Journal of NeuroVirology (1998) 4, 560–568

http://www.jneurovirol.com

Short Communication

Lactate dehydrogenase-elevating virus variants: cosegregation of neuropathogenicity and impaired capability for high viremic persistent infection

Zongyu Chen[1], Kehan Li[1], Raymond RR Rowland[2], Grant W Anderson[3] and Peter GW Plagemann[1]

[1]*University of Minnesota, Department of Microbiology, Box 196 UMHC, Minneapolis, Minnesota 55455, USA*

Neuropathogenic isolates of lactate dehydrogenase virus (LDV) differ from non-neuropathogenic isolates in their unique ability to cause a paralytic disease (age-dependent poliomyelitis, ADPM) in immunosuppressed C58 and AKR mice by cytocidally infecting their anterior horn neurons. We have recently reported that an original neuropathogenic LDV isolate, LDV-C-BR, contained a low level of a coexisting non-neuropathogenic LDV which, in a mixed infection of mice, rapidly outcompeted the former resulting in apparent loss of neuropathogenicity of the reisolated LDV. This correlated with an impaired ability of the neuropathogenic LDV to establish a viremic persistent infection. In the present study we identified the presence of three different quasispecies in another original neuropathogenic LDV by sequence analysis of cDNA clones of ORF 5 (encoding the primary envelope glycoprotein VP-3P) obtained from the isolate. Successful development of differential reverse transcription-polymerase chain reaction assays allowed us to biologically clone all three quasispecies through repeated end point dilutions. Only one of the quasispecies (LDV-v) was neuropathogenic. The other two, LDV-vP (probably the same as LDV-P) and LDV-vx (a novel LDV quasispecies that had not been previously identified), were non-neuropathogenic and found to be the common LDV quasispecies associated with almost all LDVs originally isolated from mice carrying various other transplantable tumors. The neuropathogenic LDV-v became selectively amplified in the spinal cords of paralyzed mice, but possessed an impaired ability to establish a persistent viremic infection and was rapidly out-competed by LDV-vP and LDV-vx in mixed infections, just as reported previously for LDV-C-BR. The results further support our hypothesis that neuropathogenicity and impaired capability for viremic persistence of LDV are determined by the same molecular feature. The only consistent and biologically relevant molecular difference we have observed between neuropathogenic and non-neuropathogenic LDVs is the number of polylactosaminoglycan chains associated with the ectodomain of VP-3P.

Keywords: Lactate dehydrogenase-elevating virus; neuropathogenic LDV; LDV variants

Introduction

Lactate dehydrogenase-elevating virus (LDV), a murine arterivirus (Plagemann, 1996), generally establishes a life-long infection with high viremia in mice in spite of significant host immune responses (Plagemann *et al*, 1995; 1996; van den Broek, 1997). The persistent infection is sustained by replication of LDV in a renewable subpopulation of macrophages and is not associated with any overt clinical symptoms (Plagemann and Moennig, 1992; Plagemann, 1996). However, in certain strains of mice (e.g. AKR and C58) that harbor replication-competent endogenous ecotropic retroviruses, some LDV isolates, such as LDV-Ib (Nawrocki *et al*, 1980),

Correspondence: PGW Plagemann, Department of Microbiology, Medical School, University of Minnesota, Box 196 UMHC, 420 Delaware St. S.E., Minneapolis, MN 55455.
Current addresses: [2]Department of Biology and Microbiolgy, South Dakota State University, Brookings, SD 57007. [3]Department of Medicine, Medical School, University of Minnesota, Minneapolis, MN 55455, USA
Received 6 March 1998; revised 27 April 1998; accepted 11 May 1998

LDV-C-BR (Martinez *et al*, 1980) and LDV-VIR (Anderson *et al*, 1995c) were found to interact with the endogenous retroviruses to cause paralytic disease (age-dependent poliomyelitis, ADPM) but only when the mice were immunosuppressed as a result of old age or artificial treatments (Murphy *et al*, 1983, 1986; Stroop and Brinton 1983; Anderson *et al*, 1995a, b; Plagemann, 1996). It has been hypothesized that a high level of retroviral expression in the glial cells of these mice somehow induces the expression of a surface molecule(s) on the anterior horn motor neurons of the spinal cord that can function as an alternative receptor for the neuropathogenic LDVs (Anderson *et al*, 1995a, b).

In the course of studying the mechanism of LDV neuropathogenicity we discovered that populations of the original neuropathogenic LDV-C-BR consisted of two quasispecies (Chen *et al*, 1997): a neuropathogenic LDV-C (Godeny *et al*, 1993) and a non-neuropathogenic quasispecies that seems to be identical to LDV-P (Palmer *et al*, 1995). In infections of mice with LDV-C-BR, LDV-P rapidly outcompeted LDV-C, explaining the apparent loss of neuropathogenic potential of LDV-C-BR during long term persistent infections (Chen *et al*, 1997). LDV-C was cloned free of LDV-P and proven to be neuropathogenic, but was found to possess an impaired ability, relative to LDV-P, to sustain a high viremic persistent infection (Chen *et al*, 1997). To further elucidate the correlation between neuropathogenicity and impaired ability to establish a viremic persistent infection we have examined the molecular and phenotypic properties of another neuropathogenic LDV isolate, LDV-VIR.

