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Age-dependent poliomyelitis in mice is associated with respiratory failure and viral replication in the central nervous system and lung

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Age-dependent poliomyelitis (ADPM) is a virally induced neuroparalytic disease of mice and a model for amyotrophic lateral sclerosis (ALS). ADPM is triggered in genetically susceptible mice by immunosuppression and infection with lactate dehydrogenase-elevating virus (LDV). Both ADPM and ALS are characterized by progressive degeneration of anterior horn motor neurons, and death in ALS is usually associated with respiratory failure. To assess respiratory function in ADPM, we investigated ventilation in conscious control and LDV-infected C58/J mice breathing air and then 6.5% CO_2 in O_2. Three days after LDV infection, ventilation in response to CO_2 was half of that compared to the uninfected state, but become normalized by 10 days. Administration of cyclophosphamide alone (200 mg/kg, ip), an immunosuppressant, had no effect on ventilation. Induction of ADPM by concomitant administration of LDV to cyclophosphamide-treated mice resulted in altered gait, hindlimb paralysis, wasting, decreased metabolism, and decreased body temperature by 4°C relative to controls. Compared to baseline values, mice with ADPM had decreased tidal volume and ventilation while breathing air, and while exposed to the CO_2 challenge they were unable to increase tidal volume, frequency of breathing, or ventilation. Using *in situ* hybridization, LDV replication was noted within the spinal cord, brain, and lung, but not in the diaphragm. Thus, respiratory failure is a contributory mechanism leading to death in ADPM and is associated with LDV replication in the CNS and lung. This animal model may be useful to investigate physiological and molecular mechanisms associated with the development of respiratory failure in neurodegenerative diseases. *Journal of NeuroVirology* (2001) **7,** 265–271.

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Introduction

Age-dependent poliomyelitis (ADPM) is a motor neuron disease of mice (reviewed in Contag *et al*, 1989) that involves the interaction of two viruses. In many respects, ADPM is similar to amyotrophic lateral sclerosis (ALS) because both diseases are age-related, have genetic and environmental risk factors, and share anterior horn neurons as the primary pathological targets (Sillevis Smitt and de Jong, 1989; Bromberg, 1999). The etiology of ALS is not known, although recent evidence suggests the presence of enterovirus (probably echovirus 7) RNA in the spinal cords of ALS patients, raising the possibility that ALS is a viral disease (Berger *et al*, 2000; Karpati and Dalakas, 2000). Respiratory failure is the common cause of death in ALS (Ferguson *et al*, 1996; Bromberg, 1999; Arnulf *et al*, 2000), but the cause of death in ADPM is not known, despite previous speculation that respiratory paralysis may play a role (Sillevis Smitt and de Jong, 1989; Anderson *et al*, 1995a).

ADPM has a complex etiology. It results from lactate dehydrogenase-elevating virus (LDV) infection of genetically susceptible strains of mice (e.g., C58 and AKR), which express two copies of the Fv-$1^{n/n}$ allele that regulates permissiveness to endogenous retrovirus replication (Martinez *et al*, 1980; Murphy *et al*, 1980; Nawrocki *et al*, 1980; Contag *et al*, 1989; Plagemann and Moennig, 1992; Anderson *et al*, 1995a; Anderson *et al*, 1995b; Plagemann,

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1996). As these mice age, they develop susceptibility to ADPM induction by LDV infection, due to immunosuppression associated with aging. Treatment with cyclophosphamide or X-irradiation can induce immunosuppression of younger mice and enhance susceptibility to ADPM (Contag *et al*, 1989; Plagemann and Moennig, 1992; Plagemann, 1996). Infection of susceptible mice with neuroparalytic strains of LDV induces a systemic infection and entry of LDV into the CNS. This is followed by a subclinical phase of about 10 days, during which LDV is not present at pathologic levels in the CNS. At about 10–12 days postinfection (p.i.) with LDV, LDV replication emerges in the CNS. There is a brief prodrome during which mice appear sluggish and begin to lose weight (wasting). Between about 12–18 days, p.i. mice develop frank paralysis (typically hindlimb) and die within a few days to weeks.

