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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

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Age-dependent poliomyelitis is a paralytic disease of C58 and AKR mice caused by cytocidal infection of anterior horn neurons with neuropathogenic strains of lactate dehydrogenase-elevating virus (LDV). The motor neurons are rendered LDV-permissive via an unknown mechanism through the expression of ecotropic murine leukemia virus (MuLV) in central nervous system (CNS) glial cells. Only old mice develop paralytic disease after LDV infection, but mice 5–6 months old or older can be rendered susceptible by suppression of anti-LDV immune responses by a single treatment with cyclophosphamide or X-irradiation before LDV infection. Younger mice appeared to be resistant in spite of this immunosuppresive treatment. The present results confirm that mice as young as 1 month of age possess CNS cells expressing ecotropic MuLV and show that these mice are susceptible to paralytic LDV infection provided their anti-LDV immune responses are blocked for an extended period of time by repeated cyclophosphamide treatments or by a genetic defect. Furthermore, old mice become naturally susceptible to paralytic LDV infection because of an impaired ability to mount a motor neuron protective anti-LDV immune response.

Keywords: lactate dehydrogenase-elevating virus; age dependent poliomyelitis; antibody protection of motor neuron infection

Introduction

Age-dependent poliomyelitis (ADPM) is a unique paralytic disease of mice, which is caused by an interaction between two unrelated, normally harmless viruses, an endogenous N-tropic, ecotropic murine leukemia virus (MuLV) and lactate dehydrogenase-elevating virus (LDV; Murphy *et al*, 1983, 1987; Plagemann and Moennig, 1992; Plagemann, 1995). Paralysis is caused by the cytocidal infection of anterior horn neurons by LDV (Contag *et al*, 1986; Brinton *et al*, 1986; Contag and Plagemann, 1989; Anderson *et al*, 1995b). The disease is induced by infection of neuropathogenic strains of LDV only in mice that possess at least one endogenous replication-competent N-tropic, ecotropic MuLV provirus and the Fv-$1^{n/n}$ genotype, such as mice of strains AKR, C58, PL/J, C3H/Fg and AKXD-16 (Murphy *et al*, 1983, 1987; Contag and Plagemann, 1988; Anderson *et al*, 1995a). In these mice, the ecotropic MuLV is expressed in glial cells throughout the CNS, and anterior horn neurons are susceptible to LDV infection (Anderson *et al*, 1995a). Otherwise LDV replicates in all strains of mice only in a subpopulation of macrophages and does not cause any overt disease (Plagemann and Moennig, 1992).

The causal relationship between the expression of N-tropic, ecotropic MuLV in CNS cells and susceptibility of mice to paralytic LDV infection was directly proven by infection of ADPM-resistant, endogenous ecotropic MuLV provirus-less, Fv-$1^{n/n}$ CE/J mice *in utero* with N-tropic, ecotropic MuLV (AKR-623, emv-11). All progeny mice that had become infected with the ecotropic MuLV developed susceptibility to paralytic LDV infection and this susceptibility correlated with an extensive

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expression of the ecotropic MuLV in cells of the CNS (Anderson *et al*, 1995a). Similarly in AKR and C58 mice, a replication competent ecotropic MuLV germline provirus becomes first activated during embryogenesis, probably in a rare cell (Rowe and Pincus, 1972), and progeny virus then infects cells in other tissues including the CNS. Virus infection and replication requires the Fv-$1^{n/n}$ genotype which permits the efficient replication of N-tropic, ecotropic MuLVs (Coffin, 1985). Infection of cells in the CNS by these viruses seems to be largely restricted to the time of fetal development (Jaenisch 1980; Anderson *et al*, 1995a). Only anterior horn neurons seem to become susceptible to cytocidal LDV infection as a result of ecotropic MuLV expression in glial cells, but how this effect is mediated is still unclear.

