucleic acid probes. RNA was extracted from frozen spinal cords and spleens essentially as described by Chirgwin et al. (5) with modifications as described by Lewis et al. (19) and as previously reported (10). Total RNA (5 μg) was glyoxylated and electrophoresed in 1% agarose as previously described (10) by the method of McMaster and Carmichael (21) and subsequently transferred to nylon membranes. An ecotropic MuLV-specific oligonucleotide (10) was hybridized to Northern blots at 65°C by the protocol of Collins and Hunsaker (8) with previously described modifications (10). The oligonucleotide was labeled at the 3′ end by using terminal deoxynucleotidyl transferase by the method of Collins and Hunsaker (8). An LDV-specific cDNA probe, representing 1.5 kb of the 3′ terminus (J. T. Harty and P. G. W. Plagemann, unpublished data), and the full-length proviral clone AKR-623 (obtained from Doug Lowy, National Institutes of Health) were labeled by nick translation (29) using either [^{32}P]dCTP (for Northern blot analysis) or [^{125}I]dCTP (for in situ hybridization). Nick-translated probes were hybridized to Northern blots at 42°C in 50% formamide by using standard protocols (20).

In situ hybridization. Aldehyde-fixed tissue sections were prepared by the method of Blum et al. (1) with modifications (9). The ^{125}I-labeled LDV-specific cDNA and proviral clone AKR-623 were hybridized at 37°C to tissue sections as previously described (9, 10).

RESULTS

Time course of ecotropic MuLV and LDV expression in the spinal cord. In the experiment depicted in Fig. 1A and B, we determined the levels of MuLV and LDV RNA in the spinal cords of C58/M mice as a function of time after cyclophosphamide treatment and infection with LDV. Six-month-old C58/M mice were treated with cyclophosphamide and, 2 days later, infected with a neuropathogenic strain of LDV (LDV_v). Cyclophosphamide treatment increased the incidence of ADPM in 6- to 10-month-old C58/M mice raised in our colony from <10% (3 out of a total of 35 infected) to 94% (176 out of a total of 187 infected). In these experiments, there was no apparent difference in susceptibility to ADPM between 6- and 10-month old mice. The onset of paralytic symptoms ranged from 10 to 26 days p.i. with a mean onset (plus or minus standard error of the mean) of 16 ± 1 days p.i. In the experiment depicted in Fig. 1A and B, spinal cord tissue was removed at various times after cyclophosphamide treatment and LDV infection and RNA was extracted from three mice per time point. Levels of specific RNAs in total spinal cord RNA were analyzed by Northern blot hybridization using, sequentially, an ecotropic MuLV-specific oligonucleotide probe, 27 nucleotides in length (Akv 27-mer), and an actin-specific oligonucleotide probe (Fig. 1A; 10). A 1.5-kb cDNA fragment representing the 3′ end of the LDV genome was hybridized to a Northern blot of the same RNAs analyzed in Fig. 1A as well as RNA from 10-day-infected mice (Fig. 1B).

The data in Fig. 1A show that ecotropic MuLV RNA appeared in the spinal cord of C58/M mice after cyclophosphamide treatment and LDV infection in a biphasic manner.

