Inhibition of Replication of Lactic Dehydrogenase Virus by Actinomycin

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Lactic dehydrogenase virus replicated rapidly in monolayers of primary mouse embryo cells and reached a titer of 10^8 mean infective dose per ml within 18 h after infection. Despite the high virus yield, cytopathology was not observed. Examination of the tissue culture media failed to reveal any evidence of interferon, but the virus was found to be as sensitive to mouse interferon as vesicular stomatitis virus. Incubation of mouse embryo cells with actinomycin D markedly inhibited viral replication, whereas cytosine- β -D-arabinofuranoside and 5-fluorodeoxyuridine had no effect on replication. These findings indicate that new DNA synthesis is not required but suggest that the intact function of cellular DNA may be required for lactic dehydrogenase virus replication.

Lactic dehydrogenase virus (LDV) is an ether-sensitive RNA virus about 40 nm in diameter (19). Infection of mice with LDV results in a life-long viremia and an increase in the activity of a number of enzymes in the plasma. The increase in activity is at least in part due to the impaired clearance of these enzymes from the circulation (19). The infection also is characterized by alterations in the function of the immune system, circulating infectious virus-antibody complexes, and the development of a mild form of immune complex-type of glomerulonephritis (19, 21, 22, 24). Little is known about the replication of LDV or the factors responsible for the persistence of the infection. The present investigation was undertaken to study the effect of metabolic inhibitors on the production of infectious virus and the sensitivity of the virus to interferon.

MATERIALS AND METHODS

Cell culture and media. Primary cultures of mouse embryo (ME) cells were prepared by trypsinization of minced 17- to 19-day-old CAF-1 embryos. The monolayers were grown to confluency in plastic petri plates (50-mm diameter) or plastic flasks (75-cm² surface) with Eagle minimal essential medium (MEM) supplemented with 10% calf serum. Cells cultured for 24 to 48 h were used throughout the experiments.

L-929 mouse fibroblasts were purchased from Flow Laboratory, Rockville, Md. and were grown in Eagle MEM with 5% calf serum.

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Viruses. The source of LDV and the virus assay have been described elsewhere (20). Infected plasma obtained from CAF-1 mice 24 h after they had been injected with LDV were diluted and inoculated onto ME cell monolayer cultures. The culture media were harvested 24 h after infection, clarified by low-speed centrifugation, and stored at -60°C until used. Four- to six-week-old CAF-1 mice were used to determine the titer of the virus. Serial 10-fold dilutions of the virus were made in MEM, and each dilution was injected intraperitoneally into 10 mice. The mice were bled from the orbital plexus 72 to 96 h later and tested for the characteristic five- to tenfold increase in plasma lactate dehydrogenase enzyme activity. Infectivity titers by this method ranged from $10^{7.5}$ to $10^{8.5}$ ID₅₀ (mean infectious dose) per ml of the stock virus pool.

The Indiana serotype of vesicular stomatitis virus (VSV), provided by Robert R. Wagner (University of Virginia School of Medicine, Charlottesville, Va.), was grown and assayed on monolayers of L cells, and the results were expressed as PFU per ml. VSV was used as a control virus for the characterization of LDV and as a challenge virus in the interferon assay.

Interferon. The procedures for production and assay of interferon are similar to those reported previously (34), except that the mouse interferon used in this study was not purified. In brief, primary cultures of ME cells were infected with the CG strain of Newcastle disease virus (640 hemagglutinin units per ml). After 1 h of virus adsorption, the cells were washed twice and incubated in MEM without calf serum. Media from the cultures were collected at 16 h after infection, clarified by low-speed centrifugation, and then recentrifuged at $100,000 \times g$ for 4 h. The

supernatant fluid was dialyzed against 0.85% NaCl solution at pH 2.0 for 24 h and then redialyzed against MEM. The titer of this crude interferon preparation, as measured by plaque inhibition of VSV on L cells, was about 640 PDD₅₀/ml (mean plaque-depressing doses). The interferon was stored at 4 C and appropriately diluted in MEM before use.

Chemicals. ³H-uridine (26.1 Ci/mmol) and ³H-thymidine (18.4 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Actinomycin D was purchased from Merck, Sharpe and Dohme, West Point, Pa. Cytosine- β -D-arabinofuranoside (ara-C) and 5-fluorodeoxyuridine (FUdR) were obtained from Roche Company, Rahway, N. J.