Nevertheless, these findings explain why only mice of strains such as AKR and C58, that possess at least one endogenous replication-competent ecotropic MuLV provirus and the $Fv1^{n/n}$ genotype are susceptible to paralytic LDV infection (Anderson *et al*, 1995a). However, AKR and C58 mice generally develop motor neuron disease after LDV infection only when infected with LDV in old age (Murphy *et al*, 1983, 1987; Stroop *et al*, 1985), thus the name ADPM. Younger mice seem resistant probably because they rapidly develop anti-LDV immune responses that protect motor neurons from LDV infection without affecting LDV replication in macrophages (Plagemann and Moennig, 1992). This conclusion was suggested by the finding that C58 and AKR mice 6 months of age and older could be rendered susceptible to paralytic LDV infection by a single sublethal treatment with X-irradiation or cyclophosphamide given at about the time of LDV infection (Murphy *et al*, 1983, 1987). Such mice then could be protected from the motor neuron disease by passive transfer of neutralizing or non-neutralizing polyclonal or monoclonal anti-LDV antibodies (Harty *et al*, 1987; Harty and Plagemann, 1990). However, only limited information was available on the anti-LDV antibody response in C58 and AKR mice and it was unclear why mice younger than 5 months of age were resistant to paralytic LDV infection in spite of receiving this immunosuppresive treatment (Murphy *et al*, 1983, 1987; Stroop *et al*, 1985). The latter question gained further importance in light of the recent finding that ecotropic MuLV expression in CNS glial cells can be detected in these mice soon after birth and that in older mice, expression of ecotropic MuLV in the CNS correlated with the susceptibility to paralytic LDV infection (Anderson *et al*, 1995a). The present study provides an explanation for this discrepancy and detailed information on the relationship between anti-LDV antibody formation and neuron protection as a function of age of the mice. We found that young mice are, in fact, susceptible to LDV infection but to demonstrate the development of paralysis a more prolonged inhibition of anti-LDV immune responses is required. In contrast, old mice exhibit an impaired ability to mount a motor neuron protective anti-LDV immune response and are thus naturally susceptible to the disease. At all ages, LDV induces disease only in the absence of an anti-LDV immune response during a critical time after infection.

Result

One purpose of the present study was to clarify further the role of anti-LDV immune responses in blocking the development of paralytic LDV infec-

Table 1 Effects of immunosuppressive treatments on incidence of ADPM, LDV replication, and anti-LDV antibody formation in C58/M mice[a]

Treatment	*Plasma LDV (ID_{50} ml^{-1}) days p.i.*			*Paralysis*		*Anti-LDV FA titer (days p.i.)*			
	1	*2*	*5*	*/Total*	*Onset (days p.i.)*	*8*	*12*	*16*	*42*
None	$10^{9.5}$	$10^{9.5}$	$10^{7.5}$	0/10		128	1024	2048	2048
CY	$10^{10.5}$	$10^{8.5}$	$10^{8.0}$	7/7	15, 15, 15, 16, 16, 16, 17	<32	<32	<32, <32, <32 256, 1024, 2048[b]	
X-irradiation	$10^{9.0}$	$10^{9.5}$	$10^{8.0}$	8/9	12, 15, 16, 17 18, 19, 19, 20	<32	<32	<32, <32, <32 64,128[b]	128
CsA	$10^{10.0}$	$10^{9.5}$	$10^{8.5}$	4/8	17, 17, 23, 26	<32	128	128, 256, 2048[b]	128[c]

[a]Groups of 6 to 7-month-old C58/M mice were injected, where indicated, with 200mg cyclophosphamide (CY)/kg or X-irradiated (500 rads) 1 day before infection with 10^6 ID_{50} of LDV-v. One group of mice received daily injections of 0.2 ml solution of cyclosporin A (CsA) in olive oil (5 mg ml^{-1}) beginning 3 days before LDV infection until the end of the experiment. The mice were monitored for paralytic symptoms for 63 days p.i. Five mice of each group were bled at intervals (1, 2, 5, 8, 12, 16 days p.i. and surviving mice at weekly intervals thereafter) and their plasma was pooled and assayed selectively for infectious LDV (ID_{50} = 50% infectious dose) and anti-LDV antibodies. FA: fluorescent antibody

[b] Individual mice were bled at the time of paralysis. Some of the paralyzed mice died before blood samples could be collected and thus could not be assayed for anti-LDV antibodies

[c] FA titer of the plasma pooled from the surviving mice

tion in C58 and AKR mice. Some experiments suggested that cyclophosphamide and X-irradiation might enhance the susceptibility of C58 mice to paralytic LDV infection by inducing the expression of ecotropic MuLV proviruses in spinal cord cells (Contag and Plagemann, 1989). However, in recent experiments we found that cyclophosphamide treatment had no effect on the expression of ecotropic MuLV in the CNS of 6 to 7-month-old C58 mice as measured by both Northern blot hybridization of spinal cord total RNA and *in situ* hybridization of spinal cord sections using an ecotropic MuLV-specific probe (Plagemann and Moennig, 1992; Anderson *et al,* unpublished results). Thus, the results suggested that the function of cyclophosphamide in rendering mice susceptible to paralytic LDV infection is solely by suppressing anti-LDV immune responses. The results in Table 1 support this conclusion.