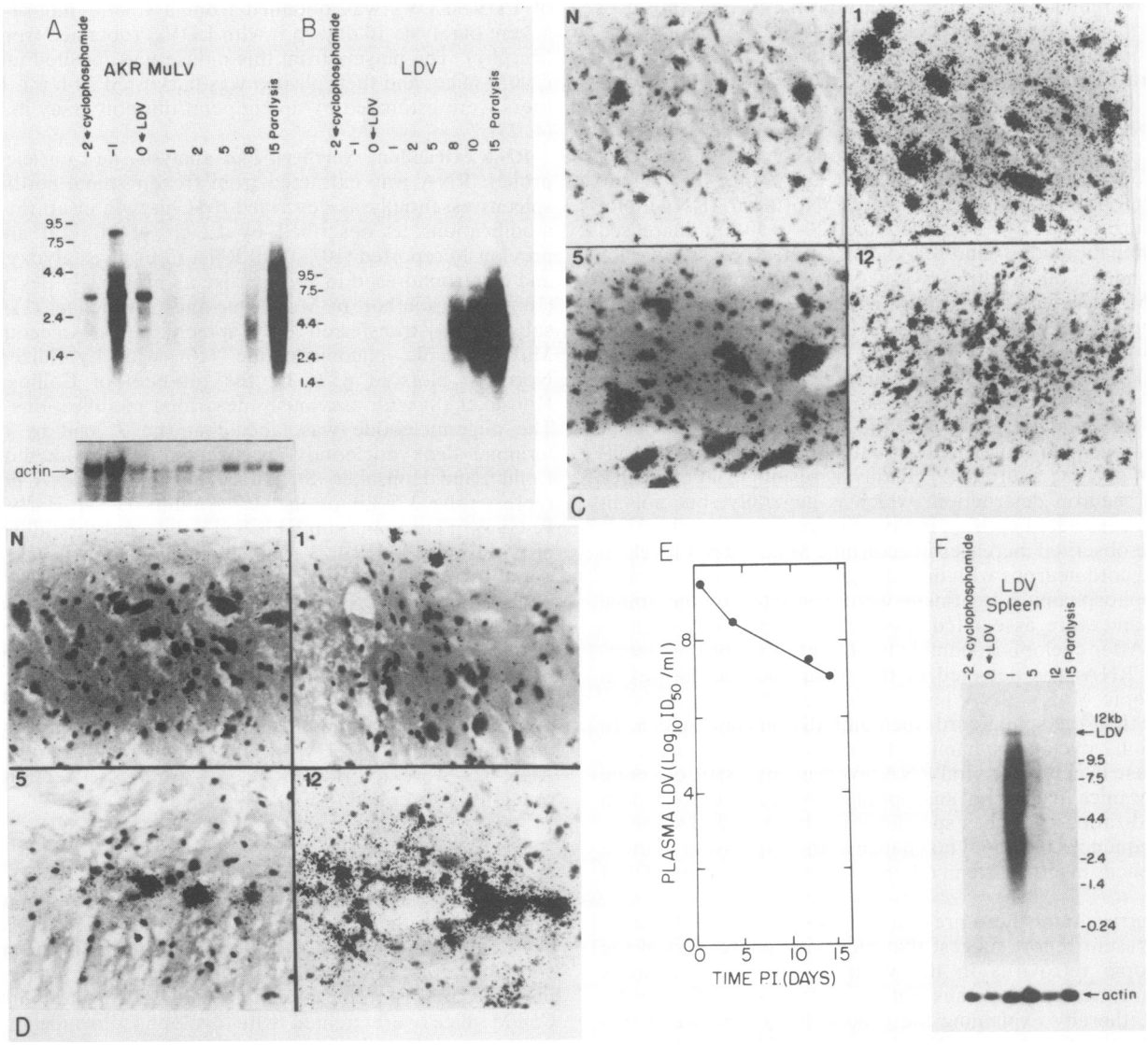

FIG. 1. Composite analyses of MuLV and LDV RNA levels in the spinal cord (A and B), LDV titers in plasma (E), LDV RNA levels in the spleen (F), and cellular localization of MuLV and LDV RNA in spinal cord tissue (C and D) of 6-month-old C58/M mice after cyclophosphamide treatment and LDV infection. The mice were injected with 200 mg of cyclophosphamide per kg and 2 days later (zero time) with 10^6 ID_{50} of LDV_v per mouse. At the indicated times (in days p.i.), RNA was extracted from the spinal cords (panels A and B) and spleens (panel F) of groups of three mice and total RNA was analyzed by Northern blot hybridization. In panel A, the Northern blot was hybridized with a ^{32}P-labeled 27-mer oligonucleotide probe specific for the gp70 *env* region of the ecotropic MuLV genome and subsequently with a ^{32}P-labeled actin-specific oligonucleotide probe. In panel B, a ^{32}P-labeled LDV-specific cDNA probe was hybridized to a different blot of the same samples of RNA and RNA from 10-day-infected mice. In panel F, the blot of spleen RNA was hybridized with the LDV-specific cDNA probe and subsequently with the actin-specific oligonucleotide probe. Panels C and D indicate in situ hybridization analyses of spinal cord tissue obtained from single mice at the indicated times p.i. by using the full-length proviral clone, AKR-623 (panel C), and an LDV-specific cDNA probe (panel D). N, Samples from untreated, uninfected, age-matched control C58/M mice. Magnification in panels C and D, ×100. Panel E shows plasma LDV titers in C58/M mice over the time period p.i. for which results from the Northern blots and in situ hybridizations are presented.

