Journal of NeuroVirology (1999) 5, 200–208

http://www.jneurovirology.com

Short Communication

Selective antibody neutralization prevents neuropathogenic lactate dehydrogenase-elevating virus from causing paralytic disease in immunocompetent mice

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

[1]*University of Minnesota, Department of Microbiology, Box 196 FUMC, Minneapolis, Minnesota 55455;* [2]*Department of Biology and Microbiology, South Dakota State University, Brookings, South Dakota 57007, USA*

Neuropathogenic lactate dehydrogenase-elevating viruses (LDV) cytocidally infect anterior horn neurons in C58 and AKR mice via interaction with endogenous murine retroviruses to cause a paralytic disease, age-dependent poliomyelitis (ADPM). The induction of ADPM requires a suppressed host immune system as a result of old age, genetic defects (such as nude mice) or any immunosuppressive treatment. Previous results have shown that the infection of anterior horn neurons by neuropathogenic LDV isolates and the subsequent development of ADPM are prevented by anti-LDV antibodies either induced actively during infection or when passively administered. However, the mechanism of protection was unclear since both neutralizing and non-neutralizing polyclonal antibodies seemed protective, whereas only neutralizing monoclonal antibodies were protective. Furthermore, the protection of motor neurons from infection occurred in the absence of any apparent effect on LDV replication in a subpopulation of macrophages known to be the primary permissive host cells. These paradoxes have now been resolved. We have recently reported that the neuropathogenic LDV isolates contain both neuropathogenic and non-neuropathogenic quasispecies that differ in their ability to establish a high viremia persistent infection. Using biological clones of both neuropathogenic and non-neuropathogenic quasispecies, we now demonstrate that both replicate in the same subpopulation of permissive macrophages, but that the neuropathogenic quasispecies are about 100 times more susceptible to *in vitro* antibody neutralization than the non-neuropathogenic ones, and that antibodies that neutralize the neuropathogenic but not the non-neuropathogenic quasispecies develop as soon as 7 days after infection with neuropathogenic LDVs and selectively suppress the replication of the neuropathogenic LDVs *in vivo* in FVB, BALB/c, C57 BL/6 and C58 mice. The previously observed lack of neutralizing effect of early polyclonal anti-LDV antibodies and the apparent ineffective antibody control of LDV replication in macrophages were due to outgrowth of the non-neuropathogenic quasispecies that are also present in the neuropathogenic LDV inoculum and are highly resistant to antibody neutralization. Using cloned neuropathogenic LDV quasispecies, we demonstrate a clear relationship in the development of neutralizing antibodies, replication suppression of the neuropathogenic LDVs and the prevention of ADPM in C58 mice. Our results therefore establish an inseparable relationship between the neuron-protective effect of an antibody and its neutralization of the neuropathogenic LDV quasispecies and explain why neuropathogenic LDVs cause paralytic disease only in immunosuppressed mice.

Keywords: neutralizing antibody; LDV; paralytic disease

Correspondence: PGW Plagemann
Received 6 March 1998; revised 17 August 1998; accepted 27 August 1998

Infection of mice of certain strains, such as C58 and AKR, with neuropathogenic quasispecies of lactate dehydrogenase-elevating virus (LDV) can result in a fatal paralytic disease (age-dependent poliomyelitis or ADPM) through interaction with murine endogenous retroviruses (Murphy *et al*, 1983, 1987; Anderson *et al*, 1995b; Chen *et al*, 1998). However, the paralytic disease occurs only if the mice are immunosuppressed as a result of old age (thus the name ADPM), a genetic defect (e.g. nude mice) or artificial treatment such as X-irradiation and cyclophosphamide injection (Murphy *et al*, 1983, 1987; Anderson *et al*, 1995a). In these mice, the neuropathogenic LDVs not only replicate in the primary permissive cells of a subpopulation of macrophages (Chen *et al*, 1997, 1998) but also spread through the central nervous system (CNS) during the first day post-infection (p.i.), most likely via extensive replication in leptomeningeal cells, and LDV replication in and destruction of anterior horn neurons become apparent at about 10–12 days p.i. (Anderson *et al*, 1995c). The delay of the viral replication in anterior horn neurons is not understood but may reflect a need for the decay of some cytokine(s) (e.g. interferons) generated as a result of the massive LDV replication in macrophages during the first day p.i. (Plagemann and Moennig, 1992; Plagemann, 1996) that specifically suppresses LDV replication in anterior horn neurons (Anderson *et al*, 1995c; Cafruny *et al*, 1997). In immunocompetent C58 and AKR mice, on the other hand, little if any infection of anterior horn neurons was detectable and no paralytic disease developed in spite of comparable vigorous viral replication in macrophages (Brinton *et al*, 1986; Anderson *et al*, 1995a,b). Similarly, passive immunization with anti-LDV antibodies of immunosuppressed C58 mice either at the time of infection or repeatedly after infection prevented neuropathogenic LDV from infecting anterior horn neurons without apparently affecting viral replication in macrophages (Murphy *et al*, 1987; Harty *et al*, 1987; Harty and Plagemann, 1990). It was unclear why infection of anterior horn neurons by neuropathogenic LDVs became suppressed by anti-LDV antibodies, either generated actively or administered passively, without apparently affecting the viral replication in macrophages. Also unexplained were findings concerning the nature of the neuron-protective antibodies. For example, plasma obtained from infected mice as early as 16 days p.i., that failed to neutralize LDV *in vitro*, nevertheless protected C58 mice from ADPM, suggesting that both neutralizing and non-neutralizing anti-LDV antibodies protect anterior horn neurons from infection by neuropathogenic LDV (Harty *et al*, 1987). In contrast, only neutralizing monoclonal antibodies (MAb) to the primary envelope glycoprotein, VP-3P, possessed neuron-protective activity; non-neutralizing MAbs were ineffective (Harty and Plagemann, 1990). In this report, we provide mechanistic explanations for these apparent paradoxes based on our recently published data and our new experimental results.

