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Coexistence in Lactate Dehydrogenase-Elevating Virus Pools of Variants That Differ in Neuropathogenicity and Ability To Establish a Persistent Infection

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Neuropathogenic isolates of lactate dehydrogenase-elevating virus (LDV) differ from nonneuropathogenic isolates in their unique ability to infect anterior horn neurons of immunosuppressed C58 and AKR mice and cause paralytic disease (age-dependent poliomyelitis [ADPM]). However, we and others have found that neuropathogenic LDVs fail to retain their neuropathogenicity during persistent infections of both ADPM-susceptible and nonsusceptible mice. On the basis of a segment in open reading frame 2 that differs about 60% between the neuropathogenic LDV-C and the nonneuropathogenic LDV-P, we have developed a reverse transcription-PCR assay that distinguishes between the genomes of the two LDVs and detects as little as 10 50% infectious doses (ID_{50}) of LDV. With this assay, we found that LDV-P and LDV-C coexist in most available pools of LDV-C and LDV-P. For example, various plasma pools of $10^{9.5}$ ID_{50} of LDV-C/ml contained about 10^5 ID_{50} of LDV-P/ml. Injection of such an LDV-C pool into mice of various strains resulted in the rapid displacement in the circulation of LDV-C by LDV-P as the predominant LDV, but LDV-C also persisted in the mice at a low level along with LDV-P. We have freed LDV-C of LDV-P by endpoint dilution (LDV-C-EPD). LDV-C-EPD infected mice as efficiently as did LDV-P, but its level of viremia during the persistent phase was only 1/10,000 that observed for LDV-P. LDV-permissive macrophages accumulated and supported the efficient replication of superinfecting LDV-P. Therefore, although neuropathogenic LDVs possess the unique ability to infect anterior horn neurons of ADPM-susceptible mice, they exhibit a reduced ability to establish a persistent infection in peripheral tissues of mice regardless of the strain. The specific suppression of LDV-C replication in persistently infected mice is probably due in part to a more efficient neutralization of LDV-C than LDV-P by antibodies to the primary envelope glycoprotein, VP-3P. Both neuropathogenicity and the higher sensitivity to antibody neutralization correlated with the absence of two of three N-linked polylactosaminoglycan chains on the ca. 30-amino-acid ectodomain of VP-3P, which seems to carry the neutralization epitope(s) and forms part of the virus receptor attachment site.

Lactate dehydrogenase-elevating virus (LDV) is an enveloped positive-strand RNA virus of the family *Arteriviridae* (8, 28). Infection of mice, regardless of strain or age, with LDV leads to a lifelong viremic but asymptomatic persistent infection which is maintained by continuous rounds of cytocidal replication of LDV in a renewable subpopulation of macrophages (28, 30, 31). LDV infection of macrophages occurs via a surface protein acting as a receptor that seems to be present only on an LDV-permissive subpopulation of macrophages (20, 31). However, in certain mouse strains, such as C58 and AKR, an LDV infection may result in paralytic disease (age-dependent poliomyelitis [ADPM] [23, 24, 28, 30, 33]). The susceptibility of mice to ADPM is genetically linked to the possession of at least one replication-competent N-tropic, ecotropic murine leukemia virus (MuLV) provirus and the *FV-1*$^{n/n}$ genotype, which permits the replication of N-tropic, ecotropic MuLVs (2). Expression of the ecotropic MuLVs in glial cells in the spinal cord renders anterior horn neurons susceptible to cytocidal infection by LDV but only by certain neuropathogenic variants of the virus (1, 3, 26, 28, 30). Infection of anterior horn neurons is prevented by anti-LDV immune responses, even though these have little or no effect on LDV replication in macrophages; thus, paralytic disease is observed only in mice in which motor neuron-protective anti-LDV immune responses are suppressed either artificially by immunosuppression, such as with cyclophosphamide, genetically (e.g., nude mice), or naturally as a result of old age (1, 18, 24). The mechanism by which ecotropic MuLV expression in glial cells renders anterior horn neurons LDV susceptible is unknown. One likely possibility is that cytokines or viral proteins produced by ecotropic MuLV-infected glial cells induce the expression in anterior horn neurons of a surface component that functions as an alternate receptor for certain neuropathogenic LDV variants (1, 3, 15).

We (see below) and others (24) have noticed that neuropathogenic isolates of LDV gradually lose their ability to induce paralytic disease in ADPM-susceptible mice during a persistent infection of either ADPM-susceptible or nonsusceptible mice. The mechanism for the loss of neuropathogenicity has been unclear. Our present study shows that neuropathogenic LDVs have an impaired ability to establish a persistent

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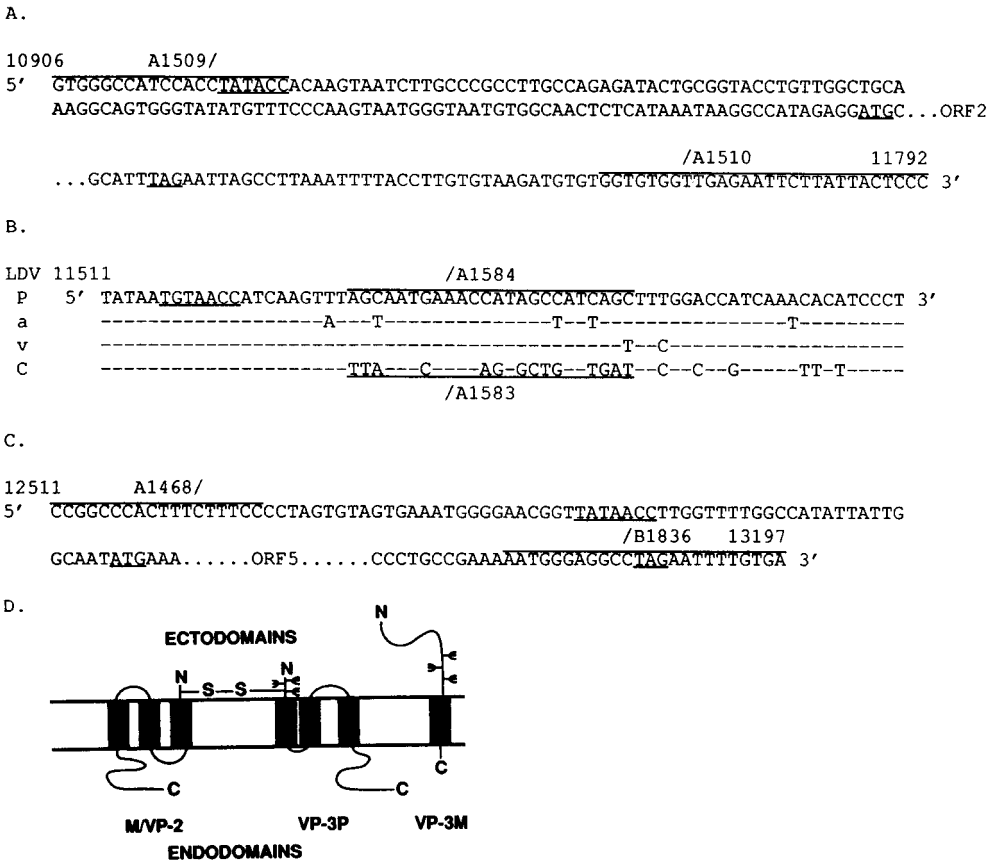