LDV-VIR consists of at least three LDV quasispecies that differ in neuropathogenicity and ability to establish a high viremia persistent infection

LDV-VIR was originally isolated in this laboratory from the spinal cord of a paralyzed C58 mouse (Anderson *et al*, 1995a, c) that had been injected with LDV-Ib. LDV-VIR was cloned by end point dilution in C58 mice and stocks were maintained by one-day passages in C58 mice and two additional isolations from spinal cords of paralyzed C58 mice injected with these stocks. When used it was in its 9th passage (LDV-VIR9). Even though LDV-VIR had been end point diluted, sequence analysis of multiple ORF 5 clones revealed that it consisted of three different quasispecies, only one of which was found to be neuropathogenic. Altogether, we sequenced 13 ORF 5 clones derived from several different reverse transcription (RT)-polymerase chain reaction (PCR) amplifications of LDV-VIR genomic RNA. Three clones (designated LDV-v) derived independently from two different genomic RNA populations were close to identical. The ectodomain of the predicted ORF 5 protein (VP-3P), like that of LDV-C (Chen *et al*, 1997), lacked the two N-terminal glycosylation sites present in the ectodomain of LDV-P VP-3P (Figure 1). However, we found that ORF 5 of three other clones (designated LDV-vP) were very similar to ORF 5 of the non-neuropathogenic LDV-P (~98% nt identity), whereas differing by 9.4% from that of LDV-v. This close identity suggests that the clones represent members of the LDV-P quasispecies. In addition, seven other ORF 5 clones (designated LDV-vx) derived independently from three genomic RNA populations differed from that of LDV-v by 8.0% and of LDV-P by 4.9%, whereas exhibiting >99% nucleotide identity among themselves. The amino acid identities of the predicted ORF 5 proteins of the individual LDV-vP and LDV-vx clones among themselves were >99%, whereas they differed from those of LDV-v by 11.1% and 10.6%, respectively, and their ectodomains pos-

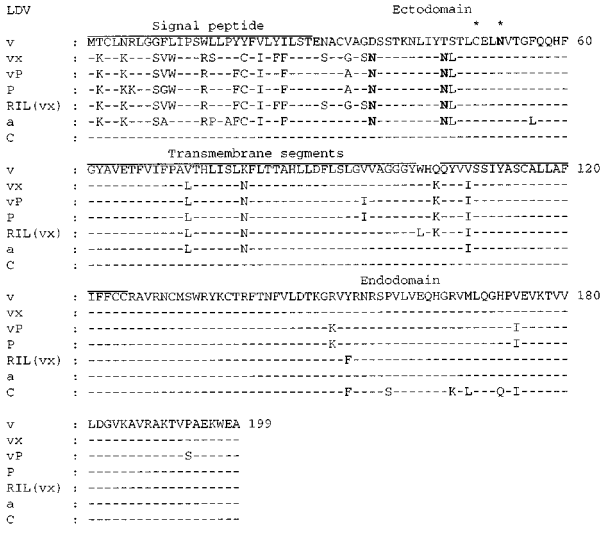

Figure 1 Comparison of the amino acid sequences of the ORF-5 proteins (VP-3P) of various LDV isolates. For LDV designations, see text. LDV-a was isolated from a WEH1 mouse carrying a transplantable tumor (Murphy *et al*, 1983) and was provided by W. Murphy. RNA was extracted from 10^6-10^7 ID_{50} of stocks of the indicated LDVs, reverse transcribed using random hexanucleotides and oligo (dT) as primers, and the first strand products were amplified by PCR using sense and antisense primers (A1468/B1836) representing conserved segments upstream and downstream of ORF 5, respectively, as described previously (Chen *et al*, 1997; Chen and Plagemann, 1997). The primers amplified ORF 5 from the genomes of all LDVs regardless of origin. The 587 bp PCR products were TA cloned and sequenced on both strands using Sequenase version 2.0 (USBC, Cleveland, OH) as described by the manufacturer. Sequence analyses were performed using Molecular Biology Information program SAM and EUGENE (Lawrence and Goldman, 1988). The signal peptide and the transmembrane segments of VP-3P are overlined and the N-glycosylation sites in the ectodomain are indicated with boldface. The highly conserved N-glycosylation site and the cysteine residue that is probably involved in disulfide linkage with M/VP-2 are indicated by asterisks.

sessed all three N-glycosylation sites (Figure 1). LDV-vx thus represents a previously unidentified LDV quasispecies. Strikingly, the N-terminal end of VP-3P of LDV-v is identical to that of LDV-C, whereas the C-terminal end is identical to that of LDV-vx (Figure 1). This suggests that LDV-v may be a recombinant of LDV-C and LDV-vx, which is further supported by their ORF 2 protein (VP-3M) sequences (see later).

To distinguish these LDV quasispecies, we developed a new differential RT–PCR assay that produces a 483 bp product specific for each of the three quasispecies. Specifically, we made a sense oligonucleotide (J1008/) to a segment in ORF 4 that is conserved for all LDVs sequenced (Figure 2A and data not shown) and three antisense oligonucleotides (/J1003, /J1004 and /J1007) to the 5′ end of ORF 5 (encoding the signal peptide of VP-3P) that are specific for LDV-v (also LDV-C), LDV-vP (also LDV-P) and LDV-vx, respectively (Figure 2A). These oligonucleotides differ from each other by at least two nucleotides toward the 3′ end. The sense oligonucleotide (J1008/) was used in pair with each of the three antisense oligonucleotides for PCR amplification of first strand products generated by RT from genomic RNAs of various LDV isolates according to procedures described previously (Chen *et al*, 1997; Chen and Plagemann, 1997). The J1008/J1003 primer pair was consistently specific for the LDV-C and LDV-v genomes under the specified experimental conditions; no cross-amplification was observed when individual LDVs were analyzed that had been cloned (see later) by repeated end point dilutions (Figure 3 lanes 16–18 and 28–33).

This was also generally the case for the LDV-P and LDV-vx genomes using the primer pairs J1008/J1004 and J1008/J1007, respectively (Figure 2A and Figure 3 lanes 16–18 and 28–33), but the results were not always consistent probably because /J1004 and /J1007 differ by only two nucleotides. We have therefore designed additional differential RT–PCR assays to specifically distinguish the LDV-P and LDV-vx genomes, that are based on recently established sequence differences in ORF 2 (Figure 2B). The ORF 2 sequence differences allowed the design of two sets of antisense primers for LDV-P and LDV-vx that differed by four nucleotides. However, these primers are not designed for distinguishing between neuropathogenic and non-neuropathogenic LDVs because, although ORF 2 of LDV-C is quite different from that of LDV-P and LDV-vx (Figure 2B), that of LDV-v is close to identical to that of LDV-vx.