Although LDV appears to have the primary role in neuropathology, ADPM is clearly a dual-virus disease (Contag *et al*, 1989; Plagemann and Moennig, 1992; Plagemann, 1996) that requires some degree of immunosuppression. Thus, ADPM provides an attractive small animal model for degenerative neurological diseases that involve aging and potential virus etiologies. The present study documents the development of respiratory failure in ADPM, which correlated with LDV replication in lung cells and central nervous system neurons. To determine if depression of metabolic rates coincided with the ventilatory effects of ADPM, both CO_2 production and O_2 consumption were measured in the mice. Control studies were conducted to determine if LDV infection or cyclophosphamide alone had similar physiological effects as the combination of the two treatments.

Methods

Eight-month-old female C58/J mice (The Jackson Laboratory) were used in these experiments. Mice were housed 3–4 per cage. Food (Purina Mouse Chow) and water were available *ad libitum*. Lighting consisted of 12 h on and 12 h off. The University of South Dakota Animal Care and Use Committee approved all procedures.

Ventilatory and metabolic measurements

To evaluate ventilation, mice were placed in a Plexiglas cylindrical chamber 15 cm long and 6.35 cm in diameter. The chamber had three ports on one side for an air inlet, a Statham low-pressure transducer, and a Cole-Palmer thermometer to measure chamber temperature. On the opposite side, ports allowed air to exit the chamber to a Gilmont rotameter and another line that was either connected to gas analyzers (OM-14 Beckman oxygen analyzer or a vacuumed CO_2 analyzer) or to a leak used to stabilize ventilatory measurements.

The Statham transducer was connected to a Grass model 7 polygraph. Outputs from the polygraph were introduced to an MP100 Biopac data analysis system. The parameters evaluated included breathing frequency (f) in breaths/min, tidal volume (Vt) in ml/breath, and minute ventilation (VE), the product of Vt and f in ml/min.

Oxygen consumption and CO_2 production were measured using the flow-through technique. For both evaluations, the differences between the fractional concentrations of gases entering and exiting the chamber were measured and multiplied by the flow rate through the chamber. These methods have been reported previously (Schlenker, 1984).

Protocols

The animals were weighed prior to being placed into the plethysmographic chamber. First, the mouse was acclimated to the chamber and ventilatory and metabolic measurements were conducted. Then, the mouse was exposed for 30 s to 6.5% CO_2 in O_2 to measure the response to hypercapnia. Subsequently, the mouse was exposed to air again. After the mouse was removed from the chamber its rectal temperature was measured using a Sensortek thermometer (Clifton, NJ).

Treatment groups and virus infections

Prior to any treatments, baseline ventilatory and metabolic measurements were performed on all animals. Three treatment groups were studied. A virus-only group (10 C58/J mice total) received an intraperitoneal injection of LDV v-9 (10^6 ID_{50}). LDV-v9 is a neuroparalytic (Group B) quasispecies isolated from the spinal cord of a paralyzed C58 mouse infected with the cloned quasispecies LDV-v (Chen *et al*, 1999). LDV-v9 stocks were generated from the plasma of CF1 mice (Harlan Sprague–Dawley) 24 h p.i. with LDV-v9 and titrated by limiting dilution assay (Plagemann and Moennig, 1992). In the second group, 10 C58/J mice received an intraperitoneal injection of 200 mg/kg cyclophosphamide on day 1 and a vehicle injection on day 2. A third group of six C58/J mice received cyclophosphamide on day 1 and LDV-v9 on day 2. On day 7, cyclophosphamide was once again administered to groups 2 and 3. Physiological measurements were conducted prior to cyclophosphamide administration and on days 3, 5, 7, and 12 after virus treatment until mice in group 3 developed severe disease on 16 to 20 days after infection. Subsequently, LDV and cyclophosphamide-treated mice, and animals treated with cyclophosphamide alone, were sacrificed. Brains, lungs, and diaphragms were quickly removed, fixed in 10% buffered formalin, and prepared for LDV RNA localization.