FIG. 1. (A to C) Segments of the genome of LDV-P that were selected for designing sense and antisense oligonucleotide primers for the PCR amplification of ORF 2 (A and B) and ORF 5 (C). (D) Model for the topography of the LDV-P envelope proteins. The segments representing the oligonucleotide primers are overlined or underlined; slashes before and after the primer designation indicate antisense and sense orientations, respectively. The translation initiation and termination codons for the corresponding ORFs and the segments where the 5′ leader becomes joined to the bodies of mRNA 2 (A) mRNA 3 (B), and mRNA 5 (C) are underlined (9). (B) Segment of ORF 2 which was utilized for the design of LDV-P- and LDV-C-specific antisense oligonucleotides and comparison with the corresponding segments of LDV-a and LDV-v.

infection in mice regardless of mouse strain and rapidly become out competed by nonneuropathogenic variants that usually coexist in neuropathogenic LDV stocks.

MATERIALS AND METHODS

Mice. C58/M, AKXD-16, and BALB/c mice were bred in the animal facility of the Department of Microbiology, University of Minnesota. C58/M mice were also bred in the animal facilities of the Department of Biology and Microbiology, South Dakota State University, Brookings. Outbred Swiss mice were purchased from Biolabs (St. Paul, Minn.). FVB, C57BL/6, and (C57BL/6 × SJL)F_1 hybrid mice were supplied by the transgenic facility of the University of Minnesota.

LDV. All neuropathogenic isolates of LDV came from C58/M mice carrying transplantable Ib leukemia cells (23, 24). During repeated passages through C58/M mice, the tumor cells apparently became contaminated with LDV of unknown origin as a passenger virus. LDV-Ib (25) and LDV-C (22, 30) were independently isolated from the spleens of Ib-cell-inoculated moribund C58/M mice. LDV-v was isolated in this laboratory from the spinal cord of a paralyzed C58/M mouse that had been inoculated with LDV-Ib (30). LDV-a is a nonneuropathogenic variant (originally designated LDV-W [24]), but its origin is not clear. LDV-P was originally isolated in this laboratory from a C3H mouse carrying a transplantable tumor (5). It is also nonneuropathogenic (15). LDV concentrations were estimated by an endpoint dilution assay in FVB or Swiss mice (29). LDV titers are expressed as 50% infectious doses (ID_{50}). Stocks of LDV-P, LDV-v, LDV-a, and LDV-C consisted of plasma harvested from groups of 10 to 30 mice 1 day after inoculation with about 10^6 ID_{50} of virus. The plasma contained 10^9 to 10^{10} ID_{50}/ml. For measuring viremia, plasma was obtained from mice by the orbital bleeding method with heparinized blood collection tubes (Fisher Scientific, Pittsburgh, Pa.).

For ADPM experiments, C58/M or AKXD-16 mice were given intraperitoneal injections of about 10^6 ID_{50} of LDV. When indicated, the mice were also given intraperitoneal injections of 200 mg of cyclophosphamide per kg of body weight 1 day before infection and at the indicated times postinfection (p.i.) to continually suppress the formation of anti-LDV immune responses (1). The mice were subsequently monitored for paralytic symptoms for about 6 weeks p.i.

Differential RT-PCR. We have found a segment in open reading frame 2 (ORF 2) that differs by about 60% for LDV-P and LDV-C (Fig. 1B) and have demonstrated that antisense oligonucleotides /A1583 and /A1584 to this segment specifically hybridize to the mRNAs of LDV-C and LDV-P, respectively (27). These oligonucleotides have been used as primers in combination with the sense primer A1509/ (Fig. 1A) to specifically amplify the 719-bp 5′ end of ORF 2 of LDV-C and LDV-P by reverse transcription (RT)-PCR (see Fig. 3). We have used two different RT-PCR conditions. For condition 1, LDV genomic RNA was extracted from 20 μl of pooled mouse plasma samples (e.g., 5 μl from each of four mice) or 10-fold sequential dilutions thereof by using 200 μl of RNAzol B (Tel-Test Inc., Friendswood, Tex.) as specified by the manufacturer. Nuclease-free glycogen (10 μg; Boehringer Mannheim, Indianapolis, Ind.) was added as a coprecipitant in the isopropanol precipitation of the RNA. The RNA-glycogen pellet was resuspended in 10 μl of diethylpyrocarbonate-treated water together with 1 μl of random hexanucleotides (0.5 mg/ml), and the mixture was heated for 20 s in a boiling water bath and chilled on ice for 1 min. To this mixture were added 4 μl of 5× reaction buffer (supplied with Superscript II reverse transcriptase), 2 μl of 0.1 M dithiothreitol, 1 μl of deoxynucleoside triphosphates (25 mM each), 1 μl of RNasin (Promega, Madison, Wis.), and 1 μl of Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, Md.). The mixture was incubated at 40 to 42°C for 1.5 to 3 h. Then 10 μl of a solution consisting of 1 N NaOH and 1 mM EDTA was added, and the first-strand cDNA product was purified by passage through a self-made spin column. The spin column consisted of a 600-μl microcentrifuge tube whose bottom center had been pierced by about four-fifths of the bevel of a 20-gauge needle and which was filled with 550 μl of Sepharose CL-6B (Pharmacia, Piscataway, N.J.) diluted 1:1 in Tris-EDTA buffer (pH 7.5). The column was placed in a bottomless 1.5-ml microcentrifuge tube and centrifuged at 2,500 rpm for 3 min in a glass test tube in an IEC CRU-5000 centrifuge to predrain the column just before use. After loading of the cDNA