Analysis of LDV-VIR9 RNA by our new differential RT–PCR assays showed that all three quasispecies were present at similar levels in the LDV-VIR9 stock (Figure 3 lanes 7–9). The RT–PCR method is not quantitative, but we have demonstrated previously that the amount of PCR product formed under standard conditions decreased progressively with a decrease in the concentration of infectious virus in the plasma from which viral RNA was extracted and that the limit of detection was generally 10–100 infectious dose 50 (ID_{50}; Chen *et al*, 1997). Thus the RT–PCR results for LDV-VIR9 indicated that the LDV-v, LDV-P and LDV-vx were present in similar amounts, which was also indicated by the recovery of clones of all three from products of RT–PCR amplifications of several RNA preparations.

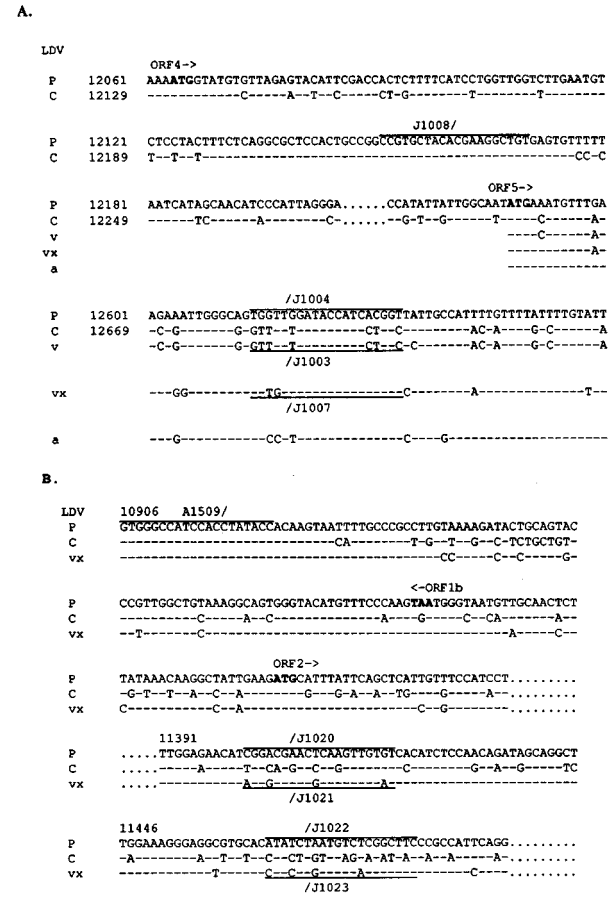

Figure 2 Oligonucleotide primers used in differential PCR amplification of an ORF 4/5 segment (**A**) and of ORF 1b/2 segments (**B**) of various LDV genomes. The primer sequences are overlined or underlined; slashes before and after the primer designation indicate antisense and sense orientations, respectively. The sense primers represent segments in ORF 4 (**A**) or ORF 1b (**B**) that are identical for LDV-C, LDV-P, and all other LDVs we have sequenced. In contrast, the antisense primers are to a segment in ORF 5 that differs by at least two nucleotides for LDV-P, LDV-C/v and LDV-vx (**A**) or to segments in ORF 2 that differ by four nucleotides for LDV-P and LDV-vx (**B**). The ATG initiation codons for ORF 2, ORF 4 and ORF 5 and the termination codon for ORF 1b are in boldface letters. The sequence for LDV-C is from Godeny *et al* (1993; GenBank accession number L13298 as modified in Figure 5B) and that for LDV-P from Palmer *et al* (1995; GenBank accession number U15146).