Part of the explanation was provided by our discovery that populations of the original neuropathogenic LDV isolates consist of both neuropathogenic LDV quasispecies (LDV-C or LDV-v) and non-neuropathogenic LDV quasispecies (LDV-P and LDV-vx), whereas original non-neuropathogenic LDV isolates, such as LDV-PLA (Brinton and Plagemann, 1975), contain only LDV-P and LDV-vx (Chen *et al*, 1997, 1998; Chen and Plagemann, 1997). We have cloned all four of these LDV quasispecies by repeated end-point dilutions in mice, which has made possible the characterization of their individual molecular and phenotypic properties (Chen *et al*, 1997, 1998). The neuropathogenic LDVs become selectively amplified in the spinal cords of paralyzed C58 mice (Chen *et al*, 1998); however, unlike the non-neuropathogenic ones, they are unable to establish a high viremic persistent infection and become rapidly outcompeted by the non-neuropathogenic ones in mixed infections (Chen *et al*, 1997, 1998).

The present study demonstrates that this difference between neuropathogenic and non-neuropathogenic LDVs in ability to establish a viremic persistent infection is not due to replication in different subpopulations of permissive macrophages, as could be suggested by the differences in their ability to productively infect anterior horn neurons in C58/AKR mice. Instead, the inability of the neuropathogenic LDVs for high viremic persistence has been shown to be due to selective antibody neutralization. Thus, the infection by neuropathogenic LDVs of both anterior horn neurons and macrophages, rather than only the anterior horn neurons, are selectively controlled by the humoral immune response. The apparent lack of effect of antibodies on their replication in macrophages observed earlier was due to outgrowth of the non-neuropathogenic LDVs that were present in the original neuropathogenic LDV isolates and are highly resistant to antibody neutralization.

The question of whether neuropathogenic and non-neuropathogenic LDVs productively infect the same or different subpopulations of macrophages was investigated in two ways. In one approach peritoneal macrophages were harvested from mice persistently infected with cloned neuropathogenic LDV-C or cloned non-neuropathogenic LDV-P and cultured *in vitro*. The cultures were examined for LDV infected cells by indirect fluorescent antibody (IFA) staining (Cafruny *et al*, 1986) before and after superinfection by LDV-C or LDV-P (Figure 1). No LDV infected macrophages could be detected in cultures of macrophages from mice persistently infected by either LDV-P or LDV-C (Figure 1A and B, respectively). Consistent with results from earlier

studies (Stueckemann *et al*, 1982; Plagemann and Moennig, 1992), the macrophages from LDV-P persistently infected mice were resistant to superinfection by LDV-P (Figure 1C), because in LDV-P infected mice new permissive macrophages become cytocidally infected as soon as they are generated. However, the macrophages from LDV-P infected mice were also resistant to superinfection by LDV-C (Figure 1E) indicating that the mice did not possess an additional pool of LDV-C permissive macrophages. In contrast, about 5% of the macrophages harvested from the mice persistently infected with LDV-C could be infected *in vitro* by either LDV-P or LDV-C (Figure 1D and F) indicating that these mice possessed a pool of macrophages that could be infected by either LDV-P or LDV-C.

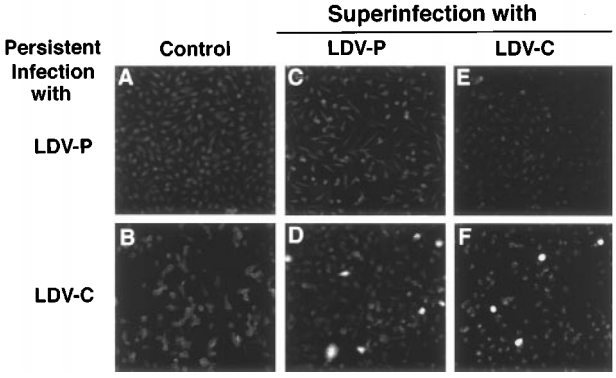

Figure 1 *In vitro* infection of peritoneal macrophages isolated from FVB mice at 220 days p.i. with cloned LDV-P (**A**, **C**, **E**) or at 178 days p.i. with cloned LDV-C (**B**, **D**, **F**). After 1 day in culture, replicate coverslip cultures were directly analyzed for LDV-infected cells by IFA staining (**A**, **B**) or 6 h after superinfection with 10^3 ID_{50} of LDV-P (**C**, **D**) or LDV-C (**E**, **F**)/cell. Peritoneal macrophages were harvested and cultured on coverslips in RPMI supplemented to 10% (v/v) with fetal bovine serum and L-cell conditioned medium (containing colony stimulating factor I) and the cultures were analyzed by IFA staining using plasma from 3 month LDV-PLA infected mice as described previously (Cafruny *et al*, 1986; Onyekaba *et al*, 1989).

The finding that the macrophages from LDV-C infected mice could be infected *in vitro* by LDV-C to the same extent as by LDV-P not only clearly indicates that both LDVs replicate in the same macrophage population but also that the reduced replication of the neuropathogenic LDV-C/v during the persistent phase of infection is due, primarily at least, to selective suppression by a factor that is lost upon *in vitro* cultivation of the macrophages. This explains the accumulation in LDV-C/v infected mice of permissive macrophages that can be infected *in vivo* by LDV-P but not LDV-C/v (Chen *et al*, 1997, 1998; see Figure 2B).