mixture, the spin column was placed in a 1.5-ml microcentrifuge tube and centrifuged as above to collect the purified cDNA. For PCR amplification, 2.5 μl of purified first-strand cDNA and 1.25 μl each of 20 μM appropriate sense and antisense oligonucleotide were added to the 20-μl reaction solution to yield final concentrations of 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% (wt/vol) gelatin, 0.2 mM deoxynucleoside triphosphates, and 0.625 U of *Taq* DNA polymerase in buffer B (Promega). The PCR was conducted in a Perkin-Elmer Cetus DNA thermal cycler with the following step-cycle programs: 94°C for 1.5 min; 40 cycles of 52 to 58°C for 1 min, 72°C for 2.5 min, and 94°C for 0.75 min; 52 to 58°C for 1 min; and 72°C for 10 min. The PCR products were electrophoresed in gels of 1% (wt/vol) agarose in Tris-boric acid-EDTA buffer. Condition 2 was the same as condition 1, except for the following modifications. RNA was extracted from 4 μl rather than 20 μl of pooled plasma (e.g., 1 μl from each of four mice), and 20 μg of glycogen was used for coprecipitation of the RNA. For RT, 0.5 μl of 0.5-mg/ml oligo(dT) was used in addition to random hexanucleotides as primers and the amount of RNasin was reduced to 0.5 μl. The final $MgCl_2$ concentration in the PCR was increased to 2 mM. A smaller volume of pooled plasma was used because of variable inhibition of RT by the heparin present in plasma samples.

Sequence analysis of ORF 2 and ORF 5 of various LDV isolates. LDV genomic RNA was extracted from 4 to 20 μl of plasma from infected mice by using 200 μl RNAzol B, coprecipitated with 10 to 20 μg of glycogen, and subjected to RT with Superscript II reverse transcriptase and random hexanucleotides plus or minus oligo(dT) as primers as described already. The first-strand products encompassing ORF 2 and ORF 5 were amplified by PCR as described above with, as primers, sense and antisense oligonucleotides (18 to 28 nucleotides in length) representing segments upstream and downstream, respectively, of ORF 2 and ORF 5 of LDV-P (Fig. 1A and C). These segments were selected because they differed by not more than 1 nucleotide from the corresponding segments of the LDV-C genome (17, 23). They were found to amplify ORF 2 and ORF 5 from the genomes of both neuropathogenic and nonneuropathogenic isolates of LDV, namely, (i) LDV-C and LDV-v and (ii) LDV-P and LDV-a, respectively (unpublished data). The size of the ORF 2 PCR product generated with the A1509/A1510 primer set is 887 bp, and that of the ORF 5 product generated with the A1468/B1836 primer set is 686 bp.

The amplified PCR products were cloned into the TA cloning vector pCR II (Invitrogen, San Diego, Calif.), and the clones were sequenced on both strands with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) as described by the manufacturer. Sequence analyses were performed with Molecular Biology Information Resources programs SAM and EUGENE (21).

RESULTS

Loss of LDV neuropathogenicity during persistent infection is due to the outgrowth of preexisting nonneuropathogenic variant. Previous results indicated that LDV-v and LDV-C were highly neuropathogenic for immunosuppressed C58/M mice whereas LDV-P and LDV-a failed to induce paralytic disease under similar experimental conditions (1, 15, 22, 24). These findings have been confirmed for LDV-P, LDV-a, and LDV-v for the presently available pools of these LDV variants (Table 1). Differences in neuropathogenicity did not seem to be related to differences in the ability of the LDVs to productively infect either immunosuppressed ADPM-susceptible mice (7, 18, 27, 30) or nonimmunosuppressed ADPM-resistant mice (Fig. 2). The time courses of replication of all four LDV isolates in Swiss and FVB mice were comparable (Fig. 2), and the same was the case for BALB/c mice (data not shown). LDV titers in plasma were highest about 1 day p.i. due to the massive cytocidal replication of the infecting LDV in the available population of permissive macrophages (28, 30, 31). The virus titers in plasma then decreased by about 2 $\log_{10}$ units during the next 10 days because continued viremia is dependent on the infection of newly generated permissive macrophages (28, 31). However, we observed that LDV isolated from the plasma of BALB/c mice 21 days after LDV-v infection or from the plasma of FVB mice 7 days after LDV-C infection lacked neuropathogenicity (Table 1). These results resemble those reported for the isolation of nonneuropathogenic LDVs by repeated passage of the neuropathogenic LDV-Ib in BALB/c mice (24). The loss of neuropathogenicity was not due to passage of LDV-C or LDV-v in ADPM-resistant mice. We observed a similar loss in C58/M mice which had been infected with LDV-v at 1 month of age when not fully susceptible to ADPM (1). These young mice generally develop only mild paralytic symptoms and survive, and we found that the LDV reisolated from such a mouse at 50 days p.i. failed to cause paralytic disease even in 11-month-old AKXD-16 mice (Table 1). Similarly, C58/M mice given injections of LDV-v at birth which became immunotolerant to LDV developed only mild

TABLE 1. Incidence of paralytic disease in ADPM-susceptible mice by various LDV isolates

LDV isolate	Infection of ADPM-susceptible mice[a]			
	Strain	Age (mo)	No. of mice paralyzed/ total no.	Time (days p.i.) of paralysis ± SEM
P	C58/M	2	0/4	
a	C58/M	2	0/3	
v	C58/M	2	3/3	22 ± 0
v	AKXD-16	11	6/6	16 ± 3
v (C58/M T50)[b]	AKXD-16	11	0/2	
v (C58/M T190)[c]	AKXD-16	11	0/4	
v (BALB/c T21)[d]	C58/M	7	0/2	
C[e]	C58/M	6	10/10	12 ± 2
C-EPD	C58/M	2	4/4	18 ± 4
C (FVB T7)[f]	C58/M	2	0/3	

[a] Mice of the indicated strains and ages were given injections of 10^5 to 10^6 ID_{50} of the LDVs indicated in column 1 and of 200 mg of cyclophosphamide/kg at −1, 7, and 14 days p.i. The mice were monitored for paralytic disease for at least 40 days. Plasma and spinal cords were obtained from some of the paralyzed mice.