Localization of LDV RNA in tissues

In situ hybridization was used to determine if LDV replication was present in the brain, spinal

Table 1 Effect of cyclophosphamide and LDV infection on O_2 consumption (VO_2) and CO_2 production (VCO_2)

	Baseline		*Day 16*	
Treatment groups	VO_2	VCO_2	VO_2	VCO_2
Cyclophosphamide alone	1.91 ± 0.39	1.38 ± 0.10	1.63 ± 0.41	1.52 ± 0.19
Cyclophosphamide alone and LDV	2.00 ± 0.46	1.65 ± 0.45	1.05 ± 0.22*	0.86 ± 0.24*

O_2 consumption (VO_2) and CO_2 production (VCO_2) are in units of ml/min. There were six animals per group. The asterisks denotes that the day 16 VO_2 is significantly less than baseline value in the same group of animals and that the day 16 VCO_2 is significantly less than baseline value in the same group of animals. Values are means ± SD.

cord, lung, or diaphragm. Tissues were fixed in formalin, embedded in paraffin, sectioned at 6 um, and then probed with a ^{35}S-labeled cDNA probe (#4-55) that hybridizes to LDV genomic RNA and all seven subgenomic RNAs (Kuo *et al*, 1992). Autoradiographically processed sections were stained with hematoxylin and eosin and examined under a light microscope for localization of LDV RNA and estimation of tissue virus burden. Uninfected tissue sections were prepared as internal experimental controls.

Quantification of tissue LDV virions
Plasma, lung, diaphragm, brain stem, and cervical spinal cord samples were obtained from mice sacrificed during paralysis at 16 days p.i. with LDV-v9. Tissue samples (except for plasma) were washed in PBS, weighed, and minced with a hollow glass rod, which was used to completely grind and disrupt the tissues in the bottom of a 12 × 75 mm tube containing cold PBS. These tissue extracts were then clarified by centrifugation at 1000 × g for 5 min. The quantitative method of choice for LDV virions is a highly sensitive end-point dilution assay, in which 10-fold dilutions of samples are injected into indicator mice, which display elevated plasma LDH after LDV infection (Rowson and Mahy, 1975; Plagemann and Moenning, 1992). Therefore, LDV titers were determined in plasma and clarified tissue extracts by end-point dilution analysis, using two mice for each $\log_{10}$ dilution.

Data analysis
To determine if each treatment had an effect of body weight, body temperature, CO_2 production and O_2 consumption or ventilatory parameters, a paired *t*-test was used to compare baseline and final values. Significance was accepted at $P < 0.05$. Values are expressed as means ± SD.

Results

Body weights, body temperatures, and metabolism
Treatment of mice with LDV alone or with cyclophosphamide alone had no significant effects on metabolic parameters, body temperature, or body weight. In contrast, animals treated with cyclophosphamide and LDV exhibited a marked decrease in body weight from 23.3 ± 0.8 to 18.3 ± 0.5 g (mean ± SD), $P < 0.01$, which occurred between about 16–20 days post infection (p.i.). Moreover, body temperature dropped from a baseline value of 37.7 ± 0.17 to 33.1 ± 2.9°C ($P < 0.01$) within the same time frame. The effects of treatment with cyclophosphamide and LDV, or cyclophosphamide alone, on CO_2 production and O_2 consumption are presented in Table 1. Baseline values were comparable between the two groups. By day 16 after treatment started (the paralytic phase of ADPM), CO_2 production and O_2 consumption in cyclophosphamide- and LDV-treated mice were markedly lower than their baseline values and also lower than those of cyclophosphamide-treated mice. The mice progressing to ADPM exhibited a wasted appearance with a slowing of gait and developed hind-limb paralysis.

Control of breathing
Control of breathing in mice exposed to air and to hypercapnia was not affected by one or two doses of cyclophosphamide (data not shown). The ventilation in response to LDV alone revealed two types of responses (Figure 1). Half of the mice given LDV showed a transient decrease of ventilation in response to hypercapnia by day 3. This decrease was due to a decreased Vt and f. Mice infected with LDV alone did not develop paralysis. The decreased ventilatory responses to hypercapnia in these mice returned to baseline values by 10 days p.i.