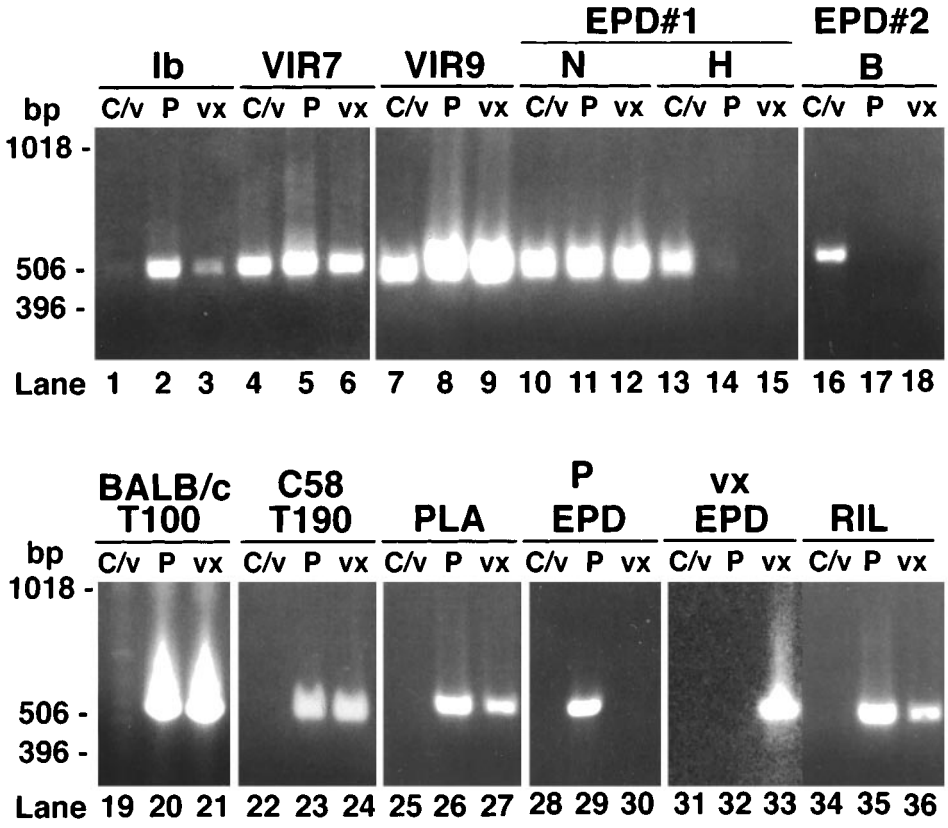

Figure 3 Differential RT–PCR analyses of the genomes of different LDV isolates. RNA was extracted from 10^6 to 10^7 ID_{50} of the indicated LDVs and reverse transcribed with random hexanucleotides and oligo (dT) as primers. The first strand products were amplified by PCR using the common sense primer J1008/ in combination with the three LDV-P, LDV-C/v and LDV-vx specific primers (/J1004, /J1003 and /J1007 respectively; see Figure 2A). The PCR products of 483 bp were electrophoresed in 1% (w/v) agarose gels. For description of individual LDVs see text. EPD stands for end point dilution.

We had empirically observed that isolation of LDV-VIR from the spinal cords of infected paralyzed mice increased the relative neuropathogenicity of the virus (increased incidence and shorter incubation period in susceptible C58/M mice measured as described by Anderson *et al*, 1995b, c). We reasoned that replication of the neuropathogenic quasispecies in anterior horn neurons resulted in its selective amplification in the spinal cord. In order to test this hypothesis, we analyzed by differential RT–PCR the genomic RNAs isolated from the original LDV-Ib isolate and LDV-VIR7 which was derived from the spinal cord of a paralyzed mouse during passage 7 of LDV-VIR. As shown in Figure 3 (lanes 1–6), LDV-v represented only a minor component in the original LDV-Ib isolate, whereas it was a major component in the LDV-VIR7 isolate. These results provide strong evidence in support of selective amplification of LDV-v in the spinal cord of paralyzed mice and point to LDV-v as the neuropathogenic quasispecies. The results also explain the finding that LDV-Ib caused paralysis in 100% of 4–7 months old C58 mice only when injected at 10^6 ID_{50}/mouse (Murphy *et al*, 1983), whereas the same was achieved by injecting 100 ID_{50} of LDV-VIR7 (data not shown). On the other hand, the composition of LDV-VIR9 derived from LDV-VIR7 by two sequential 1-day passages in C58/M mice was similar to that of LDV-VIR7 (Figure 3, lanes 4–9) indicating that all three quasispecies possessed a similar ability to acutely infect mice.

Cloning of LDV quasispecies

For determining the phenotypic properties of LDV-v, LDV-P and LDV-vx we needed to obtain each free of the others. We accomplished this by repeated end point dilutions in mice, since no LDV-permissive cell culture line is available (Plagemann, 1996). The differential RT–PCR provided for the first time a quick method for identifying the quasispecies present in the end point dilutions. An example is illustrated in Figure 3 lanes 10–18. In the first end point dilution (EPD#1) of LDV-VIR9 all mice injected with the 10^{-7} dilution of LDV-VIR9 (0.1 ml/mouse), 3/4 mice (designated H, N and HT)

injected with the 10^{-8} dilution and 0/4 mice injected with the 10^{-9} dilution became infected, yielding a titer of $10^{9.3}$ ID_{50}/ml for the LDV-VIR9 stock. Differential RT–PCR showed that the virus isolated from the N mouse, still consisted of similar concentrations of all three LDVs (Figure 3 lanes 10–12). In contrast, LDV isolated from the H and HT mice consisted largely of LDV-v, but also contained low concentrations of LDV-vP (Figure 3 lanes 13–15 and data not shown). A second end point dilution removed the latter (Figure 3 lanes 16–18). We have in a similar manner cloned LDV-P and LDV-vx. We have observed that LDV-PLA (Brinton-Darnell and Plagemann, 1975) and other LDVs originally isolated from mice carrying different LDV-contaminated transplantable tumors, such as LDV-RIL (Riley *et al*, 1960), all consisted of LDV-P and LDV-vx-like LDVs but lacked LDV-C/v-like viruses (Figure 3, lanes 25–27 and 34–36). We have cloned by repeated end point dilutions LDV-P from LDV-PLA populations in which LDV-P was the predominant quasispecies (Figure 3, lanes 28–30) and have cloned LDV-vx from a BALB/c mouse infected with LDV-VIR9 in which LDV-vx became the predominant quasispecies (Figure 3, lanes 31–33).

Phenotypic properties of LDV quasispecies

We had previously observed that C58/M mice inoculated with LDV-VIR as newborns recovered after developing only mild paralytic symptoms and that the LDV isolated from such a mouse at 190 days p.i. was non-neuropathogenic (Chen *et al*, 1997). Differential RT–PCR analysis provided an explanation for these findings; the re-isolated virus population consisted of LDV-vP and LDV-vx and lacked LDV-v (Figure 3, lanes 22–24). A loss of neuropathogenicity also occurred in ADPM-non-susceptible BALB/c mice during persistent infection with LDV-VIR (Chen *et al*, 1997) and RT–PCR analysis showed that this was also due to loss of LDV-v (Figure 3, lanes 19–21). These findings show that LDV-v can become lost during a mixed persistent infection and suggest that LDV-v is neuropathogenic, whereas LDV-vP and LDV-vx are non-neuropathogenic but possess a replication advantage over LDV-v during a long term infection. This difference in neuropathogenicity of the quasispecies was proven directly; paralytic disease developed in 4/4 immunosuppressed 6–8 week old C58/M mice after infection with cloned LDV-v with a mean onset of 16 days, whereas none of companion C58/M mice injected with LDV-PLA (consisting of LDV-P and LDV-vx only, see Figure 3, lanes 25–27) or cloned LDV-P or LDV-vx became paralyzed.