There was an initial severalfold transient increase in ecotropic MuLV RNA in the spinal cord 1 to 2 days after cyclophosphamide treatment. Results from previous (10) and additional analyses indicated that ecotropic MuLV RNA increases in spinal cords of 6- to 10-month-old C58/M mice about 10- to 20-fold after cyclophosphamide treatment. Peripheral inoculation of LDV at the time of ecotropic MuLV expression in the spinal cord resulted in a 90 to 100% incidence of paralytic disease in these mice. The levels of the two primary MuLV RNA species, an 8.2-kb full-length MuLV RNA and a 3-kb RNA (which is presumably the spliced mRNA from the *env* region), were increased. In addition, there was a corresponding increase in 4.3- and 2.0-kb MuLV RNA, but the origin of these RNAs is unclear. Whole-body X-irradiation (600 rads), which also enhances the incidence of LDV-induced ADPM (24), caused a similar increase in ecotropic MuLV RNA levels as observed after cyclophosphamide treatment (data not shown). Three days after the cyclophosphamide injection, the level of MuLV RNA relative to total spinal cord RNA (as assessed by the

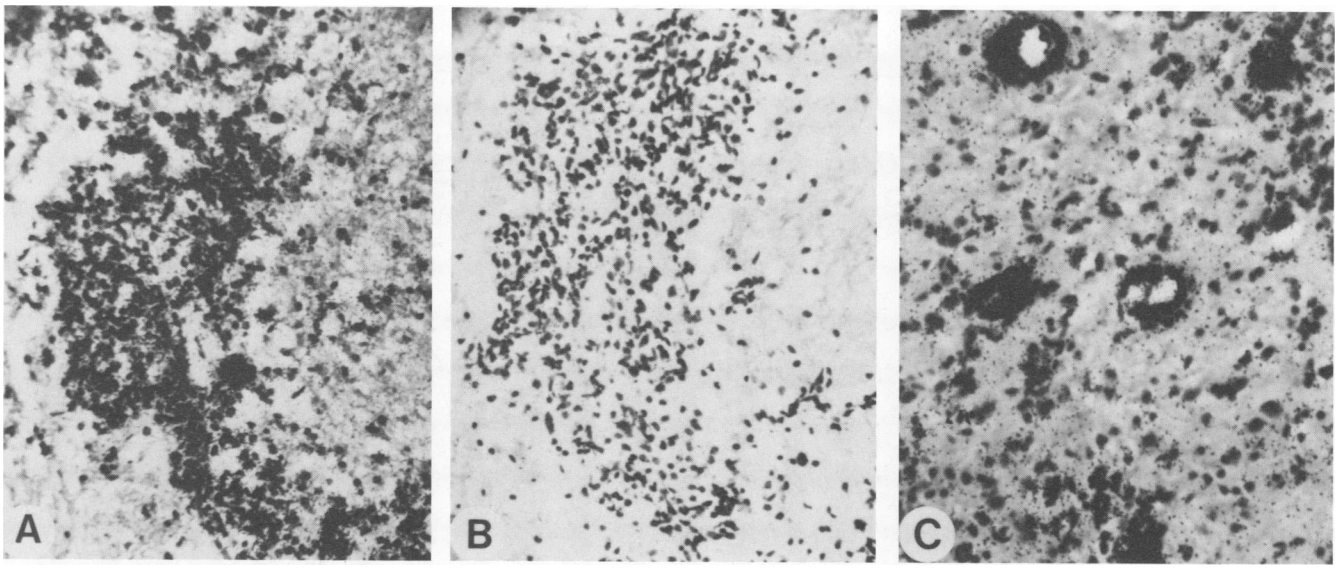

FIG. 2. Ecotropic MuLV RNA in inflammatory cells of the spinal cord of an 18-day LDV-infected paralyzed C58/M mouse. Aldehyde-fixed sections of the spinal cord sections were hybridized with an ^{125}I-labeled, ecotropic 8.2-kb AKR-623 *Pst*I fragment. The section in panel B was subjacent to that shown in panel A and was incubated with 100 μg of RNase A and 10 U of RNase T_1 per ml for 30 min at 37°C prior to hybridization. (C) Hybridization of ^{125}I-labeled AKR-623 to an anterior horn region demonstrating the presence of MuLV RNA in mononuclear cells of the perivascular cuffs. Magnification, ×200.

level of actin mRNA [Fig. 1A]) had returned to the pretreatment level. The transiency of the increase in ecotropic MuLV RNA levels after cyclophosphamide treatment was not a consequence of the LDV infection. A similar transient increase in ecotropic MuLV RNA levels was observed in uninfected, 6-month old C58/M mice after cyclophosphamide treatment in repeated experiments (data not shown).

