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Comparison of the Ability of Lactate Dehydrogenase-Elevating Virus and Its Virion RNA To Infect Murine Leukemia Virus-Infected or -Uninfected Cell Lines

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Lactate dehydrogenase-elevating virus (LDV) has a strict species specificity. Cells or cell lines other than a particular subset of mouse primary macrophages which can support LDV replication in vitro have not been identified. LDV induces neurological disorders in old C58 or AKR strains, in which the involvement of multiple copies of the endogenous N-tropic murine leukemia virus (MuLV) genome and the Fv-1 locus of the mouse has been implicated. Our previous studies have demonstrated that LDV could infect and replicate in cell lines of the mouse or other species in vitro when they were infected with MuLV. The significance of and the precise mechanism underlying this phenomenon, however, remain unclear. We demonstrated in this study the efficient infection and replication of the virus in vitro by inoculation of its RNA mixed with liposome. No significant difference either in the efficiency of RNA transfection or in the ability to support its replication was observed among the various species' cell lines examined. In addition, by RNA transfection the virus replicated with equal efficiency in MuLV-infected and -uninfected cells or in macrophages derived from mice irrespective of their age. In contrast, the pattern of the infection by virus particles was quite different; LDV replication was observed only in macrophages (particularly from newborn mice) and MuLV-infected cells. By using various LDV isolates, it was demonstrated that the capability of replication between neurovirulent, LDV type C, and the other avirulent strains was almost the same in mouse cell lines when their RNA was introduced into the cells. Higher infectivity of LDV-C to MuLV-infected cells may be due to its efficient incorporation of the particles into MuLV-infected cells.

One of the significant features of lactate dehydrogenase-elevating virus (LDV) infection is characterized by the induction of acute poliomyelitis in particular strains of mice (old C58 and AKR [21, 23]). Genetic studies have demonstrated that for the induction of the disease, mice had to contain multiple copies of the N-type ecotropic virus genome and the *Fv-1*^{n} allele, suggesting that the replication of endogenous murine leukemia virus (MuLV) is an essential requirement (28, 29). In the spinal cord of paralyzed mice, the infection by and replication of LDV and a concomitant expression of the ecotropic MuLV gene have been demonstrated in motor neurons (6, 7, 17, 30, 33). Treatment of mice with immunosuppressive drugs (e.g., cyclophosphamide) and X-ray irradiation enhanced the MuLV gene expression and the incidence of the disease (7, 8). The precise molecular mechanism of the disease induction, however, remains unclear.

The other characteristic feature of LDV is its strict host cell specificity (31, 34). Its replication is limited only in a restricted set of mouse macrophages, with no recognized infection and replication in other animal species (26, 34). The underlying mechanism of this restriction is not known. Cells or continuous cell lines, including mouse macrophage lines other than primary mouse macrophages, which are permissive for LDV have not yet been identified. Our recent studies, however, have demonstrated that LDV replication became possible after cells were infected with MuLV (16). By using various dual-, ampho-, and xenotropic virus-infected cell lines, LDV has been shown to replicate in heterologous cell lines as long as they were infected with MuLV (12). The mechanism of the induction of LDV susceptibility has not yet been elucidated.

Notkins and Scheele obtained LDV nucleic acid from infected mouse serum by phenol-ether extraction and inoculated it into mice via an intracranial or intramuscular route (25). Because LDV replication was observed and the material was sensitive to nuclease but not to DNase, they concluded that the virus contained infectious, positive-sense RNA (2, 24). Target cells for RNA inoculated in vivo can be either parenchymal cells in the brain and muscle or histiocytes, tissue macrophages present in the connective tissue of those organs (15). Precise analysis of the target cell species has not been done. In the present study, we extracted RNA from virus particles, mixed it with liposome, and inoculated it into various cell lines or macrophages in vitro in order to identify the target cells for the infectious RNA. Infection of MuLV-infected or -uninfected cells by the virus particles was also compared with that by the infectious RNA.