[b] LDV isolated from the plasma of a C58/M mouse that had been immunosuppressed and infected with LDV-v at 1 month of age. The mouse developed only transient mild paralytic disease and survived, and plasma was obtained at 50 days p.i. The virus was amplified by a single 1-day passage through Swiss mice.

[c] LDV isolated from the plasma of a mouse 190 days after infection with LDV-v at birth. The mouse did not mount an anti-LDV antibody response. It developed only mild, nonprogressive paralytic symptoms. The reisolated LDV was amplified by a 1-day passage through Swiss mice.

[d] LDV isolated from the pooled plasma of two BALB/c mice 21 days after LDV-v infection. The virus was amplified by a single 1-day passage in Swiss mice.

[e] Data are from reference 22.

[f] LDV isolated from FVB mice 7 days after LDV-C infection (Fig. 2).

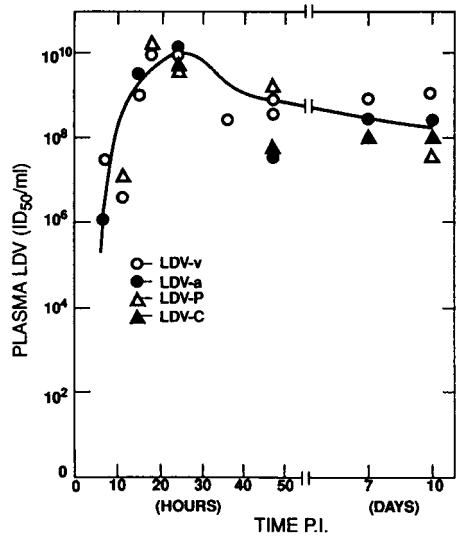

FIG. 2. Time courses of viremia of the indicated isolates of LDV in Swiss mice (LDV-v, LDV-a, and LDV-P) or FVB mice (LDV-C). Groups of two to four mice, 4 to 8 weeks of age, were infected with about 10^6 ID_{50} of LDV. The mice were bled at various times p.i., and their pooled plasma was subjected to titer determination in mice for infectious LDV.

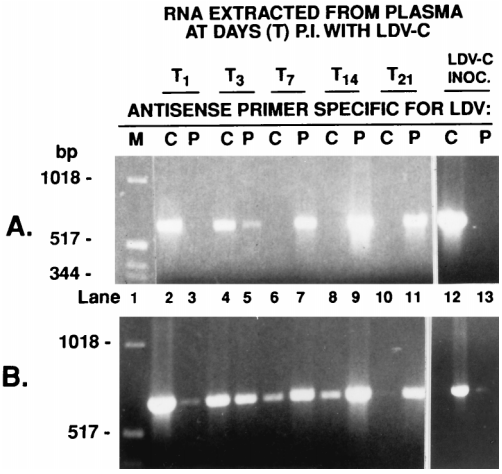

FIG. 3. LDV-C/LDV-P-specific RT-PCR amplification of ORF 2 of LDVs isolated from FVB mice at various times after LDV-C infection. RNA was extracted from the plasma of LDV-C-infected mice (Fig. 2) and from the LDV-C pool used to infect the mice and reverse transcribed. The first-strand products were PCR amplified with sense oligonucleotide primer A1509/ in combination with the LDV-C specific antisense primer/A1583 or the LDV-P-specific antisense primer /A1584, and the products were separated by gel electrophoresis. (A) Results obtained under RT-PCR condition 1; (B) results obtained under the more sensitive condition 2 (see the text for details).

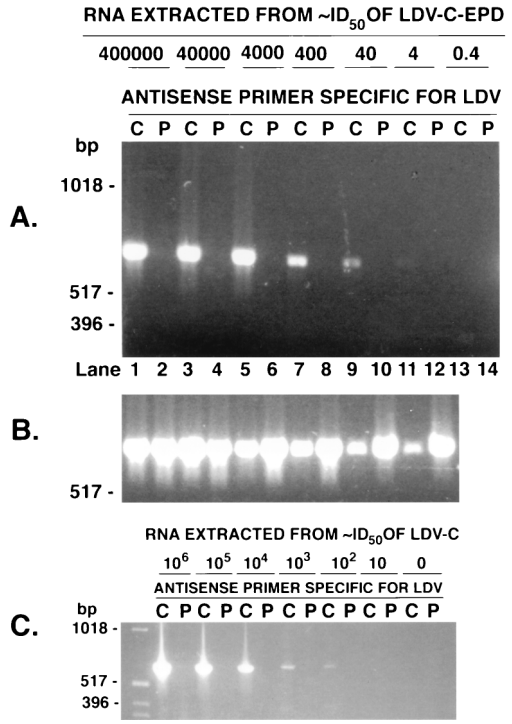

FIG. 4. Sensitivity of the differential RT-PCR procedure for detecting LDV-C in the plasma of infected mice in the absence (A) and presence (B) of an excess concentration of LDV-P and for detecting LDV-P in an original LDV-C pool (C). Plasma containing 2×10^6 ID_{50} of LDV-C–EPD (freed of LDV-P by endpoint dilution [see the text]) was serially 10-fold diluted. (A and B) RNA was extracted from 20 μl of each dilution (A) and from each dilution supplemented with 1 μl of a pool of LDV-P containing 2×10^6 ID_{50} (B). (C) RNA was extracted from 2 μl of an undiluted pool of the original LDV-C and 10-fold dilutions thereof. The RNAs were subjected to LDV-C/LDV-P-specific RT-PCR analysis under condition 2 (see Materials and Methods).

symptoms, and the LDV reisolated from these mice 190 days p.i. was nonneuropathogenic (Table 1).

The reasons for these losses of neuropathogenicity were unclear. One possibility was that novel nonneuropathogenic mutants arose from LDV-C or LDV-v that exhibited some replication advantage. Another possibility was that nonneuropathogenic variants preexisted in the original LDV-C and LDV-v populations and became selected during the persistent phase of infection.