In contrast, the second increase in MuLV RNA level in the spinal cords of paralytic mice, at about 15 days p.i. with LDV (Fig. 1A), was unique to LDV-infected mice. This increase occurred at the height of paralytic symptoms and, as shown later, reflected the influx into the spinal cord of inflammatory cells that are MuLV RNA positive. Very low levels of ecotropic MuLV RNA are also detectable in spinal cord motor neurons of 2-month old C58/M mice (10), but the levels do not increase significantly after cyclophosphamide treatment (10) and the animals remain resistant to LDV-induced paralytic disease (23, 24, 26).

We used in situ hybridization analyses with a full-length ecotropic probe (Fig. 1C) or an ecotropic-specific *Sma*I fragment from the *env* region (data not shown) to determine the distribution of MuLV RNA among cells in the spinal cords of C58/M mice. The increased levels of ecotropic MuLV RNA 1 to 2 days after cyclophosphamide injection were primarily associated with anterior horn neurons (Fig. 1C). Analysis of sections from various parts of the spinal cord and longitudinal spinal cord sections suggested that most neurons throughout the spinal cord contained similar levels of ecotropic MuLV RNA. By 5 days p.i., the MuLV RNA in neurons had decreased to approximately the pretreatment level (Fig. 1C). This observed decrease is in agreement with the results from the Northern hybridizations of total spinal cord RNA (Fig. 1A). In contrast to results for the spinal cord, no ecotropic MuLV RNA-positive cells were seen in the cerebra or cerebella of the brains of C58/M mice, which agrees with the undetectable levels of MuLV RNA in these regions of the brain by Northern blot hybridizations of ecotropic viral probes to total brain RNA (10). In young AKR mice, ecotropic viral RNA has been found in poly(A)$^+$ RNA from the brain but only at very low levels (18) which may not have been detectable in our analyses of total cerebral and cerebellar RNA. Cells containing low levels of MuLV RNA would also not have been scored as positive by in situ hybridization, in contrast to the extremely high levels of MuLV RNA following cyclophosphamide treatment. On the other hand, the ecotropic MuLV RNA detected in the brains of AKR mice could have originated primarily from the brain stem, which we found to contain low levels of ecotropic MuLV in C58/M mice (data not shown).

At the time of the first appearance of hind limb weakness in a mouse, at about 12 days p.i., few MuLV RNA-positive neurons were found in the spinal cord (Fig. 1C). Instead many MuLV RNA-positive small mononuclear cells were detected. The MuLV RNA-positive small cells are presumably cells of the inflammatory infiltrates and were found primarily in areas of neuron destruction in the gray matter (Fig. 1C) but also to some extent in the white matter of the spinal cord (data not shown). Figure 2A demonstrates a typical focus of inflammatory cells in the gray matter, along the central canal of the spinal cord of a paralyzed mouse at 15 days p.i. The hybridization signal in these cells was sensitive to pretreatment of the tissue sections with RNase A and T_1 (Fig. 2B). Inflammatory cells of perivascular cuffs also contained high levels of ecotropic MuLV RNA (Fig. 2C) suggesting that inflammatory cells contain MuLV RNA at the time of entry into the central nervous system (CNS). The appearance of MuLV RNA-positive inflammatory cells in the spinal cord correlated with the second increase in MuLV RNA as measured by Northern blot hybridizations of total spinal cord RNA (Fig. 1A). In repeated Northern hybridization analyses, both 8.2-kb full-length and 3.0-kb mRNA were detected in the spinal cord at the time of paralysis, suggestive of retrovirus production in these inflammatory cells.

Examination of fixed tissue sections by staining with a specific fluorescent monoclonal antibody indicated the widespread presence of the envelope glycoprotein of MuLV,