Eight- and 1-week-old outbred ddY mice were used for the preparation of stock virus and peritoneal macrophages and 8-week-old female BALB/c mice were used for the monoclonal antibody (MAb) preparation, as described previously (16). The cell lines NIH 3T3 and SC-1 (mouse), NRK (rat), BHK (hamster), RK13 (rabbit), LLC-MK2 (monkey), and RD and HeLa cells (human) were maintained with Eagle's minimum essential medium (MEM) plus 10% fetal calf serum (12). Resident peritoneal macrophages were taken from newborn (approximately 2-week-old) and adult (8-week-old) ddY mice and cultured in vitro as described previously (16). The neurovirulent LDV type C (LDV-C) strain (from W. G. Stroop) and avirulent LDV-N (from A. L. Notkins), LDV-Nu (from H. Sato), LDV-R (from K. E. K. Rowson),

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LDV-P (from P. G. W. Plagemann) strains were kindly donated as indicated (16). The method for titration used to determine their infectivity was described previously (16, 31). Each strain was cloned by the limiting dilution method by inoculation of mice with the diluted virus. C58 MuLV, amphotropic virus (1504A), and the titers of the stock virus were described in our previous papers (12, 16). The methods of cell culture, infection with MuLV or LDV, and immunofluorescent (IF) staining were also described previously (12, 16). The percentage of antigen-positive cells was calculated after 5,000 to 10,000 cells were counted on each culture (12).

For purification of LDV and preparation of viral RNA, sera from 100 mice infected with each LDV strain 24 h earlier were collected by heart puncture. They were centrifuged on a 25% sucrose cushion at 25,000 rpm for 5 h with a Beckman SW27 rotor as described previously (4). The pelleted virus was suspended with 10 ml of TNE (0.01 M Tris-HCl [pH 7.0], 0.1 M NaCl, 1 mM EDTA) buffer, and its RNA was extracted with phenol-chloroform. After the extraction procedure was performed twice, RNA in the supernatant was precipitated with ethanol and suspended in TNE buffer containing 0.5% sodium dodecyl sulfate (SDS). The RNA was loaded onto a prepared 15 to 30% sucrose gradient in TNE buffer containing 0.5% SDS and centrifuged at 18,000 rpm for 16 h at 22°C with a Hitachi RPS 40T rotor (1). Enterovirus 70 RNA (35S [35]) and the sample from uninfected mouse sera, which were prepared by the same procedure as that for infected mouse sera, were centrifuged in a separate tube as the control. They were fractionated into approximately 0.6-ml portions, and the optical density at 260 nm in each fraction was determined. Simultaneously, 10 μl of each fraction was electrophoresed in a 1.0% agarose gel containing ethidium bromide. The fractions containing RNA determined from the assay for the optical density at 260 nm and the electrophoresis were collected, and the RNA was precipitated with ethanol and suspended in sterile distilled water. Approximately 10 to 30 μg of RNA was obtained from 100 infected mice by this procedure. For transfection of cells with RNA, lipofectin reagent (1 mg/ml; GIBCO BRL) was mixed with an equal volume of various amounts of RNA and incubated for 15 min at room temperature (9). Cells were washed three times with MEM supplemented with serum of reduced concentration (Opti-MEM I; GIBCO), inoculated with the RNA-liposome mixture, and incubated at 37°C for 5 h in 5% CO_2 in air. After incubation, an equal volume of MEM containing 20% fetal calf serum was added, and the cells were incubated further for 7 to 10 h. The medium was removed, and the cells were dried and fixed with acetone for 10 min for IF staining. For titration to determine virus yield, the cultures in a 24-well plate were frozen and thawed three times and centrifuged, and the infectivity of the supernatant was determined as described previously (16). To remove the residual RNA, the supernatants were treated with ribonuclease A (50 μg/ml; 5prime-3prime, Inc., Boulder, Colo.) before inoculation into the mice. RNA or virus samples were treated with ribonuclease A (50 μg/ml) or DNase I (100 μg/ml; Takara) at 20°C for 30 min. The method of preparation of the MAbs to LDV-N was described previously (16). From several antibodies obtained, anti-VP1 (anticapsid antigen, clone no. 8, immunoglobulin G2a) and anti-VP3 (anti-envelope glycoprotein, no. 36, immunoglobulin G2a) were selected for IF staining because of their strong reactivity with the corresponding LDV antigens as described previously (16). The cross-reactivity of the MAbs with the other LDV strains employed in the present study has been confirmed by IF staining and immunoblotting (unpublished results). A MAb to human parvovirus B19 with the same isotype as that of clones no. 8 and 36 was used as control. Rabbit anti-MuLV (Friend virus) serum was kindly donated by H. Yoshikura, as described previously (16).