To distinguish between these possibilities, we took advantage of an ORF 2 segment that differs by about 60% between LDV-C and LDV-P. Antisense oligonucleotides (/A1583 and /A1584 [Fig. 1B]) derived from the segment have been shown by Northern blot hybridization to be specific for LDV-C and LDV-P RNA, respectively (27). We used these two oligonucleotides, together with a common ORF 2 sense oligonucleotide, A1509/ (Fig. 1A), to establish a differential RT-PCR assay. When this assay was used under the initially designed condition (condition 1 in Materials and Methods) to study LDV-C infection of FVB mice, a dramatic switch from LDV-C to a LDV-P-like virus in the plasma was observed starting as early as day 3 p.i. (Fig. 3A, lane 5). From 7 days p.i., no LDV-C could be detected in the plasma under this assay condition; only the LDV-P-like virus was detected (lanes 6 to 11). This switch was accompanied by the loss of neuropathogenicity of the virus isolated from the mice (Table 1) and occurred without apparent deflections from the normal time course of viremia (Fig. 2). The same change from LDV-C to LDV-P was observed in BALB/c, C57BL/6, and (C57BL/6 × SJL)F_1 hybrid mice after infection with the same lot of LDV-C (data not shown), indicating that the change was independent of the host mouse strain. The rapidity of the conversion from LDV-C to the LDV-P-like virus in the circulation suggested that the LDV-P-like virus probably preexisted in small amounts in the LDV-C population, but RT-PCR analyses under condition 1 failed to detect any LDV-P-like virus in the LDV-C inoculum (Fig. 3A, lanes 12 and 13). By endpoint dilution, we determined that the differential RT-PCR under condition 1 could detect approximately 10^4 ID_{50} of either LDV-C or LDV-P present in 20 μl of plasma (data not shown). Thus, the LDV-P-like virus must have been present in the initial LDV-C inoculum at a level below 10^6 ID_{50}/ml. Subsequent studies confirmed the estimation.

By systematic modification of the RT-PCR condition, we were able to significantly increase the sensitivity of the assay in detecting LDV genomes without loss of specificity. In fact, under condition 2 (see Materials and Methods), we detected in some experiments as little as 4 ID_{50} of LDV-C–EPD (LDV-C freed of LDV-P by endpoint dilution [see below]), i.e., in 20 μl of a 10^{-7} dilution of plasma containing 2×10^9 ID_{50}/ml (Fig. 4A), but in repeated experiments the detection limit was most often 10 to 100 ID_{50} of LDV (see below) (Fig. 4C). The presence of an excess of LDV-P did not interfere with the detection of minute amounts of LDV-C (Fig. 4B). In contrast, in repeated experiments, the presence of an excess of LDV-P increased the detection of LDV-C about 10- to 100-fold, most probably by aiding in the precipitation of the minute amounts of LDV-C RNA extracted from plasma dilutions.

With the improved differential RT-PCR assay, we could detect the LDV-P-like virus in the original LDV-C pool (Fig. 3B, lane 13) as well as in the plasma of the FVB mice 1 day after infection with this LDV-C pool (lane 3). Furthermore, the assay revealed that LDV-C persisted in the mice at a low level for at least 21 days p.i., although the LDV-P like virus was the predominant LDV (lanes 5 to 10).

Combined, the results indicate that although LDV-C was the

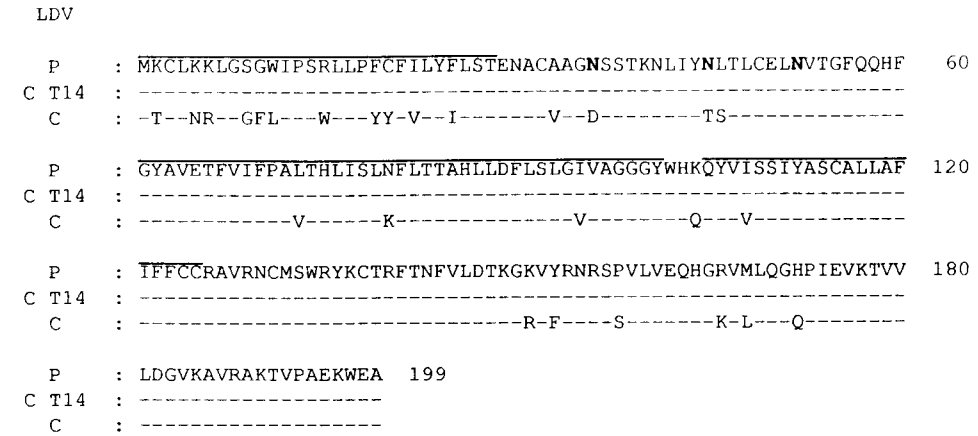

FIG. 5. Comparison of the amino acid sequence of the ORF 5 protein of the LDV isolated from 14-day LDV-C-infected FVB mice (LDV-C T14 [Fig. 2]) with those of the ORF 5 proteins of LDV-C and LDV-P. The signal peptide and the transmembrane segments are overlined, and the N-glycosylation sites in the ectodomain are in boldface letters.

predominant variant in the LDV-C pool, a small amount of an LDV-P-like virus was also present, and the latter possessed apparent growth advantage during a persistent infection. Repeated endpoint dilution assays showed that the LDV-P like virus was present in the LDV-C pool at a concentration of about 1/10,000 the level of LDV-C (e.g., Fig. 4C), i.e., about 10^5 ID_{50}/ml compared with 10^9 ID_{50} of LDV-C/ml. LDV-P genomes were just detectable by RT-PCR in RNA extracted from 2 μl of undiluted plasma but generally only very weakly, if at all, in RNA extracted from a 10-fold plasma dilution.

To further characterize the apparently nonneuropathogenic LDV-P-like virus present in small amounts in the LDV-C pool, we determined the sequences of its two envelope glycoproteins, VP-3P and VP-3M. VP-3P is the primary envelope glycoprotein of LDV and is encoded by ORF 5 (28). It is linked by disulfide bonds to a nonglycosylated protein (M/VP-2), probably involving conserved cysteine residues in their short ectodomains (Fig. 1D) (13, 14). The joined ectodomains seem to represent the virion receptor attachment site, since breakage of the disulfide bonds between them inactivates LDV infectivity. VP-3M is a second minor envelope glycoprotein (Fig. 1D) (13) and is encoded by ORF 2 (28). ORF 2 and ORF 5 were amplified by RT-PCR from RNA isolated from the plasma of the 14-day LDV-C infected mice (LDV-C T14), and the PCR products were cloned and sequenced as described in Materials and Methods. ORF 2 of LDV-C T14 differed from that of LDV-P by only 5 nucleotides, resulting in a 2-amino-acid difference in the ORF 2 protein (one in the signal peptide [data not shown]), whereas it differed from that of LDV-C by 25%. The ORF 5 protein of LDV-C T14 was identical to that of LDV-P (Fig. 5) and differed from that of LDV-C by 13%. Therefore, LDV-C T14 seems to be a member of the LDV-P quasispecies, and the results explain the apparent loss of neuropathogenicity of LDV-C during the persistent infection of FVB mice (Table 1).