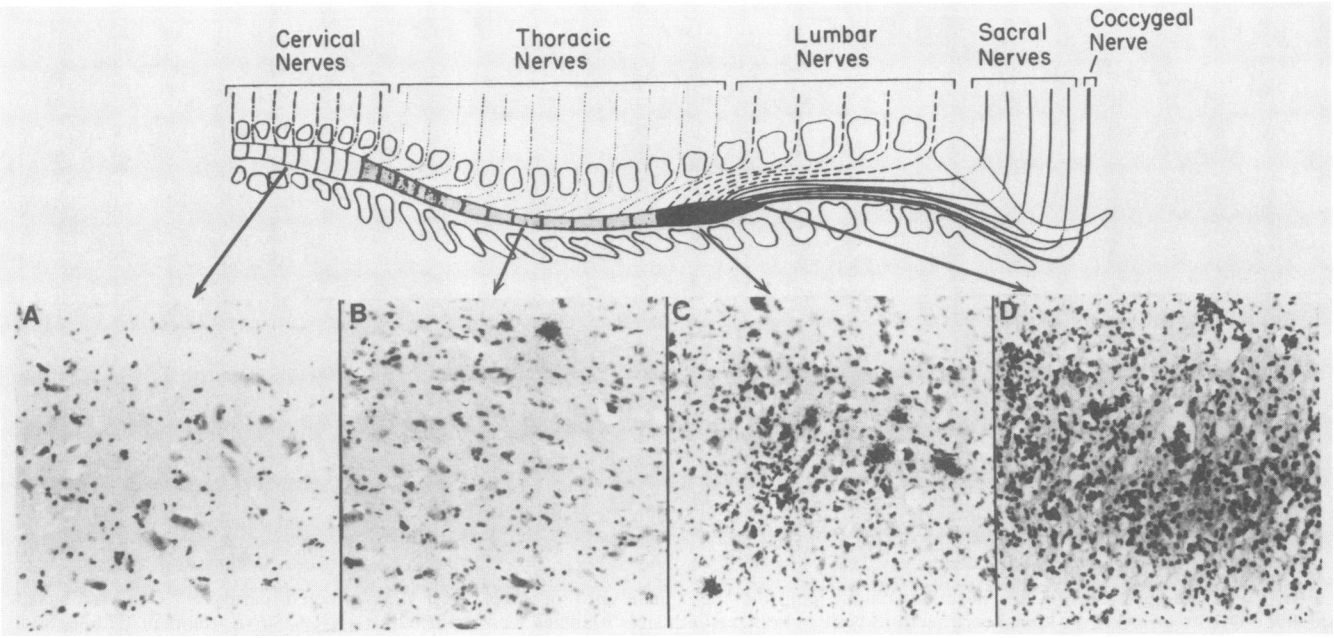

FIG. 3. Localization of LDV RNA-positive neurons and inflammatory cells in the spinal cord of a 15-day LDV-infected C58/M mouse with paralysis in one hind limb. The aldehyde-fixed spinal cord was cut longitudinally and hybridized with an ^{125}I-labeled cDNA probe to the 3′ end of the LDV genome. Representative micrographs from four regions of the spinal cord are aligned with a diagram of the spinal cord. Magnification, ×150.

gp70, throughout the spinal cord of a 9-month-old C58/M mouse 3 days after treatment with cyclophosphamide, whereas little or no gp70 was detectable in the spinal cord of a companion mouse without cyclophosphamide treatment (data not shown). The results indicate the presence of MuLV gp70 and presumably other MuLV proteins in motor neurons after the transient increase in MuLV RNA and at the time of infection with LDV in the experiment depicted in Fig. 1.

Low levels of LDV RNA became detectable in the spinal cords of cyclophosphamide-treated C58/M mice 2 to 5 days p.i. with LDV (Fig. 1B). The levels of LDV RNA then increased progressively until the development of paralysis. This slow increase in LDV RNA in the spinal cords contrasts sharply with plasma levels of infectious LDV (Fig. 1E), LDV RNA accumulation in the spleen (Fig. 1F), and LDV replication in tissue macrophages (34, 35). Plasma LDV titers peaked 1 day p.i. and then decreased several orders of magnitude during the next 2 weeks (4, 27, 30; see Fig. 1E). Peak viremia correlates with the transient appearance of LDV RNA in the spleen (Fig. 1F) and of LDV-infected macrophages in the spleen and other tissues as detected by Northern blot hybridization of total tissue RNA and fluorescent antibody staining of tissue sections (13, 28; unpublished data). LDV RNA and infected macrophages largely disappear from the spleen and other tissues, outside of the CNS, 2 to 3 days p.i. (Fig. 1F), when LDV RNA first becomes detectable in the spinal cord (Fig. 1B). The disappearance of LDV-infected macrophages is due to the rapid replication of LDV in and destruction of the majority of LDV-permissive macrophages in the animal (34, 35). Maintenance of low-level viremia after the initial burst of replication depends on the infection of the small number of newly generated permissive macrophages (34).

LDV RNA positive cells were detected in the spinal cord as early as 1 day p.i. with LDV, but their number was low and detectable levels of LDV RNA were primarily localized to smaller nonneuronal cells (Fig. 1D). LDV RNA-positive cells were detected more frequently 5 days p.i. and appeared in focal areas (Fig. 1D). There was a general increase in the number and size of foci of LDV RNA-positive cells by 12 days p.i. Although the foci were composed of both neuronal and nonneuronal LDV RNA-positive cells, the primary cell type that was LDV RNA positive at the time of paralysis was the large motor neuron. The results suggest a slow progressive spread of LDV infections, from initial foci of infection, through the spinal cord, which results in a progressive destruction of motor neurons and eventually in paralysis.