C58 mice 6–7 months of age rapidly generated an anti-LDV antibody response after infection with LDV and did not develop paralytic disease (Table 1, line 1), whereas a single injection of cyclophosphamide or sublethal X-irradiation completely blocked anti-LDV antibody formation at least until 12 days post infection (p.i.) and the mice developed paralytic disease (Table 1, lines 2 and 3). The development of paralytic disease correlated with a blockage of anti-LDV antibody formation and was not related to differences in LDV replication in the mice since the levels of LDV in the circulation were the same whether or not the mice were treated with the immunosuppresive agents (Table 1). This result is consistent with the earlier finding that anti-LDV antibodies do not affect LDV replication in macrophages the primary host cell for LDV (Harty and Plagemann, 1988, 1990). However, at the time of paralysis some, but not all of the treated mice possessed considerable levels of anti-LDV antibodies (Table 1, lines 2 and 3). This result is in agreement with those of earlier studies which indicated that the anti-LDV immune response of mice generally recovers 12–14 days after a single cyclophosphamide treatment but that the efficiency of recovery differs between individual mice (Cafruny *et al,* 1986b; Onyekaba *et al,* 1989). Regardless, the results indicate that the formation of anti-LDV antibodies must be blocked for a critical length of time after LDV infection to lead to the destruction of a sufficient number of motor neurons to result in frank paralysis. In the mice that possessed anti-LDV antibodies at the time of paralysis, these apparently became generated too late to prevent the massive cytocidal infection of the motor neurons that leads to paralysis.

Daily injections of cyclosporin A also inhibited anti-LDV antibody responses and some of the treated mice developed disease after LDV infection (Table 1, line 4). These results reinforce the conclusion that early anti-LDV antibody formation protects mice from LDV-induced paralytic disease, since it mediates immunosuppression by a different mechanism than cyclophosphamide and X-irradiation. However, the cyclosporin A treatment was less effective in inhibiting anti-LDV antibody formation and therefore fewer mice became susceptible to paralytic LDV infection than after treatment with cyclophosphamide or X-irradiation.

Since earlier studies have shown that the paralytic disease induced by LDV infection in cyclophosphamide-treated C58 mice can be prevented by passive immunization with anti-LDV antibodies (Harty and Plagemann, 1990), the results in Table 1 suggest that the blockage of the genera-

Table 2 Effect of age of C58/M mice and administration of cyclophosphamide on incidence of ADPM[a]

Age (months)	*Paralyzed mice/total number of mice after cyclophosphamide treatment at:*		
	None	*1 day before LDV infection*	*1 day before LDV infection and weekly p.i.*
1	0/6	0/6	12/25 (23 ± 5)
2	0/6	1/5 (21)	11/12 (34 ± 3)
3	0/6	1/11 (ND)[b]	4/5 (34 ± 2)
4–5	0/2	2/6 (23, 31)	7/8 (23 ± 2)
6–8	1/27 (ND)	130/140 (16 ± 2)[c]	1/1 (15)
10	2/12 (ND)	14/14 (15 ± 1)	3/3 (13 ± 2)
11–12	2/8 (32, 49)	15/16 (17 ± 2)	ND
14–20	7/11 (14 ± 2)	ND	ND

[a] C58/M mice of the indicated ages were injected i.p. with approximately $10^{6.0}$ ID_{50} of LDV-v and, where indicated, with 200 mg cyclophosphamide kg_{-1} 1 day before LDV infection or 1 day before and at weekly intervals p.i. The mice were observed for development of paralysis for at least 40 days p.i. The results have been combined from many individual experiments. Numbers in parentheses represent onset of paralysis (days p.i., mean ± SEM)