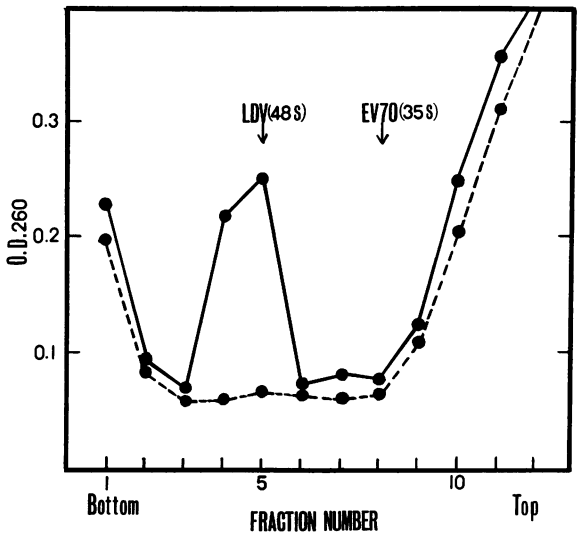

FIG. 1. LDV RNA (48S) purification profile on a 15 to 30% sucrose gradient containing 0.5% SDS. The dotted line indicates a sample obtained from uninfected mouse serum. Enterovirus 70 RNA (35S) was centrifuged in a separate tube. O.D.260, optical density at 260 nm.

The virion RNA was extracted from purified LDV particles from infected mouse sera and purified by a sucrose gradient centrifugation. Figure 1 shows the profile of the gradient purification. The fractions of the optical density peak were collected, and the RNA was concentrated. A portion of the sample (500 ng) was mixed with an equal volume of lipofectin. NIH 3T3 cells were inoculated with the mixture and stained with MAb (anti-VP1) 15 h posttransfection. Approximately 20% of the cells were LDV-antigen positive (Fig. 2A). With 2 μg of RNA, about 60 to 70% of the cells were LDV positive, and the percentage decreased in parallel with the decrease of the amount of RNA used (Fig. 3). A few positive cells were still seen when 1 ng of RNA with liposome was used (data not shown). When cells were transfected with RNA without liposome only a few positive cells were detected for 2.0 μg of RNA and no positive cells were detected for less than 2.0 μg of RNA (Fig. 3). A similar dose-response curve was obtained when mouse peritoneal macrophages were transfected with the RNA-liposome complex (Fig. 3). In contrast to the RNA transfection, LDV could not infect NIH 3T3 cells. In cultured macrophages, approximately only 5 to 7% of the cells were permissive for the virus as demonstrated earlier (13, 34). The transfection was dependent upon viral RNA but neither DNA nor viral proteins, as the ability of transfection was sensitive to nuclease A but not to DNase I (data not shown). LDV consists of three major structural proteins: capsid protein, VP1 (15 kDa), envelope protein, VP2 (18 kDa), and the envelope glycoprotein VP3 (25 to 45 kDa) (2). We examined whether envelope glycoproteins were produced as well as capsid antigens in the cells transfected with RNA. The number of antigen-positive cells observed for anti-VP3 MAb was similar to the number for the anti-VP1 MAb, though the strength of fluorescence by the former was considerably