In order to explore in more detail the fate of the three quasispecies in mice after infection, a group of FVB mice was bled at various times p.i. with LDV-VIR9, and their plasma titrated for infectious LDV and analyzed by differential RT–PCR. The time course of viremia (Figure 4A) was similar to that observed for various LDVs (Plagemann, 1996; Chen *et al*, 1997). A maximum titer of 10^9 ID_{50}/ml of plasma was observed 1 day p.i. reflecting the massive cytocidal replication of LDV in practically all available permissive macrophages. Thereafter the plasma titer decreased by 2–3 $\log_{10}ID_{50}$, the persistent infection being maintained by LDV replication in newly generated permissive macro-

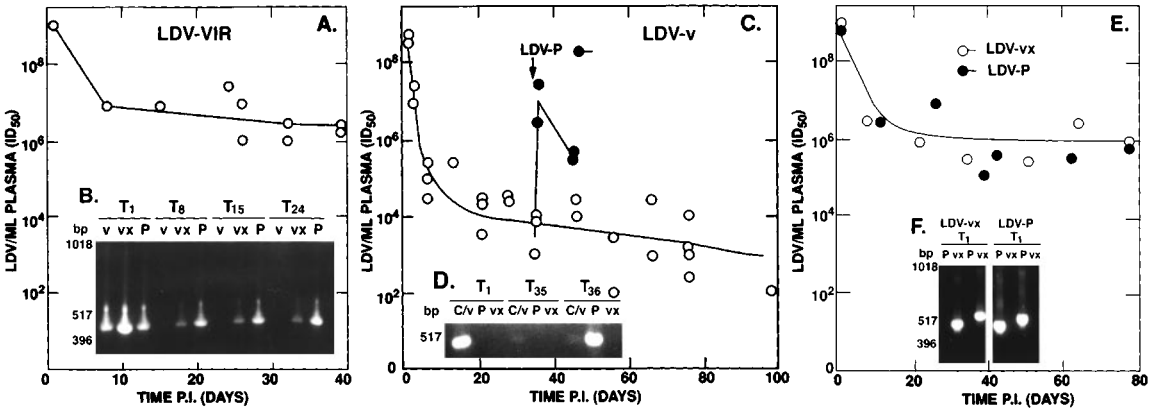

Figure 4 Time courses of viremia in FVB mice after infection with LDV-VIR9 (**A**), cloned LDV-v (**C**) or cloned LDV-P and LDV-vx (**E**) and differential RT–PCR analysis of samples of plasma of the mice (**B, D** and **F**). The FVB mice were provided by the transgenic facility of the University of Minnesota. Groups of 2 (**A** and **E**) or 4 (**C**) mice were injected with about 10^6 ID_{50} of LDV (T0). The mice were bled at intervals and their pooled plasma or individual plasma samples were analyzed for infections LDV by end point dilution titration in FVB mice (Plagemann *et al*, 1963; Chen and Pagemann, 1997) and by differential RT–PCR using primer J1008/ in combination with /J1004, /J1003 and /J1007 in **B** and **D** or in **F** primer A1509/ in combination with /J1020 and /J1021 (LDV-P and LDV-vx specific, respectively) or J1022 and/J1023 (LDV-P and LDV-vx specific, respectively). In **C/D** two mice were superinfected with LDV-P at 35 days p.i. and their plasma also analyzed (T 36) as already described.

phages (Plagemann, 1996). Differential RT–PCR analyses showed that initially all three quasispecies replicated similarly, that is the T1 PCR products were about the same as those of the inoculated LDV-VIR9 (c.f. Figure 4B and 3, lanes 7–9). Thereafter, however, only LDV-vP and LDV-vx persisted, whereas LDV-v had disappeared by 8 days p.i. (Figure 4B).

From these studies, it is concluded that the loss of LDV-v during persistent infection with LDV-VIR9 is rapid and independent of the mouse strain. Since the loss of LDV-v occurs regardless of the permissiveness of the anterior horn neurons, it reflects an advantage of LDV-vP and LDV-vx over LDV-v in productively infecting newly generated macrophages.

When FVB mice were infected with cloned LDV-P or cloned LDV-vx, the time courses of viremia were similar to that illustrated for LDV-VIR (Figure 4E) indicating that both possess the ability to establish high viremic persistent infections. In contrast, the time course of viremia for the cloned LDV-v was very different. As shown in Figure 4C, the 1-day plasma titer was comparable to that observed after infection with LDV-VIR9 and other LDVs, confirming the ability of LDV-v to efficiently establish an acute infection. However, the subsequent decrease in viremia was much more precipitous and drastic than after infection with LDV-VIR9 or cloned LDV-P or LDV-vx (c.f. Figure 4A/E and C). In fact, at 35 days p.i. the plasma LDV titer was so low that the virus was hardly detectable by our standard infectivity assay and the genomes hardly detectable by RT–PCR (Figure 4D; 4 μl of plasma with $10^{3.5}$ ID_{50}/ml used for RNA extraction contained just about 70 ID_{50}). When a subgroup of the mice was superinfected with LDV-P at 35 days p.i. with LDV-v, the plasma LDV titer rose by 3 $\log_{10}$ ID_{50} within the next day and persisted at a level comparable to that observed in mice infected with LDV-VIR9 (c.f. Figure 4A and C). Only LDV-v was detected in the plasma of the LDV-v infected mice, except after superinfection with LDV-P, when LDV-P became the predominant replicating LDV (Figure 4D). The results resembled those observed with LDV-C (Chen *et al*, 1997) and showed that both neuropathogenic LDVs lack the ability to establish a high viremic persistent infection. Macrophages that were permissive for LDV-P, but not infected by LDV-v, accumulated in the LDV-v-infected mice and became productively infected after superinfection with LDV-P (Figure 4C and data not shown).