[b] ND: not determined

[c] Some of the data were contributed by CH Contag

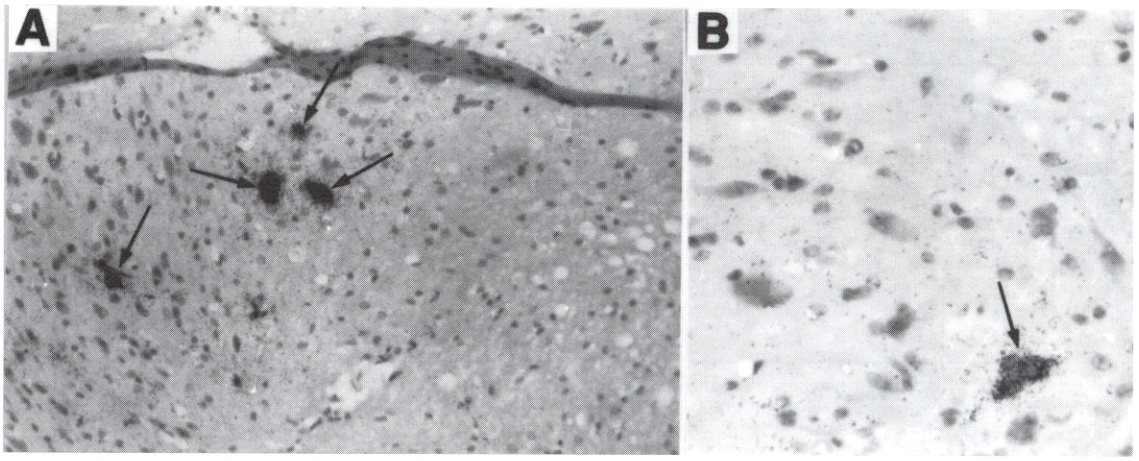

Figure 1 LDV-infected anterior horn neurons in the spinal cord of a 1-month-old C58 mouse (A and B). The mouse was sacrificed at the heights of paralysis at 16 days p.i. with LDV-v. Spinal cord sections were hybridized with the LDV-specific 4-55 cDNA probe. Magnification = 200× (A) and 400× (B).

tion of anti-LDV antibodies for at least 12 days is required to render 6 to 7-month-old C58 mice susceptible to paralytic LDV infection. This length of immunosuppression is achieved by a single treatment with cyclophosphamide or X-irradiation, but as shown below, is not sufficient to render younger mice ADPM-susceptible.

The second purpose of the present study was to inquire into reasons for the previously observed increase in susceptibility of C58 and AKR mice to paralytic LDV infection with increasing age and the apparent resistance of young mice (Murphy *et al*, 1983, 1987). The results in Table 2, column 2 confirm the observation that only old C58/M mice develop paralytic disease after LDV-v infection and that the incidence of the disease increases with advancing age of the mice. Furthermore, treatment of the mice with a sublethal dose of cyclophosphamide 1 day before LDV infection enhanced the incidence of paralytic disease; practically all mice older than 6 months of age became paralyzed after LDV-v infection, but younger mice remained relatively resistant (Table 2, column 3). We hypothesized that a single immunosuppressive treatment might be insufficient to render the younger mice susceptible and this view turned out to be correct. We found that most of these younger mice could be rendered susceptible to paralytic LDV infection by additional weekly cyclophosphamide injections after LDV infection (Table 2, column 4). This treatment continuously blocked anti-LDV antibody formation and none of the young mice treated with cyclophosphamide at weekly intervals p.i. possessed detectable levels of anti-LDV antibodies at the time of paralysis (data not shown). However, only about 50% of these young mice developed paralytic disease after LDV-v infection, even though their anti-LDV immune response was continuously blocked and those that showed signs of paralysis developed only mild non-progressive paralysis and survived for prolonged time periods. Some young mice that became partially paralyzed were still alive at 2 month p.i. and the paralysis did not progress (data not shown). Only 30–40% of 1 to 2-month-old C58/M mice with paralytic symptoms developed paralysis in more than one limb, whereas the proportion for 6 to 8-month-old mice was about 90% (combined results from several experiments, data not shown).

In situ hybridization with an LDV-specific cDNA probe demonstrated the presence of many LDV-infected motor neurons in the area of the spinal cord controlling the limb(s) that had become paralyzed in these young C58/M mice (eg Figure 1). In the segment shown in frame A most motor neurons had disappeared and there was evidence of infiltration of inflammatory cells. These results confirm that the paralysis resulted from cytocidal infection of the motor neurons.

Results similar to those presented in Table 2 for C58/M mice were obtained with AKXD-16 mice. AKXD-16 mice represent a recombinant inbred strain derived from AKR and DBA/2J mice. They possess only a single replication competent ecotropic MuLV (emv-11) (Jenkins *et al*, 1981) and are ADPM susceptible (Anderson *et al*, 1995a). Four of 12 6 to 11-month-old AKXD-16 mice were rendered susceptible to paralytic LDV infection by a single injection of cyclophosphamide and one 16-month-old mouse became paralyzed after LDV-v infection without receiving an immunosuppressive treatment (data not shown). None of 10 1 to 4-month-old AKXD-16 mice developed paralysis after LDV-infection and receiving a single injection of cyclophosphamide one day before infection, but 15/21 became paralyzed upon repeated injections of cyclophosphamide.