Additional proof for these conclusions is provided by an experiment comparing plasma LDH levels in LDV-PLA (consisting of non-neuropathogenic LDV-P and LDV-vx; Chen *et al*, 1998) and cloned LDV-C infected mice. Both LDVs caused the 5–10-fold increase in plasma LDH characteristic for LDV infections (Figure 2). As expected (Rowson and Mahy, 1975; Plagemann, 1996; Chen and Plagemann, 1997), continued high viremic persistence by LDV-PLA was associated with continued plasma LDH elevation (Figure 2A). In contrast, in LDV-C infected mice the plasma LDH level slowly returned to about normal concomitant with the decrease in viremia, but became elevated again after superinfection of the mice by cloned LDV-P (Figure 2B).

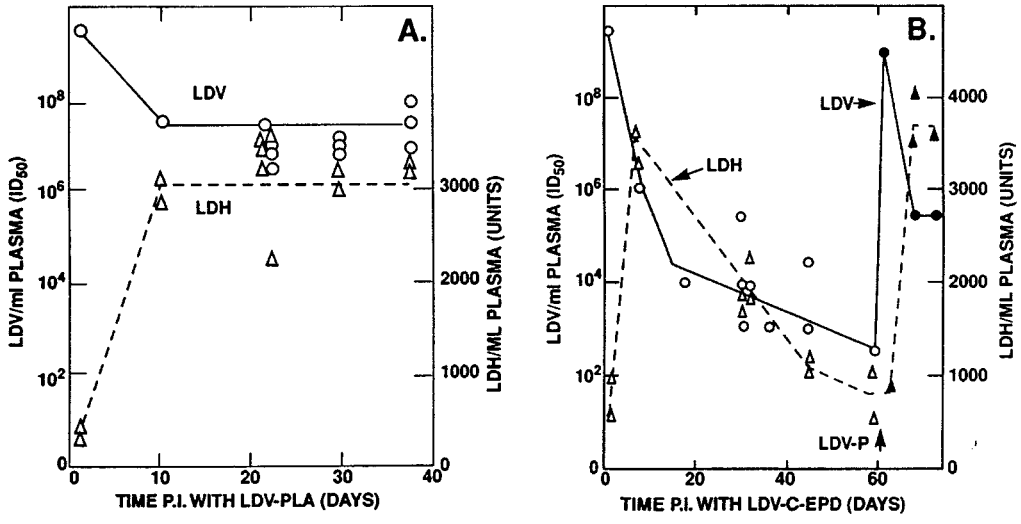

Figure 2 Viremia and LDH activity of plasma of FVB mice as a function of time p.i. with LDV-PLA (**A**) or cloned LDV-C (**B**) and after superinfection of 60-day LDV-C-infected mice with cloned LDV-P. Groups of two infected mice were bled at the indicated times p.i. and their pooled plasma was assayed for infectious LDV (○—○; ●—●) and LDH activity (△—△; ▲—▲). LDH activity was measured by a spectrophotometric method as described previously (see Chen and Plagemann, 1997). Plasma LDV titers were determined by end point dilution assay in mice (Plagemann *et al*, 1993; Chen and Plagemann, 1997).

The correlation between levels of LDV replication and plasma LDH elevation in LDV-PLA (LDV-P) and LDV-C infected mice further supports the conclusion that LDV-P and LDV-C replicate in the same macrophage subpopulation. This conclusion is also indicated by the isolation of genetic recombinants between neuropathogenic LDV-C and non-neuropathogenic LDV-P or LDV-vx (in preparation) which can only occur when the two types of virus infect the same cell.

The preceding results indicated that the replication of neuropathogenic LDVs becomes selectively suppressed in infected mice by some removable factor. The following experiments indicate that this factor is antibodies that selectively neutralize the infectivity of the neuropathogenic LDVs.

One line of evidence is provided by the finding that the neuropathogenic LDVs are about 100 times more susceptible to *in vitro* neutralization by anti-LDV antibodies than the non-neuropathogenic LDVs and that antibodies that neutralize the neuropathogenic but not the non-neuropathogenic LDVs can be detected in mice infected with the neuropathogenic LDVs as early as 7 days p.i. The *in vitro* neutralization assay was conducted as described previously (Cafruny *et al*, 1986; Plagemann *et al*, 1992). In brief, samples of 10 μl of different LDV stocks (containing 10^7-10^8 50% infectious dose, ID_{50}) were mixed with 20 μl of normal mouse plasma (NMP), polyclonal anti-LDV antibodies obtained from infected mice (IMP) or ascites fluid containing an anti-LDV MAb and 50 μl of balanced salt solution. The mixtures were incubated at 35°C for 2 h and then at 4°C for 1–2 h. The residual infectious LDV titer (in $\log_{10}$ ID_{50}) was determined by end-point dilution titration in mice (Plagemann *et al*, 1963; Chen and Plagemann, 1997). The degree of neutralization was defined as the difference in LDV titer between LDV samples incubated with NMP and those incubated with polyclonal antibody-containing IMP or anti-LDV MAbs. In this assay only differences of $\geqslant 0.5$ $\log_{10}$ ID_{50} are considered significant (Plagemann *et al*, 1992). To maximize the accuracy of the comparisons, the neutralization of neuropathogenic and non-neuropathogenic LDVs by various antibody preparations was generally measured together in individual experiments. In each individual experiment we have observed that the neuropathogenic LDV quasispecies (LDV-C and LDV-v) were reproducibly about 100 times more efficiently neutralized by IMP than LDV-PLA (Table 1) and the cloned non-neuropathogenic quasispecies LDV-P (data not shown). Equally importantly, antibodies that neutralized LDV-C and LDV-v were detectable in LDV-C infected mice as early as 7 days p.i. (Table 1). The neutralizing activity in plasma increased rapidly with time, reaching a maximum level by about 3 weeks p.i. At this time, the plasma neutralized LDV-C or LDV-v on the average about 3 $\log_{10}$ ID_{50} which is equivalent to 99.9%. This is in sharp contrast to results with the non-neuropathogenic LDV-PLA (consisting of LDV-P and LDV-vx). Previous studies have shown that the replication of LDV-PLA in mice is impervious to anti-LDV humoral and cellular immune responses (Plagemann *et al*, 1995; Plagemann, 1996) and that LDV-PLA is only poorly neutralized *in vitro* by IMP from long term LDV-PLA infected mice (Cafruny *et al*, 1986; Harty and Plagemann, 1988). Furthermore, LDV-PLA neutralization requires the binding of many antibody molecules per virion (Plagemann *et al*, 1992) and antibodies that neutralize LDV-PLA become detectable only 1–2 months p.i. (Cafruny *et al*, 1986). The