The loss of neuropathogenicity of LDV-v during the persistent infection of either ADPM-susceptible or -resistant mice (Table 1 and data not shown) also seems to have been due to a displacement of LDV-v by a nonneuropathogenic LDV that preexisted in the LDV-v inoculum, because none of several ORF 5 clones generated from RNA of LDV-v persistently infected mice was homologous to that of LDV-v; instead, they were homologous to that of either LDV-P or another previously unrecognized non-neuropathogenic LDV (in preparation). However, our attempt to develop a differential RT-PCR assay to differentiate between LDV-v and the nonneuropathogenic LDV variants has not been successful, partly because LDV-v is more closely related to the nonneuropathogenic LDV-P and LDV-a than to LDV-C (Fig. 1B) (27) and we have not yet found a reasonably long, continuous stretch of nucleotides in ORF 2 or ORF 5 that differs sufficiently between LDV-v and the nonneuropathogenic variants to allow generation of differential oligonucleotide primers.

Of special interest in relation to the lack of neuropathogenicity of the LDV variants isolated from mice persistently infected with either LDV-C or LDV-v was the possession of all three N-glycosylation sites in the ectodomains of their ORF 5 proteins (VP-3P) in all these variants (Fig. 5 and data not shown), which is a characteristic of the ectodomains of the ORF 5 proteins of the nonneuropathogenic LDV-P and LDV-a (Fig. 5) (15, 27). In sharp contrast, the ectodomains of VP-3P of the neuropathogenic LDV-C and LDV-v lack the two sites toward the N-terminus (Fig. 5) (15, 27).

Neuropathogenic LDV devoid of the preexisting nonneuropathogenic variant has an impaired ability to establish a persistent infection. To study the biological properties of LDV-C per se, we attempted to clone out LDV-C from the original LDV-C pool containing low levels of LDV-P. Since no plaque assay is available for LDV (28, 30), we turned to the endpoint dilution titer determination in mice (29). This approach seemed adequate for this purpose, since LDV-P seemed to be present in the LDV-C pool at about 1/10,000 the level of LDV-C. Groups of FVB mice were given injections of 0.1 ml each of 10-fold dilutions of an LDV-C pool, and 4 days later their plasma was assayed for a 5- to 10-fold elevation in lactate dehydrogenase activity in plasma as a sign of LDV infection (29, 30). All mice given injections of the 10^{-7} or lower dilutions, 7 of 8 mice given the 10^{-8} dilution, and 2 of 9 mice given the 10^{-9} dilution became infected, yielding a titer of 2×10^9 ID_{50}/ml. LDV was isolated from the two infected mice given the 10^{-9} dilution and amplified by a single 1-day passage through FVB mice (designated end-point-diluted LDV-C or LDV-C–EPD). LDV-C- and LDV-P-specific RT-PCR analyses demonstrated that the amplified virus was LDV-C and free of LDV-P (see Fig. 7). When LDV-C–EPD was injected into four immunosuppressed 8-week-old C58/M mice, all the mice developed fatal paralytic disease between 12 and 22 days p.i.

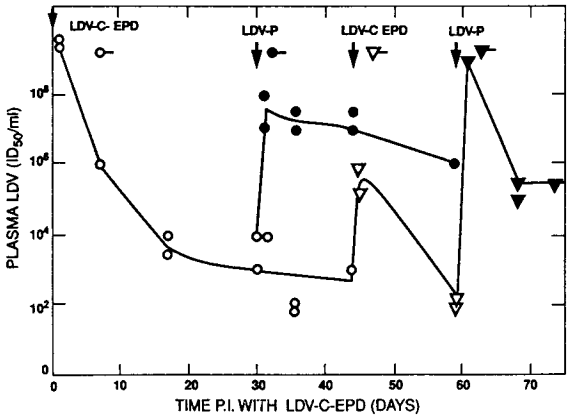

FIG. 6. Time courses of viremia in FVB mice after infection with LDV-C–EPD and subsequent superinfection with LDV-P or LDV-C–EPD. Four mice were given i.p. injections of about 10^6 ID_{50} of LDV-C–EPD (zero time of infection). Two mice were superinfected with about 10^6 ID_{50} of LDV-P at 30 days p.i. The other two mice were superinfected with about 10^6 ID_{50} with LDV-C–EPD at 44 days p.i. and then with LDV-P at 59 days p.i. The mice were bled at the indicated times p.i., and their plasma was assayed for infectious LDV by titer determination.

(Table 1), and the virus isolated from the paralyzed mice was LDV-C free of LDV-P (data not shown).

The finding that nonneuropathogenic LDVs rapidly outcompeted the neuropathogenic LDVs during a persistent infection of either ADPM-susceptible or nonsusceptible mice suggested that the neuropathogenic LDVs are impaired in their ability to establish a persistent infection. This conclusion has been directly proven by monitoring the time course of replication of LDV-C–EPD in FVB mice (Fig. 6). The LDV titer in plasma at 1 day p.i. was as high ($10^{9.5}$ ID/ml) as those observed with all LDV strains (Fig. 2), but thereafter it decreased much more rapidly and extensively than is normally observed; i.e., the titer had decreased to 10^3 to 10^4 ID_{50}/ml by 20 days p.i. (cf. Fig. 6 and 2). In fact, at 36 days p.i. (Fig. 6) no infectious LDV was detected in the plasma (i.e., the titer was $\leq 10^2$ ID_{50}/ml). Differential RT-PCR analysis of the plasma of the LDV-C-EPD-infected mice detected only LDV-C genomes between 1 and 44 days p.i. (Fig. 7A, lanes 2 to 15). No LDV-C was detected in 4 μl of plasma of the 36-day LDV-C–EPD-infected mice (Fig. 7A, lane 12), because the virus titer in plasma at this time was very low (Fig. 6), but it was detected when RNA was extracted from 20 μl of plasma (data not shown). The finding that no LDV-P genomes were detected in the plasma during the entire LDV-C–EPD persistent infection period examined confirms that the stock of LDV-C-EPD was free of LDV-P, because if LDV-P had been present, even at a very low level, it would have rapidly outgrown LDV-C and become the dominant quasispecies. In the experiment shown in Fig. 3, where the mice were given injections of 10^6 ID_{50} of LDV-C and about 100 ID_{50} of LDV-P, LDV-P genomes were detectable in plasma as early as 1 day p.i. In another experiment where the inoculum dose of LDV-P was reduced to ≤ 10 ID_{50}, LDV-P genomes became detectable by 7 days p.i. (data not shown). Furthermore, no significant change was detected in the LDV-C genome during the persistent infection; the ORF 5 of the LDV reisolated from the LDV-C–EPD-infected mice at 44 days p.i. was >99% identical to that of the inoculated LDV-C, and the predicted ORF 5 proteins were identical (data not shown). During the period of low-level LDV-C persistent infection, LDV-permissive macrophages accumulated, since superinfection of two of the four LDV-C–EPD-infected mice with LDV-P at 30 days p.i. resulted in a rapid 5 $\log_{10}$ unit increase in the LDV titer in plasma (Fig. 6) and RT-PCR analysis of the viral RNA in plasma demonstrated the predominant presence of LDV-P in these mice (Fig. 7B, lanes 2 to 9).