The susceptibility of young mice to paralytic LDV infection and the importance of the anti-LDV antibody response in protecting these mice from the

Table 3 Incidence of paralytic disease, LDV replication, and anti-LDV antibody formation in young nude AKR/J mice and their heterozygous and wild type littermates[a]

AKR/J mice	*CY*	*Plasma LDV (ID_{50} ml^{-1}) days p.i.*		*Paralysis*		*Anti-LDV (FA titer) days p.i.*		
		1	*7*	*Total*	*Onset (days p.i.)*	*7*	*14*	*35*
$nu^{str/str}$	-	ND[b]	$10^{8.0}$	2/2	37, 39	32	32	<32
	-1	$10^{9.0}$	$10^{8.0}$	2/2	18, 22	<32	<32	<32
$nu^{str/+}$ or $nu^{+/+}$	-	$10^{9.5}$	$10^{8.5}$	0/2		32	512	1024
	-1	$10^{9.5}$	$10^{7.5}$	0/2		<32	64	256
	-1, + 10	ND	ND	4/4	27 ± 2	ND	ND	<32

[a] Where indicated, groups of two 5 to 6-week-old athymic AKR/J streaker mice ($nu^{str/str}$; Shultz *et al*, 1982) and their heterozygous ($nu^{str/+}$) and wild type ($nu^{+/+}$) littermates were injected i.p. with 200 mg cyclophosphamide (CY) kg^{-1} 1 day before infection or 1 day before and 10 days p.i. with 10^6 ID_{50} of LDV-v. The mice were bled at 1, 7, 14 and 35 days p.i. (or at the time of paralysis) and their pooled plasma assayed for infectious LDV or anti-LDV antibodies or both. The mice were monitored for paralytic symptoms for 60 days p.i.

[b] ND: not determined

disease is further illustrated by the data in Table 3, which compares the incidence of paralytic disease in young nude AKR mice with that of their immunocompetent littermates. The nude mice mounted only a very low or no anti-LDV antibody response after LDV-v infection and developed paralytic disease. In contrast, the immunocompetent heterozygous and homozygous wild type littermates mounted a normal anti-LDV immune response and did not become paralyzed, even after a single injection of cyclophosphamide 1 day before LDV-v infection. Only when the anti-LDV immune response was continuously blocked in these mice by repeated cyclophosphamide injections did the mice become paralyzed.

As in C58/M mice (Table 2 and Anderson *et al*, 1995a) the increasing susceptibility of AKXD-16 mice to paralytic LDV infection with advancing age correlated with an increased expression of ecotropic MuLV in the CNS (Figure 2). Northern hybridization analysis detected only a low level of 8.2-kb genomic ecotropic MuLV RNA and of 3-kb spliced mRNA in the spinal cords of 2-month-old AKXD-16 mice, but the level increased progressively with age (Figure 2A) just as in C58/M mice (Anderson *et al*, 1995a). There was considerable binding of the probe to 28S and 18S rRNA because of the relatively low stringency of the washes necessary for visualization of the signal.

In the 1 to 2-month-old AKXD-16 mice we detected foci of ecotropic MuLV RNA-containing cells only in a few sections of white matter of the spinal cord. One such focus is shown in Figure 2B. In most sections, few, if any, positive cells were present. In contrast, all sections of the spinal cord of a 10-month-old AKXD-16 mouse contained many ecotropic MuLV RNA-positive cells, mostly in the white matter, but also some in the grey matter. A representative section of spinal cord white matter is shown in Figure 2C. As reported for C58/M mice (Anderson *et al*, 1995a) the anterior horn neurons

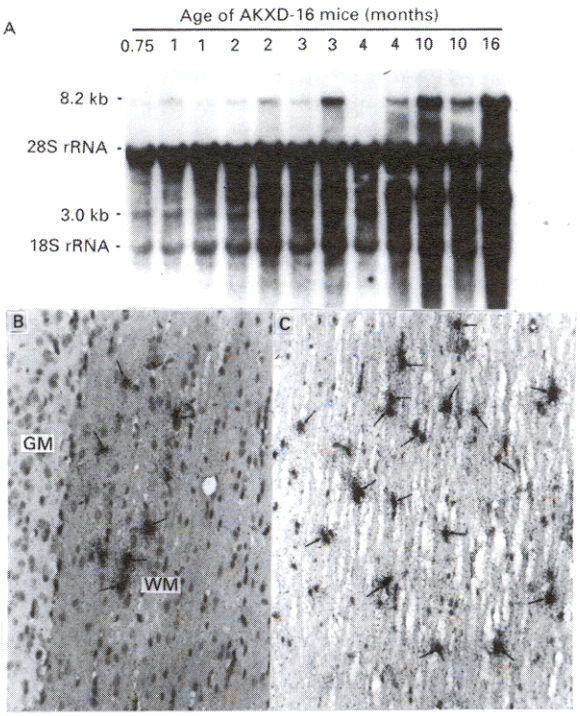

Figure 2 Expression of ecotropic MuLV RNA in spinal cords of AKXD-16 mice of various ages, as analyzed by Northern hybridization (A) or *in situ* hybridization (B and C). Total spinal cord RNA was analyzed by Northern hybridization using the ^{32}P-labeled, 168-bp Sma I ecotropic MuLV-specific fragment as probe (A). The blot was then stripped and hybridized with a mouse actin-specific oligonucleotide (Contag and Plagemann, 1988; Anderson *et al*, 1995a). The level of hybridization to actin mRNA was about the same in all samples (data not shown). Sections of the spinal cord from a 2-month-old (B) and a 10-month-old (C) mouse were hybridized wih the same ecotropic MuLV-specific probe, except that it was labeled with ^{35}S instead of ^{32}P. Magnification = 200×.