Mice given cyclophosphamide plus LDV decreased their Vt, but not f, while breathing air by day 15 after infection (Table 2). Ventilatory responses to hypercapnia were similar to those of the mice while breathing air or blunted (Figure 2). The inability to respond to hypercapnia was due to a marked reduction of Vt and f. The time period for this blunted response to hypercapnia ranged from 15 to 21 days after infection. Mice either died or were sacrificed shortly after decreased ventilation was recorded, and all mice displayed a wasted appearance and a halting gait.

Localization of LDV RNA and tissue virions
In situ hybridization demonstrated LDV RNA in neurons within the brain and spinal cord, and in the lung coincident with the onset of LDV-induced

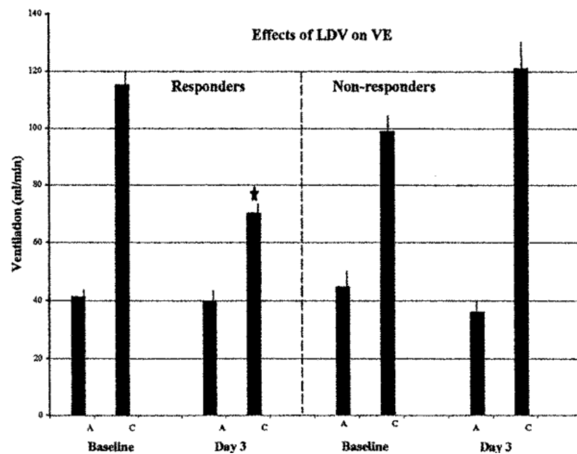

Figure 1 Effects of lactate dehydrogenase-elevating virus (LDV) on ventilation in mice exposed to air (**A**) and CO_2 (**C**) at baseline and 3 days after infection. The left panel denotes the five animals that exhibited a significant ($P < 0.01$) depression of ventilation on day 3 on response to CO_2 (as denoted by the asterisk). These are called the responders. The right panel denotes the five animals (nonresponders) that did not exhibit a depression of ventilation 3 days after being infected with LDV.

paralysis (representative data from five mice with ADPM are shown in Figure 3). The occasionally elongated pattern of LDV RNA in spinal cord sections (arrows, Figure 3C and F) is suggestive of nerve axon localization. In contrast, LDV RNA was not detected in the diaphragms of any of the mice studied but was detected within a lymph node located in the diaphragm (arrows, Figure 3B and E). The lung and lymph nodes within the lung contained LDV RNA.

Table 2 Effect of cyclophosphamide and LDV infection on tidal volume (Vt) and frequency of breathing (f)

	Baseline	*Paralysis*
Vt (air)	0.25 ± 0.05	0.16 ± 0.03*
Vt (CO_2)	0.39 ± 0.08	0.19 ± 0.03*
f (air)	223 ± 28	227 ± 53
f (CO_2)	318 ± 44	255 ± 49*

Values are means ± SD. The asterisks indicate that the values are significantly different ($P < 0.05$) from the corresponding baseline value. Paralysis decreases Vt (ml/b) in air and both Vt and F (b/min) in response of mice to hypercapnia (CO_2). There were six animals per treatment group.

The proportion of lung cells expressing LDV RNA ranged from about 5–8/10000. Since these cells are productively infected, they represent a theoretical tissue virus burden of 5–8× 10^4 virus particles/10000 lung cells (Plagemann and Moennig, 1992). As reported previously (Anderson *et al*, 1995a), brain and spinal cord LDV RNA was detected in multiple foci (3–7 per hemisphere or spinal cord cross-section), but due to the inherent lack of histological resolution, no estimation of the proportion cells productively infected was possible for these neural sites of virus replication. Cyclophosphamide-treated mice that were used as controls did not exhibit LDV RNA in any of the tissues examined. Tissue virions were also quantitated in two mice during LDV-induced paralysis at 16 days p.i. with LDV (Table 3). By this approach, large viral burdens were seen in all tissues examined, potentially reflecting local replication or virus from the circulation.