Measurement of DNA and RNA synthesis. ME monolayers in plastic plates were covered with 2 ml of MEM (without serum) containing either ³H-thymidine (2 μ Ci/ml) or ³H-uridine (5 μ Ci/ml). After incubation at 37 C for 1 h, incorporation of labeled precursors was stopped by washing the cells with ice-cold Eagle balanced salt solution. The cells then were suspended in 1 ml of the same solution and disrupted by freezing and thawing and by sonic treatment. The cell contents were precipitated and washed twice with 5% trichloroacetic acid and once with ethanol-ether (1:1). After drying, the precipitate was dissolved in 0.5 ml of *N*-chlorosuccinimide at 60 C for 30 min, diluted in toluene-based fluors, and the radioactivity was counted by scintillation spectrometry.

RESULTS

Replication of LDV in ME cells. The replication of LDV at three different multiplicities of infection is shown in Fig. 1. After a lag period of several hours, the virus multiplied exponentially and, at the higher multiplicities (2 and 21), reached a maximum titer within 24 h. More detailed experiments (data not shown) revealed that at a multiplicity of 10 the virus began to multiply within 9 h and reached a maximum titer of 10⁸ ID₅₀/ml at 16 to 18 h after infection. Despite the high virus yield, the cells did not show any signs of cytopathology. At lower multiplicities, maximum virus yield was not reached until 48 h after infection.

Attempts to detect interferon in LDV-infected cell cultures. At 12, 24, and 48 h after infection of ME cells with LDV, medium was removed, treated as described in Materials and Methods, and tested for antiviral activity by reduction of either VSV plaques or total VSV yield. Both ME cells and L cells were used to titer interferon because preliminary observations showed that the titer of a known preparation of mouse interferon (induced by Newcastle disease virus in ME cells) was at least 12-fold greater when it was assayed in L cells than ME

FIG. 1. Replication of LDV in ME cells infected at different multiplicities. ME monolayers containing 5×10^6 cells were infected with LDV at a multiplicity of 20, 2.0 or 0.2 ID₅₀ per cell. After adsorption for 1 h, monolayers were washed three times, replenished with 3 ml of MEM containing 10% calf serum, and then incubated at 37 C. At the indicated times, media obtained from triplicate cultures at each multiplicity were assayed for virus infectivity in mice.

cells. None of these methods, however, revealed detectable levels of interferon in media from LDV-infected cultures. Moreover, there was no evidence of viral interference. Preinfection of ME cells for 20 h with LDV did not interfere with superinfection by VSV as measured by plaque reduction or virus yield.

Sensitivity of LDV to interferon. The failure to find interferon in the culture fluid did not exclude the possibility that if LDV were extremely sensitive to interferon, small amounts of endogenous interferon might affect the extent of viral replication both in vivo and in vitro (9, 11, 20). Sensitivity of LDV to interferon was examined and compared to that of VSV which is known to be very sensitive to mouse interferon (29). As shown in Table 1, different concentrations of mouse interferon suppressed the replication of LDV to about the same extent as that of VSV.

In view of the high sensitivity of LDV to interferon, the possibility that endogenous interferon might inhibit LDV replication was further investigated. Actinomycin was used to inhibit the induction by LDV of hypothetical endogenous interferon. It was reasoned that if actinomycin itself did not interfere with viral replication, then the inhibition of endogenous interferon by actinomycin might result in enhancement of virus yield (16, 30). Monolayers of ME cells were treated for 1 h at 37 C with 1 μ g of actinomycin in 1 ml of Eagle MEM and then washed. Control

TABLE 1. *Comparative sensitivity of LDV and VSV to mouse interferon^a*

Concentration of interferon	LDV ^b		VSV ^c	
	(PDD ₅₀ /ml)	ID ₅₀ /ml	Control (%)	PFU/ml
0		1.1×10^7	100	7.0×10^6
15		2.6×10^6	23	1.1×10^6
30		2.1×10^6	19	7.0×10^5
60		1.3×10^6	12	9.0×10^5
120		8.0×10^4	<1	6.5×10^5

^a Duplicate ME monolayers containing 4.5×10^6 cells were exposed to each concentration of interferon for 18 h prior to being infected either with LDV or with VSV at a multiplicity of 10.

^b LDV was collected 18 h after infection and titrated in mice.