Table 1 *In vitro* neutralization of LDV-PLA[a], LDV-C and LDV-v by polyclonal antibodies to LDV-C or LDV-PLA and by neutralizing MAbs to VP-3P

IMP		*Neutralization[b] (log_{10} ID_{50}) of*		
Days p.i.	*To:*	*LDV-PLA*	*LDV-C*	*LDV-v*
7	LDV-C	0	1.0, 0.5	1.0
10	LDV-C	N.D.[c]	1.0	N.D.
17	LDV-C	N.D.	1.5	2.5
27	LDV-C	0, 0.5	3.0, 4.0	2.0, 3.0
44	LDV-C	0.5	3.0	3.0
58–73	LDV-C or PLA	1.0, 0.5, 2.0	2.0, 2.5, 2.5	2.5, 2.0, 3.0, 3.0, 3.0, 3.0
>100	LDV-PLA	1.0, 1.0, 1.0, 0.5, 1.0	1.5, 3.0, 2.0, 2.5, 2.0, 3.0	4.0, 2.5
MAb 159-12/19		0.5, 1.0	2.0, 3.0	N.D.
MAb 159-18		0, 1.5	3.0	2.0
MAb C3904-H12		1.0	2.0	1.0
MAb B6505-H9		1.0	2.0	N.D.
MAb B6503-E7		0.5	1.5	1.5

[a]LDV-PLA is a mixture of non-neuropathogenic LDV-P and LDV-vx, whereas the neuropathogenic LDV-C and LDV-v are cloned quasispecies that have been freed of the non-neuropathogenic LDVs (see text). [b]*In vitro* neutralization was measured as described previously (Plagemann *et al*, 1992; and in the text). The degree of neutralization was defined as the difference in LDV titer ($\log_{10}$ ID_{50}) between LDV samples incubated with control NMP, and those incubated with polyclonal antibody-containing IMP or anti-LDV MAbs. The neutralizing MAbs were generated in previous studies (Harty and Plagemann, 1988; Coutelier and van Snick, 1988) and are specific for the ectodomain of VP-3P (Li *et al*, 1998). Each value represents an independent analysis. [c]N.D.=not determined.

present data (Table 1) confirm these earlier results (also see Figure 4B). The same conclusions were also indicated for populations of LDV-RIL, LDV-NOT and LDV-ROW (Rowson and Mahy, 1975; and data not shown), which are also composed of LDV-P and LDV-vx quasispecies (Chen and Plagemann, 1997; Chen *et al*, 1998).

The difference in neutralization efficiency between neuropathogenic and non-neuropathogenic LDV quasispecies was also observed with several MAbs to the viral primary envelope glycoprotein, VP-3P (Table 1). The short (about 30 amino acids long) ectodomain of VP-3P seems involved in host receptor interaction (Faaberg *et al*, 1995a; Faaberg and Plagemann, 1995) and carries the LDV neutralization epitope (Li *et al*, 1998).

Figure 3A illustrates the rapidity with which neutralizing antibodies are generated in immunocompetent mice infected with cloned LDV-C and the effectiveness of these antibodies to suppress the replication of the neuropathogenic LDV *in vivo*. In this experiment neutralizing antibodies were measured by their interaction in an indirect ELISA with a synthetic peptide (ORF-P5) containing the neutralization epitope of LDV-C/v (Li *et al*, 1998). The results in Figure 3A demonstrate that LDV-C replication became rapidly suppressed in BALB/c mice cocomitant with the formation of neutralizing antibodies. In fact, there was a good correlation between the time course of the neutralizing antibody response as measured by indirect ELISA (Figure 3A) and by *in vitro* neutralization of LDV-C or LDV-v infectivity (Table 1). Neutralizing antibodies for LDV-C/v became detectable as early as 4 days p.i. and reached a maximum level by 1 month p.i., that is at a time when little or no neutralizing antibodies are detectable in mice infected with the non-neuropathogenic LDV-PLA (Cafruny *et al*, 1986; Plagemann *et al*, 1995; Rowson and Mahy, 1975) or LDV-P (see Figure 4B). In contrast, in BALB/c SCID mice, which failed to generate anti-LDV neutralizing antibodies, no suppression of LDV-C replication was observed since the time course of its replication was basically the same as observed for the non-neuropathogenic LDV-PLA (composed of LDV-P and LDV-vx; Figure 3B). Differential reverse transcription (RT)-polymerase chain reaction (PCR) analysis proved that only the LDV-C genome was present in the SCID mice infected with cloned LDV-C, whereas the genomes of LDV-P and LDV-vx were present in the LDV-PLA infected SCID mice (Figure 3C). In