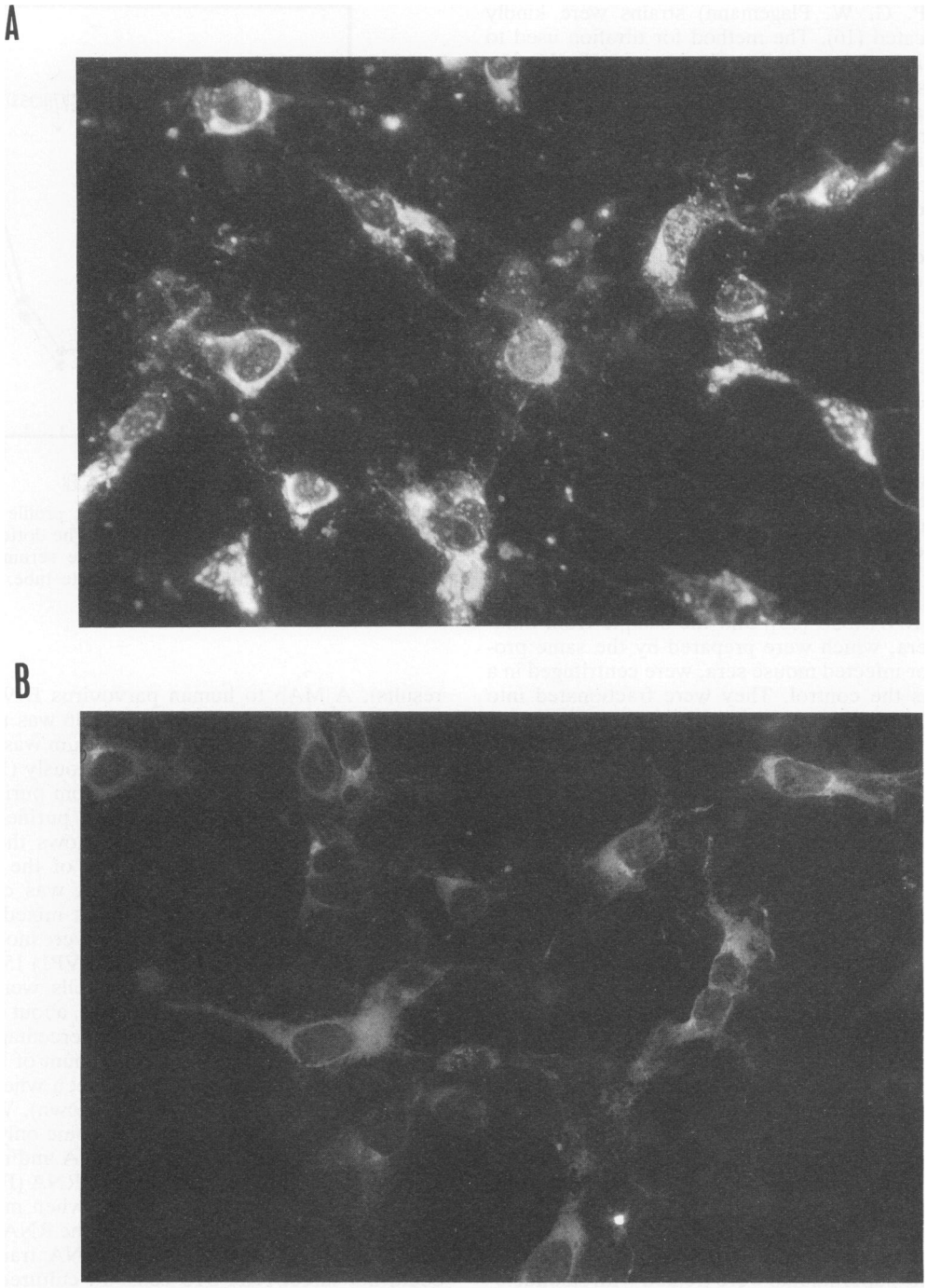

FIG. 2. IF staining of NIH 3T3 cells transfected with LDV-R RNA (500 ng) plus liposome. The cells were stained with anti-VP1 (A) or anti-VP3 (B) MAb 15 h posttransfection. The viral antigen-positive cells were not seen at all for the control MAb (anti-parvovirus B19, photograph not shown). Magnification, ×250.

weaker (Fig. 2B). The cells were transfected with RNA, and the culture supernatants were collected after being frozen and thawed 15 h after transfection. Mice were inoculated with the nuclease-treated serially diluted supernatant. The infectivity of the culture was increased up to 10^7 and 10^9 50% infective doses (ID_{50})/ml by 5 and 500 ng of RNA, respectively.