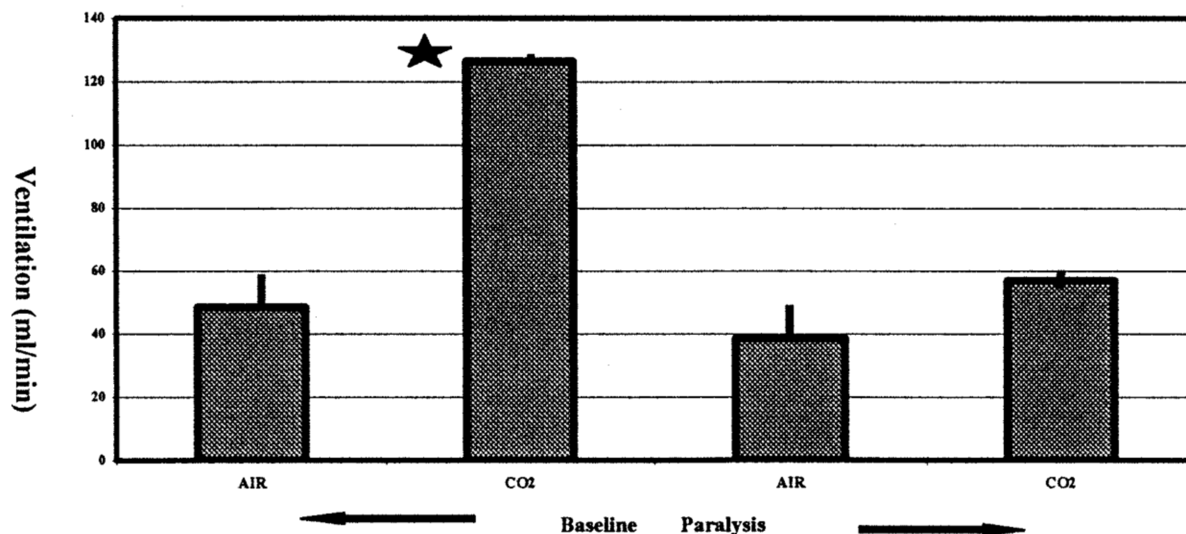

Figure 2 Effects of lactate dehydrogenase-elevating virus (LDV) and cyclophosphamide treatment on ventilation in mice exposed to air and CO_2 at baseline and 16 days after infection when paralysis or altered gait was observed. The asterisk denotes a significant ($P < 0.01$) ventilatory response to hypercapnia. There were six animals in this group.