Progression of LDV infections in the spinal cord. The progressive spread of an LDV infection through the spinal cord and destruction of neurons is also indicated by an analysis of longitudinal sections of the spinal cords of paralyzed C58/M mice. For example, in a mouse with only hind limb paralysis, hybridization of an LDV-specific cDNA probe to longitudinal sections of the spinal cord indicated an absence of LDV RNA-positive cells in the cervical region of the spinal cord and the tissues from this region were essentially normal histologically (Fig. 3A). The thoracic region contained a few neuronal and nonneuronal LDV-positive cells, but gross histopathology was not obvious (Fig. 3B). In contrast, numerous LDV-positive neurons were present in the lumbar region, accompanied by a moderate level of mononuclear cell infiltrate (Fig. 3C). LDV RNA-positive cells in this region were predominantly motor neurons. Higher-magnification micrographs from this region are shown in Fig. 4. Figure 4 demonstrates the presence of both uninfected motor neurons and motor neurons containing large amounts of LDV RNA. In addition, long stretches of LDV RNA were seen (Fig. 4C), which may represent axons of LDV-infected neurons. Figure 4C represents a region with a high proportion of LDV RNA-positive neurons. The unusually high number of autoradiographic silver grains outside cells in this region is most likely due to the presence of cell-free LDV. The hybridization signal, including that outside the cells, was eliminated by pretreatment of the sections

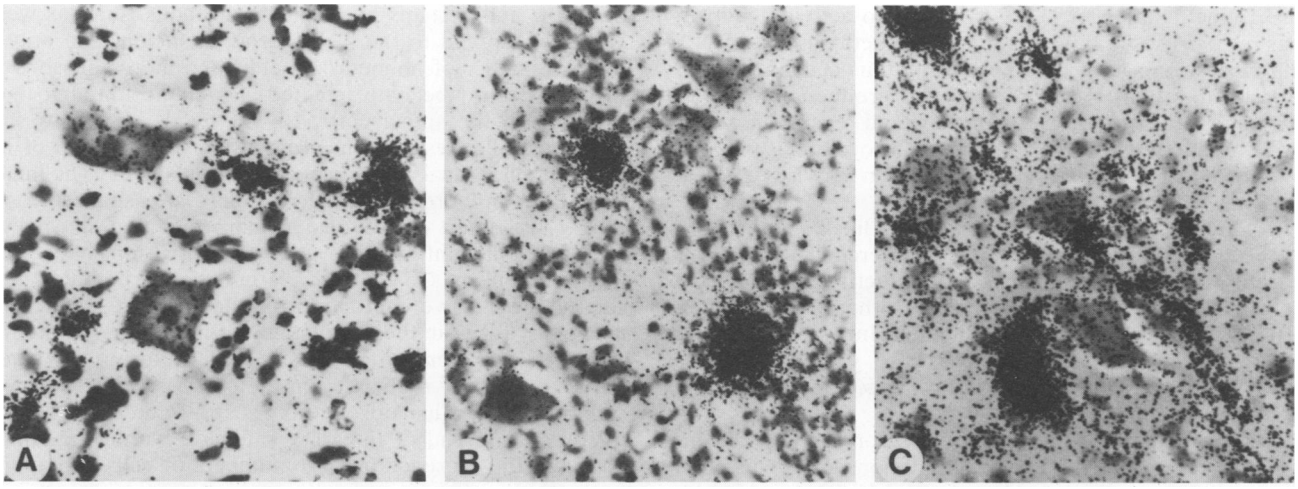

FIG. 4. Presence of LDV RNA in motor neurons of the lumbar cord anterior horns of a C58/M mouse with paralysis in one hind limb. Shown are enlargements of the spinal cord region shown in Fig. 3C. Magnification, ×400.

with RNase A and T_1 (data not shown). Some of the LDV RNA-positive neurons were surrounded by inflammatory cells (Fig. 4B). Production of LDV particles in the spinal cord is indicated by the detection by electron microscopy of mature virions in spinal cord cells of infected C58/J mice (2).