We have found that all LDVs originally isolated from mice carrying various transplantable tumors, such as LDV-RIL, LDV-NOT, LDV-ROW and LDV-SCH that are composed, just as LDV-PLA, of LDV-P and LDV-vx like viruses (Chen and Plagemann, 1997; Figure 3 lanes 25–27 and 34–36) failed to cause paralytic disease in groups of three 6-month old C58/M mice that were immunosuppressed by injection of cyclophosphamide at −1, 7, 14, 21 and 28 days p.i., whereas 100% of companion mice injected with LDV-VIR7 became paralyzed (data not shown). On the other hand, they all established viremic persistent infections comparable to that illustrated for LDV-VIR, LDV-P and LDV-vx in Figure 4A and E). Non-neuropathogenicity and ability to establish high viremic persistent infections have also been demonstrated for LDV-a (which differs slightly from LDV-P and LDV-vx; see Figure 1) and five LDVs isolated from wild house mice (in preparation) and the two properties thus always coincided. Conversely, the only two identified neuropathogenic quasispecies LDV-C and LDV-v both possess an impaired ability to establish a viremic persistent infection. We therefore conclude that there is a cosegregation of neuropathogenicity and impaired capability for high viremic persistent infection.

LDVs that are neuropathogenic and unable to establish a high viremic persistent infection lack two of the three N-glycosylation sites present in the VP-3P ectodomains of all LDVs that are non-neuropathogenic and able to establish a high viremic persistent infection

This observation holds for the VP-3P ectodomains of all LDV quasispecies we have analyzed (Figure 1) including the LDVs isolated from wild house mice (in preparation). These sites are the place of attachment of large polylactosaminoglycans (Faaberg and Plagemann, 1995). Except for the lack of the two N-glycosylation sites, no amino acid differences in VP-3P correlated with neuropathogenicity. Most of the consistent amino acid differences between VP-3P of all neuropathogenic and all non-neuropathogenic LDVs were located in the signal peptide that represents the most variable segment of VP-3P (Figure 1) but is removed during membrane-associated synthesis (Faaberg and Plagemann, 1995) and not present in the virion. Only three additional amino acids in VP-3P were unique to the two neuropathogenic LDVs and they were located in the putative transmembrane segments (V73, K80, V108) and thus are unlikely to play a significant role in any vital viral function. It therefore seems that the number of N-glycosylation sites on the ectodomain is likely the determinant for neuropathogenicity and ability to establish a high viremic persistent infection.

There is no identifiable correlation in the ORF 2 proteins between neuropathogenic and non-neuropathogenic LDVs

The LDV envelope probably possesses a very minor glycoprotein, VP-3M (encoded by ORF 2; Faaberg

and Plagemann, 1995). Its function is unknown, but it could potentially be involved in LDV neuropathogenicity and persistence. This protein is a more conventional membrane glycoprotein with a single C-terminal transmembrane segment and a large ectodomain that possesses three potential N-glycosylation sites (see Figure 5A). Especially intriguing was the finding that the ORF 2 proteins of LDV-C and LDV-P seemed to contain a 10-amino acid segment (amino acids 120–129, Figure 5B) that differed completely. These observations led us to extend our sequence comparisons to the ORF 2 proteins of the two neuropathogenic and three non-neuropathogenic LDV quasispecies. We noted that the 10-amino acid divergence between LDV-C and LDV-P was due to an apparent compensating single nucleotide deletion/insertion (see *, Figure 5B). Such viable frame-shift mutations seem to be rare in biology, and, in fact, we were unable to confirm any of six similar apparent frame-shift mutations in ORF 1a of LDV-C (Palmer *et al*, 1995). We therefore re-sequenced the 5′ terminal 511 nucleotides of LDV-C ORF 2 using clones generated via RT–PCR and TA cloning (Chen *et al*, 1997). Only a total of two nucleotide differences were observed among five independent LDV-C clones that were sequenced. The consensus sequence (designated LDV-C-PCR) exhibited high nucleotide identity with the previously reported LDV-C ORF 2 sequence, except for the segment comprising nucleotides 11470–11504 which encode the divergent amino acids (Figure 5B). The apparent nucleotide deletion/insertion was not observed in LDV-C-PCR, indicating that the 10 amino acid divergence in the ORF 2 protein was due to errors in the original LDV-C sequence (Figure 5B). The segment was identical to that of the LDV-P protein, in spite of some differences in the coding nucleotide sequences (Figure 5A).

A comparison of the ORF 2 proteins of the two neuropathogenic and three non-neuropathogenic LDV quasispecies revealed no amino acid differences that seem related to neuropathogenicity (Figure 5A). The greatest amino acid differences between the ORF 2 proteins of the LDVs were observed in the signal peptides (Figure 5A), which are removed during membrane-associated synthesis (Faaberg and Plagemann, 1995). Other amino acid differences between the ORF 2 proteins of the quasispecies seem random. Most convincing was the finding that the ORF 2 protein of the neuropathogenic LDV-v was close to identical to that of the non-neuropathogenic LDV-vx (99.1% amino acid identity) and differed significantly from that of the other neuropathogenic LDV-C (81.9% amino acid identity), suggesting that LDV-v is indeed a recombinant of LDV-vx and LDV-C with a 5′ crossover occurring 3′ to ORF 2 and a 3′ crossover occurring in ORF 5. These results further support the conclusion that the number of N-glycosylation sites on the ectodomain of VP-3P is the determinant