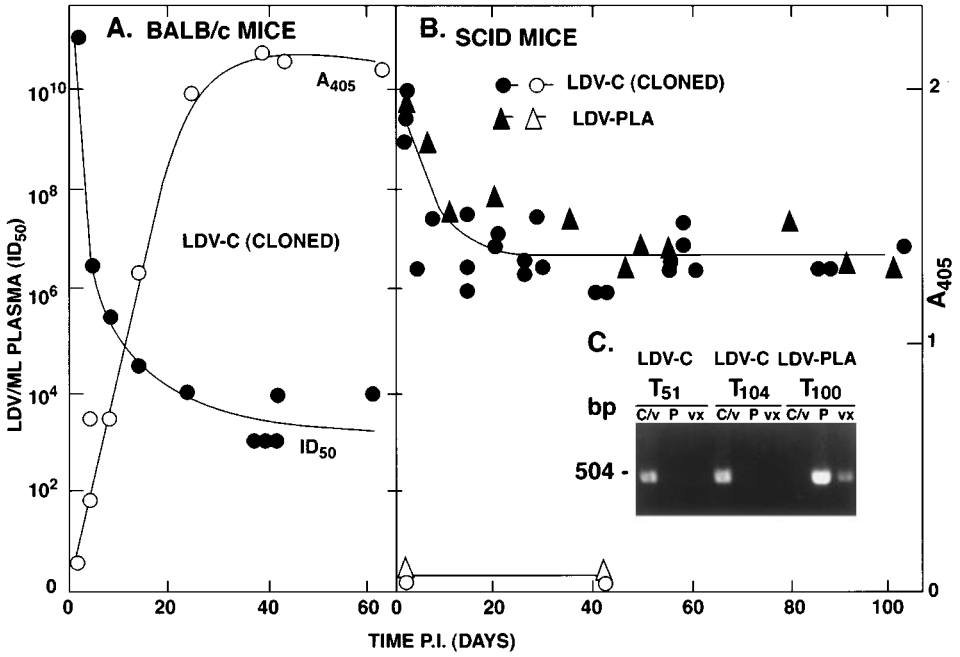

Figure 3 Time courses of viremia and of the formation of neutralizing antibodies in BALB/c mice infected with biologically cloned LDV-C (**A**) and in BALB/c SCID mice infected with cloned LDV-C or LDV-PLA (**B**). Groups of two mice were infected with about 10^6 ID_{50} of the indicated LDVs (Chen *et al*, 1997, 1998). The mice were bled at the indicated times p.i. (T in days) and their pooled plasma was assayed for infectious virus by an end point dilution titration in mice (Plagemann *et al*, 1963; Chen and Plagemann, 1997), for neutralizing antibodies by indirect ELISA using a synthetic peptide (ORF 5-P5) that contains the neutralization epitope of LDV-C/v as described by Li *et al* (1998) and for LDV RNA by differential RT–PCR using in the PCR sense primer J1008/ in combination with antisense primers /J1003, /J1004 and /J1007 (specific for LDV-C/v, LDV-P and LDV-vx, respectively) as described by Chen *et al* (1998). In the indirect ELISA, the second antibody was alkaline phosphatase-conjugated goat anti-mouse IgG. The ELISA plates were developed with SIGMA 104 substrate and the absorbency of the reaction solution was measured at 405 nm (A405). Two fold dilutions of plasma from infected mice from 1 : 50 to 1 : 1600 were analyzed, but only results from the 1 : 200 plasma dilution are reported.

another experiment, we observed that cloned LDV-C and LDV-v established a viremic persistent infection equivalent to that of LDV-P also in B-cell deficient C57BL/6 mice (Figure 4A) whereas in wild type B57BL/6 mice the replication of LDV-C and LDV-v became selectively suppressed concomitant with the formation of neutralizing antibodies (Figure 4B). In contrast, the viremia of cloned LDV-P was comparable in B cell deficient and wild type C57BL/6 mice (c.f. Figure 4A and B). As expected, the generation of neutralizing antibodies was delayed and slow in the LDV-P infected wild type mice as compared to that observed in the LDV-C/v infected mice (Figure 4B). Differential RT–PCR confirmed that the mice were infected with LDV-C/v or LDV-P (Figure 4A and data not shown). Combined the results in Figure 4 indicate that the suppression of replication of the neuropathogenic LDVs is mediated by the humoral immune response since the cellular immune response should be unimpaired in the B-cell deficient mice. This conclusion is further supported by the finding that the passive transfer of neutralizing antibodies transiently suppressed the plasma LDV titer in LDV-C infected SCID mice (Table 2).

Injection of a 13-day LDV-C infected BALB/c SCID mouse with a mixture of ether extracted IMP from LDV-C infected mice and a neutralizing mAb reduced viremia by 2.5 $\log_{10}$ ID_{50} in 5 h and another $\log_{10}$ ID_{50} by the next day (Table 2, the H mouse), whereas injection of a companion 21 day LDV-C infected BALB/c mouse with a mixture of NMP and a non-neutralizing anti-VP-3P mAb had no significant effect on viremia (Table 2, the T mouse). Suppression of viremia in the H mouse correlated with the appearance of a high level of neutralizing antibodies in the circulation, whereas no neutralizing antibodies were detected in the H mouse before passive immunization or in the T mouse injected with the non-neutralizing NMP-mAb mixture (Table 2). Viremia in the H mouse had increased again by 21 days p.i. concomitant with a decrease in the level of neutralizing antibodies in the circulation, but became again suppressed after a second passive immunization at 23 days p.i. (Table 2). Injection of the T mouse with the neutralizing antibody mixture duplicated the suppression of viremia observed in the H mouse after passive immunization at 13 days p.i. (Table 2). In contrast to the LDV-C infected mice, passive immunization of an LDV-P infected SCID mouse had no significant effect on viremia (see legend to Table 2). Similar to the results with the SCID mice we found that passive immunization of immunocompetent FVB mice with neutralizing anti-LDV antibodies re-