In the most sacral region of the lumbar spinal cord, cells with typical motor neuron morphology were essentially absent (Fig. 3D). The majority of motor neurons seemed to be destroyed, presumably as a consequence of cytocidal LDV infection. This extensive neuron destruction in the lumbar region correlated with the hind limb paralysis observed in this mouse and was probably responsible for the severe inflammation observed in this region (Fig. 3D). The progression of the LDV infection over the length of the spinal cord of a single paralyzed mouse (Fig. 3) appears to recapitulate the progression of LDV infection over time (Fig. 1D).

It seems clear that mice developed muscle weakness and paralysis only after the destruction of substantial numbers of anterior horn neurons. This is also indicated by the finding that LDV infection of cyclophosphamide-treated 2-month-old C58/J mice leads to the infection of some neurons (2) but that the number of affected neurons seems too low to result in any paralytic symptoms. In human patients with amyotrophic lateral sclerosis, it has been estimated that about one-half of the anterior horn neurons must be lost before muscle weakness becomes apparent (22).

Relationship between route of LDV inoculation and location of initial paralysis. When susceptible C58/M mice are injected with LDV intraperitoneally, hind limb paralysis most often develops first, which then usually progresses to front limb paralysis before the animal dies. However, we found that the location of initial paralysis can be modulated by the route of LDV inoculation. When groups of cyclophosphamide-treated, 6- to 7-month-old C58/M mice were injected with LDV in the rear or front footpads, the mean onset of paralysis (15 to 16 days p.i.) was comparable to that observed after intraperitoneal (14 days p.i.) and intracerebral (13 days p.i.) infection. However, four of the five mice injected in the rear footpads developed first hind limb paralysis, whereas four of the five mice injected in the front footpads developed first front limb paralysis. The results suggest that LDV, like some other viruses (15, 36), can spread to the spinal cord by axonal transport, although a hematogenous route of spread cannot be excluded, and for LDV infections, both routes of spread to the CNS may be involved. Paralytic mice generally died between 2 and 4 days after onset of paralytic symptoms, presumably because of asphyxiation, but some mice developed symptoms more slowly and, if adequately supplied with food and water, survived for a week or longer.

DISCUSSION

All evidence suggests that LDV infection of neurons in mouse strains that are susceptible to ADPM is dependent on the expression of the ecotropic retrovirus in these cells. Very little MuLV RNA is observed in motor neurons of young C58/M mice whether or not they are treated with cyclophosphamide, and these mice are resistant to LDV-induced paralytic disease. MuLV expression in motor neurons increases with increase in age but apparently to levels that are insufficient to render more than a low percentage of 6- to 10-month-old C58/M mice susceptible to LDV infection. The more substantial increase in MuLV RNA, induced in motor neurons by cyclophosphamide treatment or X-irradiation, seems to be required to render close to 100% of the mice susceptible to ADPM. The involvement of the ecotropic MuLV in LDV-induced ADPM was initially indicated by the genetic linkage between the susceptibility of mice to ADPM and both the presence of multiple proviral copies of ecotropic MuLV and the permissive genotype ($Fv\text{-}1^{n/n}$) for replication of ecotropic MuLV (24). Our demonstration of a strong correlation between the accumulation of high levels of MuLV RNA in spinal cord motor neurons and infection with LDV in these mice corroborates the results from the genetic studies. More importantly, the results suggest a direct interaction, at the cellular level, between two relatively nonpathogenic viruses as the cause of a fatal, paralytic, neurological disease, and furthermore they suggest potential molecular mechanisms for this interaction.

A specific increase in the levels of MuLV RNA in motor neurons with an increase in age and after cyclophosphamide treatment and X-irradiation is a novel observation, but the mechanisms involved have not been resolved. It seems most likely that MuLV expression in motor neurons results from provirus induction, perhaps involving two levels of regula-

tion. Demethylation of DNA is known to activate endogenous retroviruses (12), and increased demethylation of DNA with increasing age of the animal may thus account for the age-related provirus expression. The production of transcription factors due to DNA damage and repair as a result of X-irradiation and the alkylating agent cyclophosphamide may account for the transient increase in provirus expression after these treatments.

An alternate possibility, although less likely, is that it is not the inherited provirus that becomes deregulated in motor neurons but that the cells become productively infected by ecotropic MuLV that is produced in other tissues of the mouse, such as the spleen and thymus. Increasing age and cyclophosphamide treatment, in this case, would predispose motor neurons to MuLV infection, which in turn would allow for LDV infection of these cells.