^c VSV was collected 12 h after infection and titrated by plaque assay on L cells.

monolayers were treated similarly without actinomycin. Both actinomycin-treated and control cultures then were infected with LDV and incubated at 37 C in MEM with 10% calf serum. Titration of the virus at 12 h after infection showed that actinomycin did not enhance the viral yield but instead significantly suppressed the viral yield. These preliminary observations prompted us to perform more detailed experiments to confirm the inhibitory effect of actinomycin on LDV replication.

Effect of actinomycin on LDV replication.

The data in Fig. 2 show that actinomycin at a concentration of 0.125 $\mu\text{g/ml}$ inhibited RNA synthesis of ME cells by approximately 90% and also reduced the yield of infectious LDV to less than 2% of that from cells not exposed to actinomycin. The same amount of actinomycin did not inhibit VSV replication. Moreover, the cells remained intact during the whole test period (20 h), suggesting that actinomycin was not exerting a toxic effect on the ME cells. The inhibitory effect of actinomycin on LDV was not increased with higher concentrations of actinomycin, but the higher levels of actinomycin did cause a slight change in the morphology of uninfected ME cells. These changes, however, was associated with little or no inhibition of VSV replication.

The dependence of LDV replication on DNA-dependent RNA was further investigated by adding actinomycin to ME cells at various times after infection. Based on the data obtained from Fig. 2, actinomycin at a concentration of 0.1 $\mu\text{g/ml}$ was used because it suppressed the synthesis of DNA-dependent RNA but did not inter-

fere with the replication of actinomycin-resistant VSV. The data in Table 2 show that the greatest suppression of LDV replication occurred when actinomycin was introduced immediately after virus adsorption (0 h). Significant inhibition of replication also occurred when actinomycin was introduced at 3, 6, and 9 h, but relatively little inhibition was detected thereafter. These results indicate that the replication of LDV in ME cells is specifically suppressed by actinomycin during the early stage of the infection, suggesting that the virus requires some function of DNA for the initiation of its replication in ME cells.

Effect of ara-C and FUDR on LDV replication. To see whether the synthesis of new DNA was needed in the replication of LDV, ME cells

FIG. 2. *Comparative effects of various concentrations of actinomycin on cellular RNA synthesis and replication of LDV and VSV. ME monolayers containing 5×10^6 cells were incubated for 1 h at 37 C in MEM containing various concentrations of actinomycin. Cells not incubated with actinomycin served as controls. Triplicate cultures in each group were washed and infected with LDV at a multiplicity of 10 ID₅₀ per cell or VSV at a multiplicity of 10 PFU per cell. After adsorption for 1 h at 37 C, the cultures were washed and reincubated in 5 ml of MEM containing 10% calf serum. Virus yields were determined at 18 h after infection. Another group of cultures similarly treated with each concentration of actinomycin was not infected but exposed for 1 h to MEM containing ³H-uridine, 5 $\mu\text{Ci/ml}$. Incorporation of ³H-uridine into the acid-precipitable fraction was measured, and the results were expressed as percentage of control at each concentration of actinomycin. In the absence of actinomycin, ³H-uridine incorporation was 136,668 counts per min per culture, LDV titer was 1.7×10^6 ID₅₀/ml and VSV titer was 5.3×10^6 PFU/ml.*

TABLE 2. *Effect of time of addition of actinomycin on LDV replication^a*

Addition of actinomycin (h after viral adsorption)	Virus titer (ID ₅₀ /ml, log 10)		Inhibition of viral replication by actinomycin (%)
	Control	Actinomycin	
0	8.0	6.3	98
3	8.0	6.9	92
6	7.9	7.2	80
9	7.6	7.0	75
12	7.4	7.3	20
15	7.2	7.0	37
18	7.2	7.0	37

^a ME cell monolayers were infected with LDV at a multiplicity of 10 ID₅₀ per cell. After adsorption for 1 h, monolayers were washed and incubated at 37 C in MEM supplemented with 5% calf serum. Immediately after adsorption (0 h) and at 3-h intervals thereafter, six monolayers were washed and three of them were reincubated in media containing actinomycin (0.1 µg/ml). The other three monolayers (controls) received media without actinomycin. All the culture media were harvested 24 h after infection and titrated for virus.