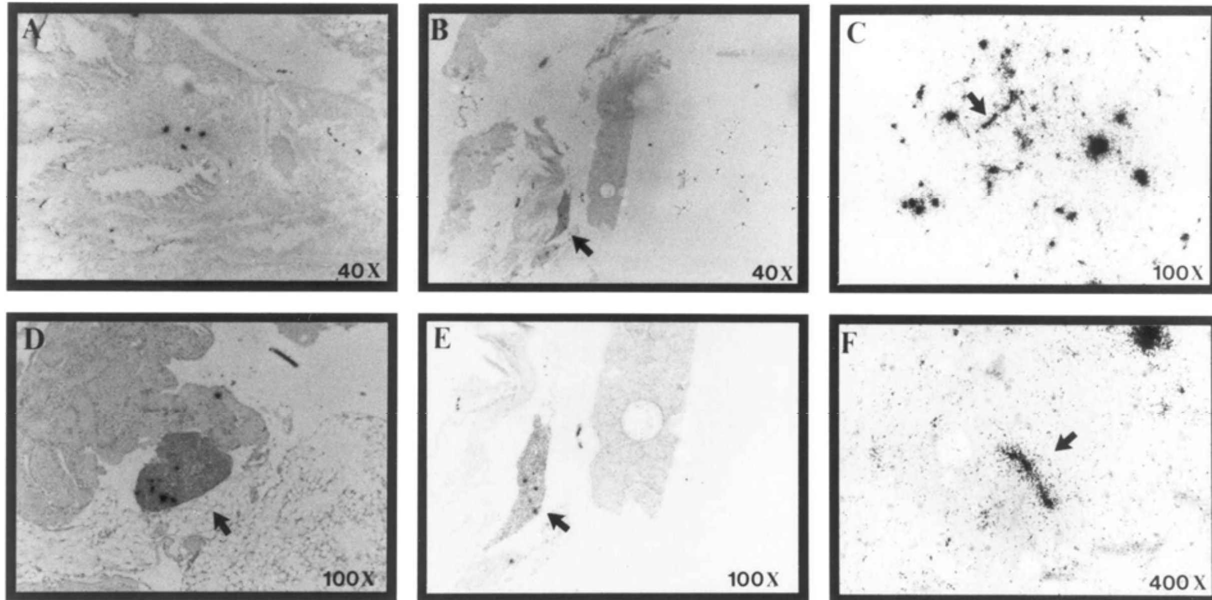

Figure 3 Detection of lactate dehydrogenase-elevating virus (LDV) RNA in lung, diaphragm, and spinal cord by *in situ* hybridization. Tissues of paralyzed C58 mice 16 days postinfection with LDV. LDV RNA was consistently found in the lung (**A** and **D**). No LDV RNA was detected in the diaphragm (**B** and **E**), although LDV RNA-positive lymph nodes were present in both diaphragm (**E**) and lung (**D**). Detection of LDV RNA in spinal cord (**C** and **F**) suggested a pattern consistent with axonal LDV replication.

Discussion

We report here the first description of respiratory failure in ADPM, extending the similarities between murine ADPM and human ALS (Sillevis Smitt and de Jong, 1989; Ferguson *et al*, 1996). Mice treated with a combination of cyclophosphamide and LDV developed wasting, altered gait, and hind-limb paralysis (ADPM as previously described). Accompanying these manifestations were decreased body weight, body temperature, O_2 consumption and CO_2 production, and ventilation in air and in response to hypercapnia. In contrast, treatment of mice with LDV or cyclophosphamide alone did not elicit these changes. Moreover, during paralysis, LDV RNA was located in the spinal cords, brains, and lungs but not within the diaphragms of these animals, demonstrating productive LDV infection at all sites but the diaphragm. Consistent with previous studies (Rowson and Mahy, 1975), LDV virions were found in all tissue sites including diaphragm, but the absence of LDV replication in diaphragm illustrates the limitations of virion detection, which may reflect virus delivered from the circulation as well as locally produced virus. While these data raise the possibility of synergy between site-specific productive infection and passive accumulation of virions in contributing to pathology, they primarily emphasize the significance of *in situ* detection of LDV replication, which is cytocidal and therefore directly pathologic.

Table 3 Tissue virus levels during ADPM

	Tissue Virion Concentration[1]	
Tissue	*Mouse A*	*Mouse B*
Plasma	$10^{7.0}$ ID_{50}/ml	$10^{6.0}$ ID_{50}/ml
Lung	$10^{5.9}$ ID_{50}/g	$10^{5.4}$ ID_{50}/g
Diaphragm	$10^{6.4}$ ID_{50}/g	$10^{6.4}$ ID_{50}/g
Spinal cord	$10^{6.2}$ ID_{50}/g	$10^{6.6}$ ID_{50}/g
Brain stem	$10^{5.3}$ ID_{50}/g	$10^{6.8}$ ID_{50}/g

Two 7–8-month-old female C58/J mice were treated with cyclophosphamide 200 mg/kg (days −1 and 7) and LDV infected on day 0. Both mice were sacrificed on day 16 p.i., at which time both exhibited limb paralysis, wasting, and respiratory dysfunction. After washing of individual solid tissue samples, virus was extracted by extensive mincing in cold PBS.
[1]Tissue LDV titers were determined by limiting dilution titration, and are reported as ID_{50}/ml (plasma) or ID_{50}/g of wet weight (all other tissues).

The lungs as a reservoir for LDV replication

The emergence of LDV replication in the CNS just prior to onset of paralysis has been described previously as a likely neuropathogenic mechanism (Chen *et al*, 1999). This is significant since after the acute phase of infection, virus replication in most mouse tissues is almost undetectable, due to exhaustion of LDV-permissive cells about 24 h after infection (Plagemann and Moennig, 1992). Our data demonstrate the lung is an additional target organ for persistent LDV replication in ADPM. Although *in situ* hybridization does not yield a precise quantitative result, the proportion of LDV RNA-producing cells was estimated to approach 1/1000, representing a significant virus reservoir. The physiological significance of LDV replication in the lung is not known, but it may affect lung mechanics (and thereby the work of

breathing) and gas exchange characteristics (Arnulf *et al*, 2000). Future studies will investigate these possibilities throughout the course of infection.