Regardless of the origin of the ecotropic retrovirus involved, it seems likely that retroviral virions are produced in these cells since both full-length MuLV RNA and 3-kb mRNA, as well as MuLV proteins, are produced in spinal cord neurons of cyclophosphamide-treated old C58/M mice. The induction of MuLV RNA accumulation occurs in essentially all spinal cord neurons, which contrasts with the low or undetectable levels of MuLV RNA found in brain tissue. In addition, the levels of MuLV RNA in other tissues such as the spleen, thymus, and lymph nodes are not affected by the age of the mice and seem slightly suppressed rather than enhanced by treatment with cyclophosphamide (10).

The mechanism by which replication of the ecotropic MuLV predisposes motor neurons to LDV infection remains to be resolved. The predisposition to LDV infection seems to be unique to motor neurons, since ecotropic MuLV replicates in many cells of many different tissues other than the spinal cord in C58/M mice apparently without rendering these nonneuronal cells susceptible to LDV infection. Outside the CNS of C58/M mice, as in all other mouse strains, LDV replication is probably restricted to a subpopulation of macrophages bearing a LDV-specific receptor (3, 16, 35). This suggests that a motor neuron-specific factor(s) that is coincidentally expressed with the retrovirus may be involved in the susceptibility of these cells to LDV infection. This factor(s) may act either extra- or intracellularly. We favor the view that a MuLV protein or a MuLV-induced neuronal surface protein functions as a surrogate LDV receptor.

Any novel neuronal LDV receptor must differ from that functioning in the infection of permissive macrophages, since C58/M mice can be immunized against the paralytic disease without the infection of macrophages by LDV being affected (11, 24). In addition, the development of neuronprotective, anti-LDV antibodies may explain the finding that C58/M mice, when persistently infected with LDV at a young age, rarely develop paralysis even at 11 to 12 months of age (24). Similarly, we have found that C58/M mice that were infected with LDV at 6 to 7 months of age without prior cyclophosphamide treatment and did not develop paralytic symptoms remained symptomless when injected with cyclophosphamide about 1 month later, whereas 100% of companion mice that were treated with cyclophosphamide and then infected with LDV developed paralysis and died.

Our results suggest the following scenario for the neuropathogenesis of LDV in C58/M mice. Upon infection of susceptible C58/M mice, as in all other strains of mice, LDV rapidly infects all permissive macrophages, resulting in high levels of viremia (up to 10^{10} ID_{50}/ml of plasma) (Fig. 1E). LDV also spreads to the CNS, most likely via axonal transport and perhaps by the hematogenous route as well. However, spread to the CNS seems inefficient and only a limited number of nonneuronal cells and motor neurons in the spinal cord become infected. From the initial foci of infection in the spinal cord, LDV spreads slowly to surrounding cells, resulting in progressively larger foci of infected nonneuronal and especially neuronal cells. The infection of motor neurons is limited to cells expressing ecotropic MuLV. Infection of neurons seems productive since both LDV RNA and antigens as well as mature virions have been found in neurons (2, 9, 10). LDV infection is cytocidal and causes progressive motor neuron destruction which will eventually result in the development of paralytic symptoms.

LDV replication in the spinal cord seems to be much slower than LDV replication in the permissive macrophage subpopulation. This observation is indicated by the relatively slow spread of the LDV infection within the spinal cord and the presence of large numbers of cells containing LDV RNA and antigens for long periods of time p.i. (Fig. 3 and 4). In contrast, maximum LDV RNA and protein synthesis occurs in macrophages between 6 and 8 h p.i.; at 12 to 20 h p.i., viral macromolecular synthesis in either macrophage cultures or spleen macrophages of infected mice has ceased, and the infected cells have largely disappeared by 48 h p.i. (13, 28, 35). A slow transmission of LDV among permissive cells in the spinal cord is likely to contribute to the slow progression of the LDV infection. Systemic infections are rapidly disseminated via the blood stream, whereas transmission within the CNS most likely occurs via diffusion of viral particles through the extracellular spaces of the neuropil or perhaps by cell-cell interaction (14).

The questions that remain to be answered in relation to the interaction of the endogenous ecotropic MuLV and LDV in causing ADPM and the immune mechanisms by which the neurological disease may be prevented are pertinent to human neurological diseases. The widespread presence of endogenous retroviruses in other animals and the novel observation that expression of these viruses can be altered in specific cells of the CNS by X-irradiation, chemical treatment, or increased age suggests that similar interactions between endogenous retroviruses and other exogenous viruses may play roles in diseases of other animals and possibly in humans. Most striking are some similarities between ADPM of mice and amyotrophic lateral sclerosis of humans. The similarities include a comparable age relatedness in susceptibility to each disease, the implication of both hereditary and environmental factors in pathogenesis, and the restriction of cell damage to motor neurons in the absence of any demyelination.

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