Superinfection of the two remaining LDV-C–EPD-infected mice at 44 days after infection with 10^6 ID_{50} of LDV-C–EPD resulted in a transient small increase in the viral titer in plasma (Fig. 6). However, this increase was most probably caused by the injected LDV-C per se and did not involve its replication. By day 59, the LDV titer in plasma had again decreased to $\leq 10^2$ ID_{50}/ml (Fig. 6). Superinfection of the mice with LDV-P at this time resulted in a 7-$\log_{10}$-unit increase in the LDV titer in plasma (Fig. 6), which was clearly due to LDV-P replication (Fig. 7B, lanes 10 to 15). Thus, these mice possessed a pool of LDV-permissive macrophages almost as large as that in naive mice. However, throughout the experiment, some LDV-C was detectable in the infected mice, and this included the LDV-P-superinfected mice in which LDV-P was the predominant quasispecies (Fig. 7B, lanes 2 to 15). In a reciprocal experiment in which FVB mice persistently infected with LDV-P for 23 days were superinfected with either LDV-C or LDV-P, the viral titer in plasma after superinfection remained the same as just before superinfection at about 10^7 ID_{50}/ml.

Although we have tried several times to obtain an LDV-v stock free of nonneuropathogenic variants by endpoint dilution, we have not been successful to date. We suspect that LDV-v and a nonneuropathogenic variant(s) are present in the original LDV-v stock at similar concentrations. However, the findings that the LDV isolated from persistently LDV-v-infected mice was a nonneuropathogenic variant (Table 1) and that only LDVs with an ORF 5 characteristic of nonneuropathogenic LDVs could be detected in their plasma indicate that the neuropathogenic LDV-v also has an impaired ability to establish a persistent infection, just like LDV-C.

From the above data and the data presented in the preceding section, we conclude that neuropathogenic LDVs have an

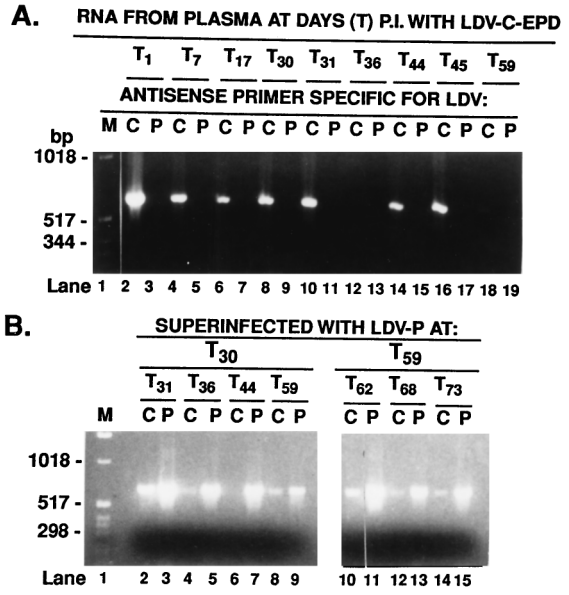

FIG. 7. RT-PCR analysis of LDV isolated from FVB mice at various times after infection (in days; T1 to T73) with LDV-C–EPD (A) and after superinfection with LDV-P (B) (see Fig. 6 for experimental details and time course of viremia). RNA was extracted from 4 μl of plasma and subjected to differential RT-PCR analysis of LDV-C and LDV-P ORF 2 under condition 2 (see Materials and Methods).

impaired ability to establish a persistent infection compared with nonneuropathogenic variants and therefore become replaced by the latter as the predominant quasispecies during the persistent phase of infection if the initial inoculum contains low concentrations of a nonneuropathogenic variant(s).

DISCUSSION

Our results indicate that available pools of the neuropathogenic LDV-C contained low concentrations of the nonneuropathogenic LDV-P quasispecies. LDV-C and LDV-P seem to be equally capable of productively infecting the LDV-permissive cells in naive mice, since the LDV titers in plasma 1 day after infection with either variant are about the same (10^9 to 10^{10} ID_{50}/ml [Fig. 2 and 6]), but LDV-P apparently is capable of maintaining a significantly higher viremia than LDV-C in the persistent phase of infection. Thus, upon infection of a mouse with an LDV-C pool that contains low concentrations of LDV-P, LDV-P rapidly outcompetes LDV-C and becomes the dominant quasispecies. In fact, the levels of LDV-C in persistently infected mice are so low (Fig. 6) that the virus isolated from mice dually persistently infected with LDV-C and LDV-P fails to induce paralytic disease in ADPM-susceptible, immunosuppressed mice (Table 1). The low ability of LDV-C to establish a persistent infection probably also applies to the other neuropathogenic LDV, LDV-v, since the LDV isolated from LDV-v persistently infected ADPM-susceptible or resistant mice also failed to induce paralytic disease (Table 1) and the ORFs 5 of such LDV isolates differ from that of LDV-v and are predicted to encode a VP-3P protein with three N-glycosylation sites on its ectodomain that are thus far characteristic of nonneuropathogenic LDVs.