We next examined whether the cell lines of other species can be permissive for the RNA-liposome complex. There were LDV antigen-positive cells after RNA transfection (500 ng) in the cell lines of all species studied (Table 1). The percentage of LDV-positive cells was not significantly different among mouse cells, heterologous cell lines, and mouse macrophages. It has been reported that resident peritoneal macrophages from newborn mice (1 to 2 weeks old) are highly susceptible to LDV infection compared with those from mice of advanced age (14, 27). The percentage of

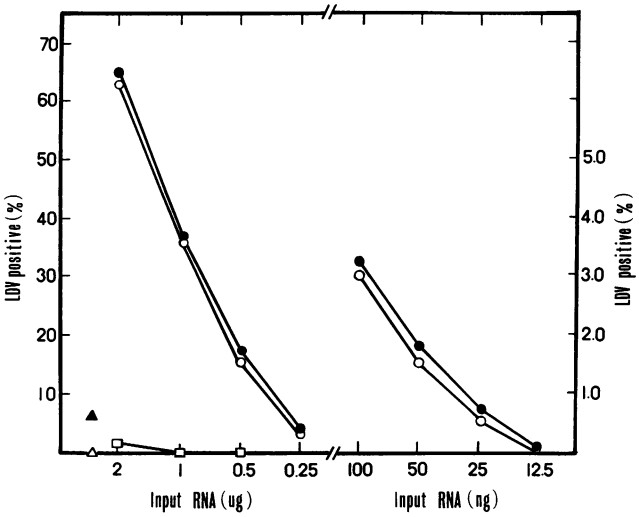

FIG. 3. Dose response between input RNA and percentage of LDV antigen-positive cells. NIH 3T3 cells (open symbols) or mouse peritoneal macrophages (from adult mice) (closed symbols) were either transfected with LDV-R RNA with (circles) or without (□) liposome or infected with LDV-R (10^4 ID_{50} per cell for NIH 3T3 cells [△] and 10^3 ID_{50} per cell for macrophages [▲]). The cells were stained with anti-VP1 MAb 15 h after transfection or infection. The percentage of positive cells was calculated after 5,000 to 10,000 cells were counted. Each point represents the mean percentage calculated from counts of two cultures.

antigen-positive cells, however, was approximately the same for those two macrophages after RNA transfection (Table 1).

Since cells became permissive for LDV when they were acutely infected with MuLV (16), we examined whether enhanced replication of LDV occurs in MuLV-infected cells by RNA transfection. There was no significant difference in the susceptibility to RNA between the MuLV-infected and -uninfected cell lines (Table 2). Among several isolates (LDV-N, -Nu, -R, and -P), LDV-C isolated from Ib leukemia cells was the most neurovirulent (21). Virion RNA was extracted from each virus and purified by centrifugation on the sucrose gradient (Fig. 1). C58 MuLV-infected or -uninfected NIH 3T3 cells were transfected with RNA (50 or 500 ng) from each isolate coupled with liposome or were infected with LDV. Table 3 shows that no significant difference in either the percentage of LDV-positive cells or the virus yield was seen among RNAs from the LDV isolates employed. In contrast, when the virus was used for infection of MuLV-infected cells, the LDV-C strain was the most infectious as long as an equivalent amount of input virus was used (multiplicity of infection [MOI], 10^4 ID_{50} per cell). There was no significant difference in the infectivity among the avirulent strains. This difference in the infectivity of LDV isolates, however, was not seen when primary cultured macrophages were used (Table 3).