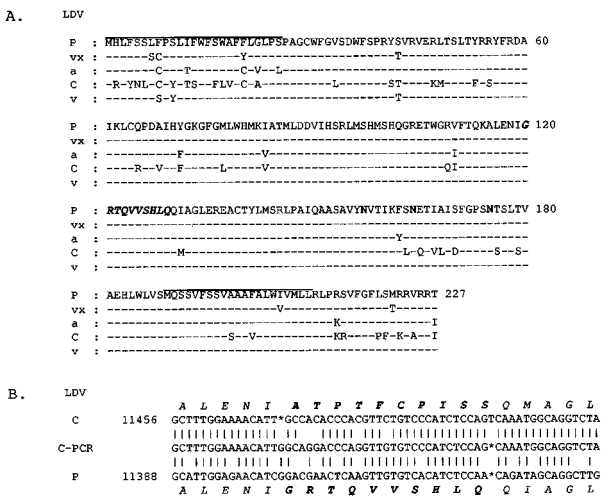

Figure 5 Comparison of the amino acid sequences of the ORF 2 proteins of LDV-P, LDV-vx, LDV-a, LDV-C (corrected according to LDV-C-PCR; the corrected segment corresponds to that shown in boldface italics) and LDV-v (**A**) and comparison of an ORF 2 segment of the original LDV-C nucleotide sequence and its encoded amino acids with those of the LDV-P genome and of a RT–PCR product of this segment of LDV-C (LDV-C-PCR; **B**). The LDV-vx, LDV-a, LDV-v and LDV-C-PCR sequences were generated by RT of genomic RNAs using oligo (dT) and random hexanucleotides as primers. The first strand products were amplified using as primers sense oligonucleotide A1509/ and antisense oligonucleotide /A1510 that represent segments upstream and downstream of ORF-2, respectively (Chen *et al*, 1997). The PCR products were TA cloned and sequenced as described in the legend to Figure 1. In **B**, stars denote apparent gaps in the nucleotide sequences and the resulting diverging amino acids in the predicted proteins are shown in boldface letters. The LDV-C and LDV-P nucleotide sequences are from Godeny *et al* (1993) and Palmer *et al* (1995), respectively.

for LDV neuropathogenicity and capability for high viremic persistent infection.

In summary, we have characterized another neuropathogenic LDV isolate (LDV-VIR) and found that it consisted of at least three different quasispecies (LDV-v, LDV-vP and LDV-vx), only one (LDV-v) of which was found to be neuropathogenic. Using differential RT–PCR assays, we were able to differentiate and biologically clone each of these quasispecies through repeated end point dilutions and to follow their fate in mixed and solo infections. We have found that all non-neuropathogenic LDVs (including LDV-P, LDV-vP, LDV-vx, LDV-a and five LDV isolates from wild mice) are capable of high viremic persistence, whereas the neuropathogenic LDV-v and LDV-C are not, even though they become selectively amplified in the spinal cords of paralyzed mice due to their unique ability to productively infect anterior horn neurons in immunosuppressed C58 and AKR mice. It is quite apparent from our results that there is an inverse relationship between neuropathogenicity of an LDV and its ability to establish a high viremic persistent

infection although only two neuropathogenic LDV quasispecies are available for study. However, the scarcity of neuropathogenic LDV quasispecies in itself supports our conclusion that neuropathogenic LDVs are unable to persist. If neuropathogenic LDVs were able to persist, we would probably have found more neuropathogenic LDV isolates, especially from ADPM-resistant mice since such LDVs would not have been selected against by the paralytic disease. However, LDV-C and LDV-v remain the only neuropathogenic quasispecies identified to date. Even these quasispecies are always associated with non-neuropathogenic ones in natural isolates and have been isolated only after many years of repeated viral passages in immunosupressed C58 mice (Nawrocki *et al*, 1980). Therefore, they are almost certainly mutants of non-neuropathogenic LDVs that have adapted to productively infecting the anterior horn neurons of the C58 mice under immunosuppression. The emergence of neuropathogenic LDVs during repeated passages in immunosuppressed mice provides an interesting example of pathogenic variants arising from non-pathogenic viruses in immunocompromised hosts.

Why neuropathogenic LDVs possess an impaired capability for high viremic persistence has been unclear. In an accompanying report (Chen *et al*, 1998), we provide an explanation to this question by demonstrating that the impaired capability of neuropathogenic LDVs to persist is mainly due to selective neutralization by antibody. Thus in any immunocompetent mouse regardless of strain, the neuropathogenic LDVs persist at a very low, sometimes hardly detectable level in solo infections and are rapidly outcompeted in mixed infections by non-neuropathogenic LDVs which are highly resistant to antibody neutralization. The selective sensitivity of neuropathogenic LDVs to antibody neutralization also explains why immunosuppression is a requirement for these viruses to cause paralytic disease in C58 and AKR mice.

The inverse relationship between neuropathogenicity and ability to establish a viremic persistent infection correlated with the number of N-linked polylactosaminoglycan chains associated with the very short ectodomain of the primary viral envelope glycoprotein VP-3P which seems involved in host-receptor recognition (Faaberg and Plagemann, 1995; Faaberg *et al*, 1995) and carries the LDV neutralization epitope (Li *et al*, 1998). The number of polylactosaminoglycan chains associated with the VP-3P ectodomain is the only consistent difference we have observed between neuropathogenic and non-neuropathogenic LDVs. No other amino acid differences in VP-3P and VP-3M between neuropathogenic and non-neuropathogenic LDVs correlated with their differences in phenotypic properties. The same applies to the non-glycosylated envelope protein (M/VP-2) and the nucleocapsid protein (N/VP-1) since those of LDV-C and LDV-P are highly homologous (95 and 98% amino acid identity, respectively, compared with 81 and 87% for the ORF 2 and ORF 5 proteins, respectively). We therefore postulate that LDV neuropathogenicity and ability to establish a high viremic persistent infection are determined by the same molecular feature on the ectodomain of VP-3P. Whereas the presence of three large polylactosaminoglycan chains in non-neuropathogenic LDVs limits their tropism to the primary permissive cells in a subpopulation of macrophages and renders them very resistant to antibody neutralization hence able to persist, the loss of the N-terminal 2 chains in the neuropathogenic LDVs expand their tropism to the anterior horn neurons of ADPM-susceptible mice but at the same time renders them much more susceptible to antibody neutralization and therefore unable to establish a high viremic persistent infection. Further experiments are in progress to confirm our hypothesis.

The finding that LDV-v is likely a recombinant between LDV-C and LDV-vx suggests that high frequency recombination between different quasispecies during mixed infections also occurs in LDV just as in coronaviruses (Lai and Cavanagh, 1997). Because LDV is an arterivirus that has a strategy of genome replication and subgenomic transcriptions very similar to that of coronaviruses, a similar mechanism may be involved. More studies are needed to further assess the frequency of recombination in arteriviruses.