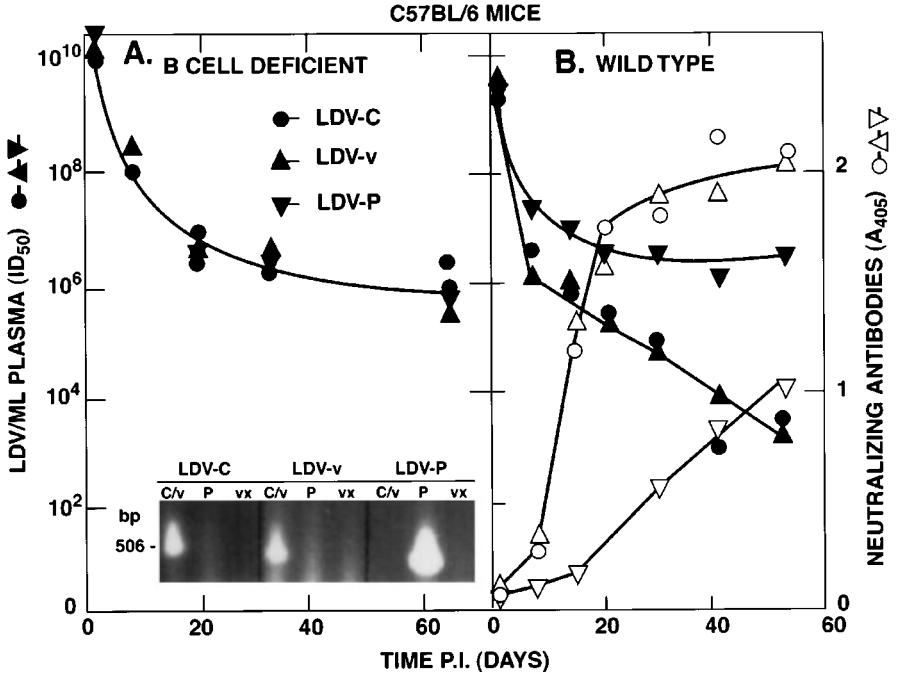

Figure 4 Time courses of viremia and of the formation of neutralizing antibodies in B-cell deficient (**A**) and wild type (**B**) C57BL/6 mice infected with cloned LDV-C, LDV-v or LDV-P. Single mice infected with the indicated LDVs were bled at various times p.i. and their plasma assayed for infectious LDV by mouse titration and by indirect ELISA for neutralizing antibodies (A_{405}) using the LDV-C/v specific synthetic peptide ORF 5-P5. No neutralizing antibodies were detected in the B-cell deficient mice (**A**, data not shown). In **B**, only ELISA values for the 1 : 200 plasma dilutions are shown. In **A** and **B**, plasma samples from the 21-day infected mice were also analyzed by differential RT–PCR (see insert in A) as described in the legend to Figure 3.

Table 2 Suppression of viremia in LDV-C infected BALB/c SCID mice by passive administration of neutralizing antibodies[a]

	H mouse		*T mouse*	
Days p.i.	ID_{50}/ml	A_{405}	ID_{50}/ml	A_{405}
13	$10^{7.0}$	0.07	N.D.[b]	N.D.[b]
13+5 h	$10^{4.5}$	1.96	N.D.[b]	N.D.[b]
14	$10^{3.5}$	1.53	N.D.	N.D.[b]
21	$10^{5.0}$	1.20	$10^{6.5}$	0.05
21+5 h	N.D.	N.D.[b]	$10^{7.0}$	0.05
22	N.D.	N.D.[b]	$10^{7.0}$	0.05
23+5 h	$10^{4.5}$	2.39	$10^{3.0}$	2.36
24	$10^{3.5}$	2.34	$10^{3.0}$	2.00

[a]Two 4-week old BALB/c SCID mice (H and T) were infected with cloned LDV-C. At 13 days p.i. the H mouse was injected with 0.3 ml of ascites fluid containing neutralizing mAb 159-18 (Harty *et al*, 1988) plus 0.3 ml of pooled plasma from 1–2 month LDV-C infected FVB mice (see Figure 2B) that had been treated thrice with ethyl ether in order to inactivate residual LDV (Cafruny *et al*, 1986). The T mouse was injected with 0.6 ml of a similar mixture of NMP and non-neutralizing anti-VP-3P mAb 159-4 (Harty *et al*, 1988) at 21 days p.i. Both mice were bled just before and at the indicated times after passive immunization and the plasma was assayed by mouse titration for infectious LDV and by indirect ELISA for neutralizing antibodies (A_{405}) using the LDV-C/v specific ORF 5-P5 peptide. Only the values for the 1 : 100 plasma dilution are reported. At 23 days p.i. both mice were injected with the neutralizing antibody mixture. Again the mice were bled after antibody injection and the plasma assayed for infectious LDV and neutralizing antibodies. Injections of the neutralizing antibody mixture into a 23 day LDV-P infected SCID mouse had no effect on viremia. The plasma LDV titer was $10^{6.5}$ ID_{50}/ml before and $10^{6.5}$ ID_{50}/ml 1 day after passive immunization. [b]N.D.=not determined.

duced the initial replication of LDV-C in these mice by 90–99%, but did not completely protect them from infection (unpublished data). All these observations document the effectiveness of the neutralizing antibodies in selectivity suppressing the replication of the neuropathogenic LDVs *in vivo* in various strains of mice.