that became infected with LDV (Figure 1) did not contain detectable levels of ecotropic MuLV RNA.

In the course of these studies we observed considerable differences in ADPM susceptibility

between substrains of C58 and AKR mice. For example, 10/10 6 to 7-month-old AKR/Boy mice became paralyzed after LDV-v infection and a single cyclophosphamide treatment 1 day before infection. In contrast, none of 65 6 to 7-month-old AKR/J mice developed paralytic disease under the same experimental conditions. However, 100% of the latter mice became paralyzed when receiving additional weekly injections of cyclophosphamide. The reasons for these differences have not been elucidated. They could be due to differences in the expression of the ecotropic MuLV in the spinal cord of these mice or differences in the ability of the mice to mount motor neuron protective anti-LDV immune responses.

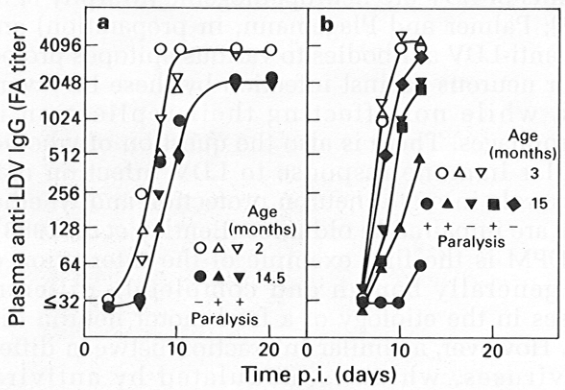

Figure 3 Time course of anti-LDV antibody production in young and old C58/M mice. C58/M mice at the ages of 2–3 (open symbols) or 14.5–15 (solid symbols) months were infected with approximately $10^{6.0}$ ID_{50} of LDV-v in two independent experiments (A and B). Plasma was collected by orbital bleeding from individual mice at the indicated times p.i. and titrated for total anti-LDV IgG by a fluorescent antibody (FA) staining technique. The plasma harvested at 20 days p.i. or at the time of paralysis was also analyzed by the FA staining assay for anti-LDV IgG1, IgG2a, IgG2b and IgG3 (data not shown). The mice were monitored for paralytic symptoms until 35 days p.i. (+ or –). The onset of paralysis in the 14.5 to 15-month-old mice was 14 ± 3 days p.i. None of the 2 to 3-month-old mice developed any paralytic symptoms.

The finding that very old C58/M, AKR and AKXD-16 develop paralytic disease after LDV-v infection without requiring immunosuppressive treatments suggested that old mice may be impaired in their ability to mount a motor neuron protective anti-LDV immune response. The following results support this conclusion. In two independent experiments, most 14- to 15-month-old C58/M mice exhibited delayed and/or reduced anti-LDV antibody responses when compared to those of 2- to 3-months-old C58/M mice (Figure 3, A and B). The anti-LDV antibody response was only slightly delayed in the older mice. However, the time of generation of the anti-LDV immune response after LDV infection seems important in determining whether the motor neuron infection progresses to frank paralysis or becomes blocked before a sufficient number of motor neurons are destroyed to result in symptoms. Thus a 2-day delay in the formation of anti-LDV antibodies in the old mice or a reduction in the initial response in the old mice may be critical in failing to stop motor neuron infection by LDV before paralysis develops, especially since the latent period in the old mice is relatively short (Table 2; Murphy *et al*, 1983, 1987; Stroop *et al*, 1985). The final anti-LDV titer at the time of paralysis might be unimportant in this respect (Table 1). The isotype distribution of the anti-LDV antibodies generated in young and old C58/M (Figure 3) was similar. Most of the antibodies were of the IgG2a and IgG2b isotype (data not shown), similar to other mouse strains (Hu *et al*, 1991). However, we noticed that in addition to the quantitative and temporal differences in anti-LDV antibody formation, the ability of the anti-LDV antibodies from old mice to protect immunosuppressed C58/M mice from paralytic LDV infection was lower than that of anti-LDV antibodies from young mice (Table 4). Intravenous injection of plasma from 3-week LDV-infected, 2-month-old C58/M mice 1 day before LDV infection almost completely protected immunosuppressed 10-month-old C58/M mice from developing paralytic disease, similarly to