Acknowledgements

We thank Brent Langley for excellent secretarial assistance. Z.C., K.L., and G.W.A. were supported by USPHS training grant CA 09138.

References

Anderson GW, Even C, Rowland RRR, Palmer GA, Harty JT, Plagemann PGW (1995a). C58 and AKR mice of all ages develop motor neuron disease after lactate dehydrogenase-elevating virus infection but only if antiviral immune responses are blocked by chemical or genetic means or as a result of old age. *J Neurovirol* **1:** 244–252.

Anderson GW, Palmer GA, Rowland RRR, Even C, Plagemann PGW (1995b). Infection of central nervous system cells by ecotropic murine leukemia virus in C58 and AKR mice and *in utero* infected CE/J mice predisposes mice to paralytic infection by lactate dehydrogenase-elevating virus. *J Virol* **69:** 308–319.

Anderson GW, Palmer GA, Rowland RRR, Even C, Plagemann PGW (1995c). Lactate dehydrogenase-elevating virus entry into central nervous system, and replication in anterior horn neurons. *J Gen Virol* **76:** 581–592.

Brinton-Darnell M, Plagemann PGW (1975). Structural and chemical-physical characteristics of lactate dehydrogenase-elevating virus and its RNA. *J Virol* **16:** 420–433.

Chen Z, Plagemann PGW (1997). Detection of lactate dehydrogenase-elevating virus in transplantable mouse tumors by biological assay and RT–PCR assays and its removal from the tumor cells. *J Virol Meth* **65:** 227–236.

Chen Z, Li K, Plagemann PGW (1998). Selective antibody neutralization prevents neuropathogenic lactate dehydrogenase-elevating virus from causing paralytic disease in immunocompetent mice. *J Neurovirol.* Submitted.

Chen Z, Rowland RRR, Anderson GW, Plagemann PGW (1997). Coexistence in lactate dehydrogenase-elevating virus pools of variants that differ in neuropathogenicity and ability to establish a persistent infection. *J Virol* **71:** 2913–2920.

Faaberg KS, Plagemann PGW (1995). The envelope proteins of lactate dehydrogenase-elevating virus and their membrane topography. *Virology* **212:** 512–525.

Faaberg KS, Even C, Palmer GA, Plagemann PGW (1995). Disulfide bonds between two envelope proteins of lactate dehydrogenase-elevating virus are essential for viral infectivity. *J Virol* **69:** 613–617.

Godeny EK, Chen L, Kumar SN, Methven SL, Koonin EV, Brinton MA (1993). Complete genomic sequence and phylogenetic analysis of the lactate dehydrogenase-elevating virus (LDV). *Virology* **194:** 585–596.

Lawrence CB, Goldman DA (1988). Definition and identification of homology domains. *CABIOS* **4:** 25–33.

Lai MMC, Cavanagh D (1997). The molecular biology of coronaviruses. *Adv Virus Res* **48:** 1–100.

Li K, Chen Z, Plagemann PGW (1998). The neutralization epitope of lactate dehydrogenase-elevating virus is located on the short ectodomain of the primary envelope glycoprotein. *Virology* **242:** 239–245.

Martinez D, Brinton MD, Tachovsky TG, Phelps AH (1980). Identification of lactate dehydrogenase-elevating virus as etiological agent of genetically restricted, age-dependent polioencephalomyelitis of mice. *Infect Immun* **27:** 979–987.

Murphy WH, Mazur JJ, Fulton SA (1987). Animal model for motor neuron disease. In: (eds) WMH Behan, PO Behan, JA Aarli, *Clinical Neuroimmunology*, pp 135–155. Oxford, England: Blackwell Scientific Publications.

Murphy WH, Nawrocki JF, Pease LR (1983). Age-dependent paralytic viral infection in C58 mice: Possible implication in human neurologic disease. *Prog Brain Res* **59:** 291–303.

Nawrocki JF, Pease LR, Murphy WH (1980). Etiologic role of lactate dehydrogenase-elevating virus infection in an age-dependent neuropathic disease in C58 mice. *Virology* **103:** 259–264.

Palmer GA, Kuo L, Chen Z, Faaberg KS, Plagemann PGW (1995). Sequence of genome of lactate dehydrogenase-elevating virus. Heterogeneity between strains P and C. *Virology* **209:** 637–642.

Plagemann PGW (1996). Lactate dehydrogenase-elevating virus and related viruses. In: (eds) BN Fields, DM Knipe, PM Howley *Virology, 3rd edition*, pp 1105–1120. New York: Raven Press.

Plagemann PGW, Gregory KF, Swim HE, Chan KKW (1963). Plasma lactic dehydrogenase elevating agent of mice: Distribution in tissues and effect on lactic dehydrogenase isozyme patterns. *Can J Microbiol* **9:** 75–86.

Plagemann PGW, Moennig V (1992). Lactate dehydrogenase-elevating virus, equine arteritis virus and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. *Adv Virus Res* **41:** 99–192.

Plagemann PGW, Rowland RRR, Even C, Faaberg KS (1995). Lactate dehydrogenase-elevating virus–an ideal persistent virus? *Semin Immunopathol* **17:** 167–186.

Riley V, Lilly F, Huerto E, Bardell D (1960). Transmissible agent associated wih 26 types of experimental neoplasms. *Science* **132:** 545–547.

Stroop WG, Brinton MG (1983). Mouse-strain-specific central nervous system lesions associated with lactate dehydrogenase-elevating virus infection. *Lab Invest* **49:** 344–345.

van den Broek MT, Spöric R, Even C, Plagemann PGW, Hänseler E, Hengartner H, Zinkernagel RM (1997). Lactate dehydrogenase-elevating virus (LDV). Lifelong coexistence of virus and LDV-specific immunity. *J Immunol* **159:** 1585–1588.