Now that we have demonstrated that the neuropathogenic LDV quasispecies are more susceptible to antibody neutralization than the non-neuropathogenic LDVs, that neutralizing antibodies are very rapidly generated in mice infected with these LDVs, and that these antibodies suppress the replication of the neuropathogenic LDVs *in vivo*, we can explain why during a mixed infection of neuropathogenic and non-neuropathogenic LDV quasispecies, such as with the LDV-VIR isolate (Chen *et al*, 1998) the outcomes of viral replication differ depending on the immune status of the infected C58 and AKR mice. If the mice are immunosuppressed, the replication of both the neuropathognic and the non-neuropathogenic quasispecies are unchecked. The selective replication of the neuropathogenic quasispecies in anterior horn neurons detectable at about 10 days p.i. will lead to neuronal destruction and development of paralytic disease by 2–3 weeks. On the other hand, if the mice are immunocompetent, rapid development of anti-LDV immune responses will result in the effective control of the replication of the neuropathogenic quasispecies but not of that of the non-neuropathogenic ones. Consequently, the infection of anterior horn neurons by the neuropathogenic quasispecies is prevented and no paralytic disease develops. In fact, the infection of macrophages by the neuropathogenic quasispecies in immunocompetent mice is also rapidly suppressed by neutralizing antibodies. This is indicated by the observation that in immunocompetent mice infected with a cloned neuropathogenic LDV macrophages that are intrinsically permissive to infection by the neuropathogenic LDV quasispecies start to accumulate and remain uninfected concomitant with the generation of neutralizing antibodies (Figures 1 and 2B). This antibody neutralization explains the impaired ability of neuropathogenic LDVs to establish a persistent viremic infection. In contrast, the replication of the non-neuropathogenic LDV quasispecies in macrophages in immunocompetent mice is not affected by the immune responses and therefore remains unchecked. Consequently the neuropathogenic quasispecies become rapidly outgrown by the non-neuropathogenic ones. In fact, upon infection of mice with LDV-VIR, the neuropathogenic LDV-v was no longer detectable in plasma by the highly sensitive differential RT–PCR assay 15 days p.i. (Chen *et al*, 1998). These findings explain the earlier observation of an apparently differential protective effect of the immune responses on the anterior horn neurons and the macrophages. Our results also explain why plasma from LDV-VIR infected mice as early as 16 days p.i. possessed neuron-protective activity but failed to neutralize LDV-VIR or LDV-PLA *in vitro* (Harty *et al*, 1987). As shown in Table 1 and Figures 3A and 4B, the plasma from mice infected with cloned LDV-C for 7–10 days already contained antibodies that neutralized the cloned neuropathogenic LDV-C and LDV-v but not the non-neuropathogenic LDV-P/vx. The failure of the plasma from 16 day LDV-VIR infected mice to neutralize LDV-VIR was almost certainly due to the significant levels of coexisting non-neuropathogenic quasispecies (LDV-P and LDV-vx; Chen *et al*, 1998) which are highly resistant to neutralization. Our results therefore indicate an inseparable relationship between the neuron-protective effect of an antibody and its neutralizing effect on neuropathogenic LDV quasispecies.

To obtain further support for our conclusions and to exclude any possible competitive effects of the non-neuropathogenic quasispecies associated with the neuropathogenic LDV isolates in earlier studies, we have compared the replication of the

Table 3 Replication and induction of paralytic disease in immunosuppressed and non-immunosuppressed C58 mice[a]

LDV	CY treatment (days p.i.)	Paralysis Total	Paralysis Days p.i.	LDV/ml Plasma (ID_{50}) Days p.i. 1	LDV/ml Plasma (ID_{50}) Days p.i. 14	Ab (A405) Days p.i. 14	Ab (A405) Days p.i. 21	Ab (A405) Days p.i. 34
Cloned C	−1, 7	2/2	14, 14	$10^{8.5}$	$10^{7.0}$	0.1		
	0	0/2		$10^{8.5}$	$10^{5.5}$	0.4	1.1	2.0
Cloned v	−1, 7	2/2	14, 18	$10^{8.5}$	$10^{6.5}$	0.1		
	0	0/2		$10^{8.5}$	$10^{5.0}$	0.7	1.9	2.7
PLA	−1, 7	0/2		$10^{9.0}$	$10^{7.5}$	0.1	0.1	

[a]Two month old C58 mice were injected with about 10^6 ID_{50} of the indicated neuropathogenic LDVs or LDV-PLA and injected, where indicated, with 25 mg cyclophosphamide (CY)/kg body weight one day before and 7 days p.i. (Anderson *et al*, 1995a). The mice were bled at intervals and their pooled plasma titrated for infections LDV by end point dilution in mice (Plagemann *et al*, 1963) and by indirect ELISA for antibodies (Ab) that bind to a synthetic peptide (ORF 5-P5) containing the LDV neutralization epitope on the ectodomain of VP-3P as described previously (Li *et al*, 1998; see legend to Figure 3). ELISA data are only shown for the 1 : 200 dilution. The mice were monitored for paralytic disease until 34 days p.i.

cloned neuropathogenic quasispecies LDV-C and LDV-v in immunosuppressed and immunocompetent C58 mice with regard to generation of neutralizing antibodies, the level of viremia and the development of ADPM. The results in Table 3 show a clear relationship in the development of neutralizing antibodies, a reduction of plasma virus titer and the prevention of ADPM. Although the 1-day plasma LDV titers were comparable in both groups of mice, by 14 days p.i. the titers of the immunocompetent mice were already 1.5 $\log_{10}$ D_{50} lower than those of the immunosuppressed mice similarly as observed in BALB/c and BALB/c SCID mice, respectively (c.f. Figure 3A and B), or wild type and B cell deficient C57BL/6 mice, respectively (c.f. Figure 4A and B). This coincided with the development of a considerable neutralizing antibody response in the immunocompetent C58 mice and the prevention of ADPM. The immunosuppressed mice, on the other hand, did not generate a neutralizing antibody response, maintained higher viremia and invariably developed ADPM. These results further support our conclusion that the neuronprotective effect of an antibody is related to its neutralizing effect on neuropathogenic LDVs. As expected (Faaberg *et al*, 1995b; Chen *et al*, 1997) LDV-PLA did not induce paralytic disease in the C58 mice even though the mice were immunosuppressed and the plasma titers were comparable to those of LDV-C and LDV-v in the immunosuppressed C58 mice (Table 3).