Table 4 Effect of passive transfer of immune mouse plasma (IMP) from young and old C58/M mice on incidence of paralytic LDV infection in old C58/M mice[a]

	Paralysis		
Anti-LDV	*Total mice*	*Days p.i.*	*Anti-LDV FA titers 10 days p.i.*
None	6/6	16, 17, 20, 21, 21, 26	<32
IMP from 2-month-old mice	1/6	27	<32
IMP from 14.5-month-old mice	5/7	13, 14, 16, 19, 23	<32
mAb 159-12	0/4		≤32

[a] *10-month old C58/M mice were injected with 200mg cyclophosphamide kg^{-1}. One day later they were inoculated intravenously with 200 µl 1:10 dilution of IMP or 400 µl of a 1:2 dilution of anti-VP3 mAb 159-12 and then with $10^{6.0}$ ID_{50} of LDV-v. The IMPs consisted of pooled plasma from three 2-month or 14.5-month-old C58M/LDV-v infected mice (see Figure 3A) harvested at 20– 22 days p.i. (the anti-LDV antibody titers of the IMPs were about 2048). The mice were bled at 10 days p.i. and their pooled plasma was assayed for anti-LDV antibodies. The mice were monitored for paralytic disease for 40 days p.i.*

neutralizing anti-LDV mAb 159-12 (Harty and Plagemann, 1990). In contrast, injection of plasma with a similar titer of anti-LDV antibody pooled from 3-week LDV-infected, 14.5-month-old C58/M mice failed to protect 5/7 of the LDV-infected mice (Table 4). The results confirm the importance of anti-LDV antibodies in protection of motor neurons to LDV infection.

Discussion

Our results have shown that ADPM susceptible mice develop paralytic disease after infection with a neuropathogenic strain of LDV only if the anti-LDV immune response is blocked for a critical time p.i. by chemical treatment, a genetic defect or an impaired immune response in old age. The anti-LDV immune response functions in modulating ADPM susceptibility of mice only by protecting the anterior horn neurons from cytocidal infection by neuropathogenic strains of LDV. The primary determinant of susceptibility of mice to paralytic LDV infection is the expression of an ecotropic MuLV in spinal cord glial cells that renders the anterior horn neurons susceptible to LDV infection. This ecotropic MuLV expression only occurs in Fv-$1^{n/n}$ strains of mice that carry at least one replication competent ecotropic MuLV germline provirus or that have been infected with such virus *in utero* (Anderson *et al*, 1995a). The primary factor that plays a role in the age dependent increase in susceptibility of such mice to paralytic LDV infection is a progressive increase in the number of ecotropic MuLV RNA-containing cells in the CNS with increasing age. There is also a shift in the distribution of ecotropic MuLV expressing cells in the CNS with increasing age. In the young mice practically all ecotropic MuLV RNA-containing cells are located in white matter tracts (Anderson *et al*, 1995a), whereas in older mice a considerable number of such cells are also found in the grey matter close to anterior horn neurons that become LDV permissive (Anderson *et al*, 1995a). These changes seem to result in an enhanced susceptibility of the anterior horn neurons to LDV infection or a progressive increase in the number of susceptible anterior horn neurons. In very young mice only relatively few LDV-permissive anterior horn neurons seem available. Thus, in these young mice the destruction of a sufficient number of these motor neurons to result in frank paralysis seems to take a considerable length of time, which probably explains the relatively long latent period between LDV infection and the development of paralytic disease observed in these young mice (Table 2, column 4) and the requirement for an extended blockage of the anti-LDV immune response as occurs naturally in nude mice or is achieved in immunocompetent young mice by repeated injections of cyclophosphamide. In old age the anti-LDV immune response becomes impaired and some mice are ADPM-susceptible without requiring artificial or genetic immunosuppression.

The reason for an impaired ability of old C58 mice to generate an efficient motor neuron protective anti-LDV immune response is unclear. It could be related to a commonly observed, age-dependent decline in the competence of the immune system which seems to result from a combination of the accumulation of committed memory T cells and a decline in the generation of naive T cells (Hobbs *et al*, 1993). On the other hand, the replication of ecotropic MuLV and recombinants thereof (Stoye *et al*, 1991) in these mice could be a contributory factor as could the formation of leukemia/lymphoma that invariably occurs in these mice as they age. In relation to these uncertainties two important related, but unresolved, questions are why only certain variants of LDV are neuropathogenic (Murphy *et al*, 1987); Palmer and Plagemann, in preparation) and how anti-LDV antibodies to various epitopes protect motor neurons against infection by these LDV variants, while not affecting their replication in macrophages. There is also the question of whether cellular immune response to LDV infection may play a role in motor neuron protection and whether these are impaired in old mice (Bentley *et al*, 1983).