One of the most significant features observed in this study was the difference in the infectivity between LDV and its virion RNA for the various cell types employed. LDV replicates only in a small subpopulation of cultured mouse macrophages, and so far, no cell lines, including mouse macrophage lines, which can support LDV replication have yet been found (16, 32). LDV infection and replication, however, became possible in the cell lines of various species when the cells were infected with MuLV previously (12). When the cells were transfected with virion RNA plus liposome, replication of LDV was seen in all cell types tested, i.e., macrophages from newborn and adult mice and cell lines from mouse and heterologous species which were MuLV infected or uninfected. The number of cells in which LDV replicated was approximately the same among the various cell types transfected and increased in parallel with the increase in the amount of RNA added. The neurovirulent strain, LDV-C, among various LDV isolates was the most infectious to MuLV-infected cells, and this was due in part to the difference in adsorption rates of the virus particles (16). The present study has demonstrated that no significant difference in the infectivity to MuLV-infected or -uninfected cell lines was observed among these isolates when the cells were transfected with virion RNA from each strain. These results suggest that autonomous virus gene expression may be possible once the RNA has penetrated the cell membrane, is released, and is exposed to the cytoplasm. This expression occurs in cells irrespective of MuLV infection. Presumably, the role of the MuLV gene product(s) in the infection by LDV may be to facilitate one of the processes from the adsorption of virus to the release of its genetic material into the cytoplasm.

Even though with an inoculation of a large amount of virion RNA (2 μg per culture) there was a small number of LDV-positive cells, the efficiency of transfection was significantly enhanced when RNA was mixed with the liposome lipofectin (9). On the basis of the LDV titer in the mouse sera and the amount of RNA obtained from these materials, the efficiency of RNA transfection with lipofectin in MuLV-infected NIH 3T3 cells was estimated to be approximately 100-fold lower than that of the LDV-C virion. Rather, we anticipated that the efficiency of RNA transfection would be significantly different among the wide variety of cell types examined, because it had been noted previously that the efficiency of DNA transfection and expression is variable, depending on the cell types (9). This may indicate that the cellular capacity to uptake RNA and to support LDV repli-

TABLE 1. Replication of LDV in various species' cell lines[a]

Virus source	% LDV antigen-positive cells in:									
	NIH 3T3	SC-1	NRK	BHK	RK-13	LLC-MK2	RD	HeLa	Macrophages from mice:	
									2 weeks old	8 weeks old
LDV-R	—[b]	—	—	—	—	—	—	—	62.6	5.8
LDV-R RNA without liposome	—	ND[c]	—	ND	ND	ND	ND	ND	—	—
LDV-R RNA with liposome	18.0	17.2	15.0	19.3	21.3	18.4	16.3	18.0	24.0	22.3

[a] The cell lines from various species or mouse peritoneal macrophages (from newborn or adult mice) were infected with LDV-R (MOI, 10^3 ID_{50} per cell) or transfected with LDV-R RNA (500 ng) with or without liposome. The cells were stained with anti-VP1 MAb 12 to 15 h after infection or transfection.

[b] —, not detectable.

[c] ND, not done.

TABLE 2. Difference in infectivity between LDV and its RNA in MuLV-infected or -uninfected cell lines[a]

Cell line	% LDV antigen-positive cells for:				
	LDV infection at:		LDV RNA transfection		
	10^3 ID_{50}/cell	10^4 ID_{50}/cell	500 ng − lipofectin	50 ng + lipofectin	500 ng + lipofectin
MuLV-uninfected NIH 3T3	—[b]	—	—	2.1	17.3
NRK	—	—	—	1.5	15.1
C58-MuLV-infected NIH 3T3	1.5	26.2 (>90)[c]	—	2.5	16.0
Amphotropic MuLV-infected NRK	<0.1	4.8 (~50)	—	1.8	13.8

[a] MuLV-infected or -uninfected NIH 3T3 and NRK cells were infected with LDV-C (MOI, 10^3 and 10^4 ID_{50} per cell, respectively) or transfected with LDV-R RNA (50 or 500 ng) with or without liposome. The cells were stained with anti-VP1 MAb 15 h after infection or transfection. The cells were infected with each MuLV at an MOI of 0.1 to 1.0 PFU or focus-forming units per cell 7 days before inoculation of LDV as described in reference 17.

[b] —, not detectable.

[c] The numbers in parentheses indicate the percentage of MuLV antigen-positive cells stained with anti-MuLV serum in the separate cultures.

cation inside the cells was not significantly different among the different cell types employed in the present study.