Effects of virus infection on control of breathing

The decrement in ventilation of LDV- and cyclophosphamide-treated mice may be due to several factors. These include decreased metabolic demand, decreased body temperature, loss of respiratory muscle mass or functional capabilities, and/or abnormalities in CNS areas associated with regulation of breathing, metabolism, and regulation of body temperature (Mortola and Gautier, 1995). The underlying mechanisms by which local virus replication in the lung or within CNS contribute to respiratory failure in ADPM are unclear, but our data clearly show that the lung is an important target organ for ADPM-associated pathology, and that respiratory failure may represent the cause of death in mice with ADPM.

ADPM has interesting characteristics as a model for human degenerative diseases of viral or unknown origin: it is a dual virus disease (i.e., requires both LDV and retrovirus replication as necessary cofactors), and is dependent upon some degree of immunosuppression in the host (provided by natural aging or induced immunosuppression). Moreover, ADPM provides a model to study how viral-induced factors, nerve degeneration, and potential cytokine alterations, may contribute to the development of respiratory failure. LDV infection results in disturbance of various cytokines (Monteyne *et al*, 1993), as well as immunological alterations that may impact cytokine levels (Plagemann and Moennig, 1992). Cytokines such as tumor necrosis factor alpha and interferons regulate LDV replication (Plagemann and Moennig, 1992; Cafruny *et al*, 1997), and can also interact with hormones including leptin, growth hormone, and thyroid hormones that affect food intake, body weight, and metabolic rate (Boelen A *et al*, 1995; Wassen *et al*, 1996; Johnson *et al*, 1997; Sarraf *et al*, 1997; Raina and Jeejeebhoy, 1998; Finck and Johnson, 2000). Within the brain, cytokines influence the function of neurotransmitters and receptors such as opioids and gamma aminobutyric acid (GABA) that depress respiration and modulate metabolism (McCrimmon *et al*, 1995; Rothwell and Hopkins, 1995; Schlenker and Inamdar, 1995; Kalra *et al*, 1999; Vitkovic *et al*, 2000). Interleukins also affect neuroendocrine function causing the release of corticotrophin releasing factor that stimulates the release of adrenocorticotrophic hormone and ultimately glucocorticoids (Johnson *et al*, 1997). Both cytokines and glucocorticoids can compromise muscle function (Wilcox *et al*, 1996; Eason *et al*, 2000). The decrement in respiratory muscle function was observed in LDV- and cyclophosphamide-treated mice that exhibited a decreased tidal volume in air and in response to hypercapnia. Thus, elevated levels of circulating or locally produced cytokines, due either to the effects of virus replication or the antiviral immune response, may be responsible for a number of the physiological manifestations observed in ADMP. Further studies to investigate which cytokines are acting when and where in the CNS, lung, and on respiratory muscles are needed.

Another component to the development of ADMP may be direct targeting of neurons within the spinal cord and brain (Chen *et al*, 1999). Anderson and colleagues (1995a) showed that LDV enters the nervous system and replicates within anterior horn neurons only late in the infection process. Another potential target for LDV is the brain stem, which contains areas involved in regulation of breathing (Berger and Bellingham, 1995). The presence of lesions within phrenic and vagal nuclei, the nucleus tractus solitarius or ventral medullary centers associated with chemoreception is worth investigating. Although both ventilation and metabolism drop to a similar extent when APDM is evident, suggesting that these two parameters change in a parallel manner (Mortola and Gautier, 1995), the ventilatory response to hypercapnia was blunted. The inability of the mice with ADPM to respond to the ventilatory challenge further suggests that either chemoreception is absent and/or respiratory muscles are not functioning properly. Additional studies are needed to dissect these possibilities. Thus, this animal model may be useful to analyze the physiological and molecular mechanisms underlying the development of respiratory failure and to predict therapeutic interventions for degenerative diseases such as ALS having respiratory failure as a significant consequence.

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