The molecular basis for the increased sensitivity of neuropathogenic LDVs to antibody neutralization has not been entirely resolved. However, it seems likely to be related to a lower number of polylactosaminoglycan chains that are associated with the neutralization epitope on the ectodomain of VP-3P. This conclusion is suggested by our finding that, although the neutralizing epitopes of the neuropathogenic and non-neuropathogenic LDVs differ slightly (by two or three amino acids), all neutralizing MAbs generated to LDV-P and LDV-vx bind in indirect ELISA with similar efficiency to synthetic peptides carrying the neutralization epitope of either LDV-C/v or LDV-P/vx (Li *et al*, 1998). Furthermore, antibodies generated to LDV-PLA or cloned LDV-P reacted with the LDV-C/v specific synthetic peptide ORF 5-P5 and cross-neutralized LDV-C and LDV-v (Table 1). The only consistent difference we have detected between the VP-3P ectodomains of the two neuropathogenic and the two non-neuropathogenic LDV quasispecies is that the latter LDVs possess three closely spaced polylactosaminoglycan chains on the ectodomain, whereas the former LDVs lack the two N-terminal chains (Chen *et al*, 1998). Our present hypothesis therefore is that the three polylactosaminoglycan chains (especially the N-terminal two) associated with the neutralization epitope of the non-neuropathogenic LDVs protect them from antibody neutralization and that the absence of two of the polylactosaminoglycan chains allows the neuropathogenic LDVs to productively infect anterior horn neurons of C58 and AKR mice but at the same time increases their sensitivity to antibody neutralization thus blocking their ability to cause paralytic disease in immunocompetent C58 and AKR mice and to establish a high viremia persistent infection in any immunocompetent mice regardless of strain. Further work is required to unequivocally prove this hypothesis.

Acknowledgements

We thank Brent Langley for excellent secretarial assistance. ZC and KL were supported by USPHS training grant CA09138.

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 MA, Cavin EI, Weibel J (1986). Detection of viral-specific nucleic acid and intracellular virions in ventral horn neurons of lactate dehydrogenase-elevating virus infected C58 mice. *Microb Pathogen* **1:** 595–602.

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

Cafruny WA, Chan SPK, Harty JT, Yousefi S, Kowalchyk K, McDonald D, Foreman B, Budweg G, Plagemann PGW (1986). Antibody response of mice to lactate dehydrogenase-elevating virus during infection and immunization with inactivated virus. *Virus Res* **5:** 357–375.

Cafruny WA, Haven TR, Lawson SR, Wong GHW, Rowland RRR (1997). Inhibition of virus-induced age-dependent poliomyelitis by interferon-γ. *Antiviral Res* **36:** 1–9.

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, 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.

Chen Z, Li K, Rowland RRR, Anderson GW, Plagemann PGW (1998). Lactate dehydrogenase-elevating virus variants: cosegregation of neuropathogenicity and impaired ability for high viremic persistent infection. *J Neurovirol* In press.

Coutelier JP, van Snick J (1988). Neutralization and sensitization of lactate dehydrogenase-elevating virus with monoclonal antibodies. *J Gen Virol* **69:** 2097–2100.

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 (1995a). Disulfide bonds between two envelope proteins of lactate dehydrogenase-elevating virus are essential for viral infectivity. *J Virol* **69:** 613–617.

Faaberg KS, Palmer GA, Even C, Anderson GW, Plagemann PGW (1995b). Differential glycosylation of the ectodomain of the primary envelope glycoprotein of two strains of lactate dehydrogenase-elevating virus that differ in neuropathogenicity. *Virus Res* **39:** 331–340.

Harty JT, Plagemann PGW (1988). Formalin inactivation of the lactate dehydrogenase-elevating virus reveals a major neutralizing epitope not recognized during natural infection. *J Virol* **62:** 3210–3216.

Harty JT, Plagemann PGW (1990). Monoclonal antibody protection from age-dependent poliomyelitis: Implications regarding the pathogenesis of lactate dehydrogenase-elevating virus. *J Virol* **64:** 6257–6262.

Harty JT, Chan SPK, Contag CH, Plagemann PGW (1987). Protection of C58 mice from lactate dehydrogenase-elevating virus-induced motor neuron disease by non-neutralizing antiviral antibodies without interference with virus replication. *J Neuroimmunol* **15:** 195–206.

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.

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.

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

Onyekaba CO, Harty JT, Plagemann PGW (1989). Extensive cytocidal replication of lactate dehydrogenase-elevating virus in cultured peritoneal macrophages from 1- and 2-week-old mice. *Virus Res* **14:** 327–338.

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

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

Plagemann PGW, Harty JT, Even C (1992). Mode of neutralization of lactate dehydrogenase-elevating virus by polyclonal and monoclonal antibodies. *Arch Virol* **123:** 89–100.

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

Rowson KEK, Mahy BWJ (1975). Lactic dehydrogenase virus. *Virol Monog* **13:** Springer Verlag, Wien.

Stueckemann JA, Ritzi DM, Holth M, Smith MS, Swart WJ, Cafruny WA, Plagemann PGW (1982). Replication of lactate dehydrogenase-elevating virus in macrophages. 1. Evidence for cytocidal replication. *J Gen Virol* **59:** 245–262.