The limited susceptibility of cultured peritoneal macrophages of mice to LDV infection has been considered to be due to a receptor for the virus (3, 7, 13, 18). One candidate has been the class II major histocompatibility complex antigen (Ia antigen), but it is not known whether this is the major receptor for LDV (3, 13). Because LDV infection of macrophages could be enhanced by complex formation of the virus with antiviral immunoglobulin antibody, the Fc receptors on the cells can be alternative receptors for LDV (4, 14). While in MuLV-infected cells the infectivity of neurovirulent LDV-C was the highest among several isolates, in macrophages this difference was not observed (16, 20). This result suggests that the mode of LDV infection of MuLV-infected cells is different from that of macrophages (16). By RNA transfection, it was demonstrated that macrophages had a susceptibility to LDV infection similar to that of the cell lines of the various species tested. Macrophages from newborn mice (1 to 2 weeks old) are highly susceptible compared with those from adult mice (14, 27). However, the susceptibility of those two kinds of macrophages to RNA transfection was not significantly different.

LDV has an approximately 14-kb, single-stranded RNA as genetic material (2). Recent studies of the strategy of LDV replication have shown that its genome is expressed via formation of a 3′-coterminal-nested set of six or seven subgenomic mRNAs (19). This and other properties, i.e., genome organization and the number and size of structural proteins, resemble those of equine arteritis virus (36) and possibly simian hemorrhagic fever virus (30). Although they also share the nature of preferential replication in macrophages, LDV does not have the ability to infect continuous cell lines in which the replication of the others could be detected (11, 37). Some parts of the nucleotide sequence corresponding to individual LDV proteins have been analyzed, and the amino acid alignments of LDV isolates with different neurovirulency have been compared (10, 19). It is not known, however, which component(s) of the viral structural or nonstructural proteins contributes to the different virulency. So far, no significant difference in the capacity of replication in the peripheral organs as well as in the cultured macrophages has been noted among these strains. It is possible that the difference in virulency is due to the capacity of replication in the central nervous system (5, 20, 22, 23), though the differences in the immunogenicity among various LDV isolates and in the antiviral immune response may affect the neurovirulency (30). The isolation of a variant which has lost its virulency after repeated passages of neurovirulent LDV in BALB/c mice without significant effect on the replication in the peripheral organs (22) may indicate that the ability to replicate in the central nervous

TABLE 3. Infectivity of different LDV strains and their RNAs in MuLV-infected or -uninfected cell lines[a]

Cell line or macrophages	% LDV antigen-positive cells by infection or transfection with LDV type as indicated														
	N			C			Nu			R			P		
	LDV	RNA		LDV	RNA		LDV	RNA		LDV	RNA		LDV	RNA	
		50 ng	500 ng		50 ng	500 ng		50 ng	500 ng		50 ng	500 ng		50 ng	500 ng
NIH 3T3															
C58 MuLV uninfected	—[b]	1.0	12.5	—	1.2	13.7	—	2.2	17.9	—	1.6	15.8	—	1.8	15.5
C58 MuLV infected	0.4 (7.5)[c]	1.3	16.1 (9.0)	25.5 (9.0)	1.2	14.3 (9.0)	0.6	1.8	19.2	0.5 (7.5)	1.5	17.0 (9.0)	0.5	1.3	15.4
Macrophages	6.2	1.2	15.1	5.5	1.6	15.7	4.7	1.5	18.8	6.6	1.9	20.0	5.5	1.2	16.0

[a] C58 MuLV-infected or -uninfected NIH 3T3 cells and peritoneal macrophages (from 8-week-old mice) were infected with various LDV isolates at an MOI of 10^4 (for cells) and 10^3 (for macrophages) ID_{50} per cell or transfected with RNA from each virus. The cells were dried, fixed, and stained with anti-VP1 MAb 15 h after infection or transfection. In a separate culture, the MuLV antigens were stained by anti-MuLV serum. Over 90% of the cells were antigen positive.

[b] —, not detectable.

[c] Virus yields (log_{10} ID_{50}/ml) from MuLV-infected NIH 3T3 cells infected with LDV or transfected with RNA are indicated in parentheses and were determined as described in Materials and Methods.

system is different and separate from that in the periphery (presumably, macrophages). Our present observation that there is a discrepancy between RNA and virion infectivity among LDV strains may suggest that the difference in viral structural proteins affects the capability to replicate infection in MuLV-infected cells but not in macrophages.

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