ADPM is the first example of the interaction of two generally benign and completely different viruses in the etiology of a fatal motor neuron disease. However, a similar interaction between different viruses, which is modulated by antiviral immune responses, could play a role in motor neuron diseases of humans and other animals.

Materials and methods

C58/M and ADXD-16 mice were bred in the animal facility of the Department of Microbiology. Outbred Swiss mice were purchased from Biolabs, Inc. (St Paul, MN) and FVB mice were supplied by the transgenic facility of the University of Minnesota. AKR/Boy mice were provided by Dr W Murphy. AKR/J and C58/J mice were purchased from Jackson Laboratories (Bar Harbor, MA). Many of the mice raised in our facilities possessed antibodies to mouse hepatitis virus (MHV) when 4–6 weeks of age without that MHV could be isolated from these mice and without ever showing any clinical symptoms (Even and Plagemann, 1995). However, this fact seems immaterial in the context of the present study since ecotropic MuLV expression in the CNS and ADPM susceptibility of these mice were comparable to those of pathogen-free C58 and AKR mice purchased from Jackson Laboratories. Also, LDV replication in all mice is the same whether or not they possess anti-MHV antibodies (Even and Plagemann, 1995; see below).

LDV-v is a neuropathogenic strain of LDV which has been isolated from the spinal cord of a paralyzed C58/M mouse that had been injected with Ib-

LDV obtained from Dr W Murphy (Murphy *et al*, 1987). Stock LDV-v was prepared by injecting groups of C58, Swiss or FVB mice with LDV-v and harvesting their plasma 1 day p.i. The plasma pools contained $10^{9.0}$ to $10^{10.0}$ ID_{50} ml^{-1} regardless of the mouse strain used. For ADPM experiments, mice were also injected intraperitoneally (i.p.) with about 10^6 ID_{50} of LDV-v. Where indicated, mice were also injected (i.p.) with 200 mg cyclophosphamide/kg of body weight 1 day before and at weekly intervals after LDV-v infection or they were injected with CsA or X-irradiated as described in appropriate experiments. Mice were subsequently monitored for paralytic symptoms until at least 5 weeks p.i. Paralytic onset was noted when a mouse demonstrated a reduced ability to grasp with or splay at least one of its legs. Such mice were scored as paralytic if they subsequently developed flaccid paralysis of the weak limb(s).

Blood was collected by the retro-orbital method. Plasma was titrated for infectious LDV by an end point dilution assay in Swiss or FVB mice (Plagemann *et al*, 1963) and for total anti-LDV IgG and for anti-LDV IgG1, IgG2a, IgG2b and IgG3 by an indirect fluorescent antibody (IFA) staining assay (Cafruny *et al*, 1986a; Hu *et al*, 1991). In brief, LDV-infected mouse macrophage cultures in multiwell microscope slides were fixed in acetone at 8 h p.i and then sequentially incubated, first with 2-fold dilutions of test material containing anti-LDV mouse IgG and then with fluorescein isothionates-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3. The slides were examined in a fluorescence microscope and the antibody titer expressed as the reciprocal of the highest dilution that yielded positive staining of 5–20% of the macrophages, which represents the subpopulation of LDV-permissive cells. In antibody protection experiments, mice were injected intravenously with 0.2 ml anti-LDV antibody solution as described in appropriate experiments.

Total RNA was extracted from spinal cords and analyzed by Northern hybridization as described previously (Anderson *et al*, 1995a). The probe was a ^{32}P-labeled, 168 bp Sma I restriction fragment of the AKR-623 MuLV clone which represents a segment of the *env* gene and is specific for ecotropic MuLVs (Anderson *et al*, 1995a). Spinal cord sections were prepared and hybridized *in situ* with ^{35}S-labelled probes as described previously (Anderson *et al*, 1995a). The probes were the ecotropic MuLV-specific Sma I fragment described already or a 437 bp cDNA (4–55) representing the 3′ end of the LDV genome which hybridizes to both genomic RNA and all seven subgenomic mRNAs (Kuo *et al*, 1991). Sections from several mice were analyzed by *in situ* hybridization on the same slide. One duplicate slide was treated with RNase A and T_1 before hybridization as described previously (Anderson *et al*, 1995a). No foci of autoradiographic grains were detected in any of the RNase-treated sections or in tissue sections from uninfected mice (Anderson *et al*, 1995a, b).

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