The molecular mechanism by which nonneuropathogenic LDVs outgrow neuropathogenic variants during a persistent infection has not been established. Several non-mutually exclusive possibilities exist. One possibility is that the replication of neuropathogenic LDVs is more susceptible to suppression by anti-LDV immune responses than is the replication of nonneuropathogenic LDVs. In the case of LDV-P and some other LDV isolates, it has been demonstrated that antibodies that neutralize LDV infectivity in vitro do not appear in mice until 1 to 2 months p.i. and neutralize LDV infectivity only poorly (6, 31, 32). Cytotoxic T cells (CTLs) specific for an epitope(s) on the nucleocapsid (N) protein of LDV-P are generated in mice but lyse LDV-infected macrophages too slowly to efficiently block LDV replication in these cells (12). In any case, neither neutralizing antibodies nor CTLs seem to have a significant effect on LDV-P replication in mice, since the time course of viremia in nude mice that fail to mount anti-LDV-immune responses is about the same as that in their immunocompetent littermates (31). However, it could be argued that LDV-C is more immunogenic than LDV-P and that its replication is more efficiently controlled by anti-LDV immune responses. With regards to CTLs, the N proteins of LDV-P and LDV-C exhibit 99% amino acid identity and the two potential CTL epitopes of the protein are identical. On the other hand, the absence of two large oligosaccharide chains on the short ectodomain of VP-3P of LDV-C (Fig. 5), which carries the LDV neutralizing epitopes (31), might allow a more efficient neutralization of LDV-C than of LDV-P. Indeed, we have found that plasma from mice persistently infected with either LDV-C or LDV-P as well as anti-VP-3P monoclonal antibodies neutralized LDV-C in vitro 10 to 100 times more efficiently than it neutralized LDV-P and that antibodies that neutralize LDV-C appeared in mice as early as 7 days after infection with LDV-C (not shown). However, it has not been demonstrated that these neutralizing antibodies suppress LDV-C replication in vivo. Furthermore, the displacement of LDV-C by LDV-P in dually infected mice begins so rapidly (within 3 days p.i. [Fig. 3 and unpublished data]) that anti-LDV immune responses alone probably cannot account for the rapid suppression of LDV-C replication during such a short period. Perhaps LDV-C replication in macrophages is also more susceptible than LDV-P replication to alpha/beta interferon, which is transiently produced in large amounts during the first day after infection with LDV (30, 31).

An alternative possibility that could account for the inefficient persistence of neuropathogenic LDVs is that although the neuropathogenic LDVs possess the unique ability to infect the central nervous system (including the spinal cord anterior horn neurons of ADPM-susceptible mice and certain leptomeningeal cells of both ADPM-susceptible and nonsusceptible mice [4, 27]), they may have a reduced ability to productively infect newly generated macrophages which support the continuous rounds of LDV replication during the persistent infection. Such a difference in the tropism of the neuropathogenic and nonneuropathogenic LDVs may reside in the interaction between the LDVs and the receptor(s) on permissive cells. As mentioned above, the disulfide-linked short ectodomains of about 30 and 10 amino acids of VP-3P and M/VP-2, respectively (Fig. 1D), probably from the virion receptor attachment site, at least for the nonneuropathogenic LDV-P (13, 14). Comparison of the amino acid sequences constituting the short ectodomains revealed a striking correlation between neuropathogenicity and the lack of two of the three N-glycosylation sites on the VP-3P ectodomain (references 15 and 27 and unpublished data). This correlation is further strengthened by the finding that the nonneuropathogenic LDVs that grew out from the neuropathogenic LDV inocula possessed all three N-glycosylation sites on the ectodomain of VP-3P (Fig. 5) (unpublished data). It is therefore possible that the two additional large oligosaccharide chains on the ectodomain of VP-3P of nonneuropathogenic LDVs will interfere with binding to a receptor on certain leptomeningeal cells and anterior horn neurons of ADPM-susceptible mice (15) while facilitating the binding to a different receptor on newly generated macrophages. Further studies are required to explore the viral properties responsible for the differential tropism of neuropathogenic and nonneuropathogenic LDVs and the factors that account for the reduced ability of the former to establish a persistent infection.

Regardless of the mechanism involved, it is clear that LDV-P permissive macrophages accumulate in LDV-C–EPD persistently infected mice (Fig. 6). In fact, the burst in viremia upon superinfection of the 59-day LDV-C–EPD-infected mice with LDV-P indicates that the mice at this time point possessed about as large a pool of LDV-P-permissive macrophages as naive mice did. Interestingly, the accumulation of the LDV-P permissive macrophages occurred in the presence of a low but persistent LDV-C–EPD viremia. Again, whether the failure of LDV-C–EPD to infect these macrophages is due to an effective anti-LDV-C–EPD immune response or to a reduced tropism of LDV-C–EPD for these cells needs further investigation.

The origin of LDV-P in the LDV-C population is unclear. It might have been associated with the original LDV-C isolate, or LDV-C could have become contaminated with LDV-P during mouse passages in the laboratory. However, the fact that nonneuropathogenic LDV variants are also found in other neuropathogenic pools such as LDV-v and the very early LDV-Ib (24) suggests that the coexistence of nonneuropathogenic and neuropathogenic LDV variants may be a general phenomenon.

In fact, it was reported in earlier studies (22, 25) that several LDV isolates, including LDV-P, exhibited a low level of neuropathogenicity. Since our present LDV-P is nonneuropathogenic (15) (Table 1), it is possible that a neuropathogenic variant associated with the original LDV-P isolate at low concentrations was lost during numerous mouse passages in the laboratory or upon cloning by endpoint dilution. Indeed, we have detected low concentrations of an LDV-C-like virus in some earlier pools of LDV-P by the differential RT-PCR assay (unpublished data).

The selection and amplification of a quasispecies that is fittest under specific environmental conditions is a common feature in viral evolution and pathogenesis (reviewed in references 11 and 19). Selection may occur on the basis of resistance to host immune responses or drug therapies or advantages in cell tropism, which is probably the case with the neuropathogenic and nonneuropathogenic variants of LDV. Other known examples are human immunodeficiency virus (16) and lymphocytic choriomeningitis virus. In the case of lymphocytic choriomeningitis virus, neurotropic and lymphotropic variants selectively accumulate in the central nervous system and peripheral tissues, respectively (10, 34). The difference in tropism is linked to a single amino acid difference in the envelope glycoprotein, but its molecular basis is yet to be understood.

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