were exposed for 1 h to varying concentrations of ara-C, a potent inhibitor of DNA synthesis (2). The cells then were either labeled with ³H-thymidine (2 µCi/ml) or infected with LDV. At the end of 1 h, DNA was extracted from the appropriate monolayers, and the incorporation of ³H-thymidine was measured. Virus yield was determined 18 h after infection. The data in Table 3 show that although cellular DNA synthesis was inhibited by up to 99%, ara-C had little if any effect on viral replication. To be certain that the inhibitory effect of ara-C had not waned during the course of the experiment (2), cells were pulsed for 1 h with ³H-thymidine at 22 h after initial exposure to ara-C (10⁻⁴ mol/ml). Although the inhibitory effect of ara-C was somewhat reduced, DNA synthesis still was suppressed by approximately 60%. These observations suggest that DNA synthesis in ME cells was effectively suppressed by ara-C throughout the period of virus synthesis and that the ability of LDV to replicate in the presence of ara-C was not due to a rapid reduction in the effect of ara-C. Similarly, FUDR, which effectively inhibits DNA synthesis by acting on thymidylate synthetase (7), failed to inhibit LDV replication. Cells were treated with varying concentrations of FUDR for 1 h and then infected with LDV at a multiplicity of 10 ID₅₀ per cell. Virus titers were determined 18 h after infection. The data in

Table 3 show that although FUDR inhibited DNA synthesis, viral replication was not affected.

DISCUSSION

LDV has been successfully propagated in primary cell cultures from adult mice (e.g., macrophages, lung, spleen) and embryos (1, 9, 11, 12, 23, 32). Considerable variation, however, has been reported in viral yield and duration of replication (9, 11, 14). In our experiments, primary ME cells prepared from 17- to 19-day-old CAF-1 embryos routinely yielded 10⁸ ID₅₀/ml within 24 h after infection. Despite the high viral yield, cytopathology was not observed, and numerous attempts to detect interferon have failed. Similar attempts to detect interferon in the media from LDV-infected macrophage cultures also have failed (9, 12). Low levels of interferon, however, have been reported in the blood of LDV-infected mice for up to 48 h after infection (3, 12, 13), but little if any interferon could be

TABLE 3. *Effect of ara-C and FUDR on LDV replication and cellular DNA synthesis^a*

Concentration of inhibitors (mol/ml)	DNA synthesis (% of control)	Virus (ID ₅₀ /ml, log 10)
Ara-C		
None		8.1
10 ⁻⁶	43	8.1
10 ⁻⁵	24	8.1
10 ⁻⁴	6	8.0
10 ⁻³	1	7.9
FUDR		
None		8.3
10 ⁻⁵	100	8.3
10 ⁻⁴	11	8.2
10 ⁻³	13	8.3

^a ME monolayers containing 3 × 10⁶ cells were incubated for 1 h at 37 C in 3 ml of MEM containing various concentrations of ara-C or FUDR. Cells not incubated with inhibitors served as controls. Triplicate cultures from each group were washed and reincubated for 1 h in 2 ml of MEM containing ³H-thymidine, 2 µCi/ml. Incorporation of ³H-thymidine into the acid-precipitable fraction was determined and the results were expressed as percentage of the control at each concentration of inhibitor. In the absence of inhibitors, ³H-thymidine incorporation was 225,770 and 207,976 counts per min per culture for ara-C and FUDR, respectively. Another group of cultures similarly treated with various concentrations of inhibitors were infected with LDV at a multiplicity of 10 ID₅₀ per cell, washed three times, and incubated in MEM supplemented with 5% calf serum. At 18 h after infection, the virus titer in the medium of each culture was determined.

detected thereafter. In contrast to its inability to induce substantial amounts of interferon, LDV appears to be highly sensitive to mouse interferon; in fact, as sensitive as VSV. Thus, the poor interferon-inducing capacity of LDV may be one of the factors responsible for the life-long persistence of this infection.

Although it had been reported by others that the replication of LDV is not affected by actinomycin (10, 12), our experiments showed that treatment of ME cells with actinomycin markedly reduced the virus yield. Since the assay for LDV involves inoculation of animals, we used 10 mice for each serial 10-fold virus dilution to ensure accuracy and reproducibility (20). Moreover, it is known that the effect of actinomycin on some RNA viruses varies with viral strains (27), the cell type (15, 33), and cultural conditions (8). Therefore, the sensitivity of an RNA virus to actinomycin must be carefully evaluated by experiments in which actinomycin is used at a concentration which effectively inhibits DNA-dependent RNA synthesis but does not inhibit the capacity of the cell to support the replication of RNA viruses known to be resistant to actinomycin. Our experiments showed that 0.1 μ g of actinomycin inhibited both the synthesis of cellular RNA and the replication of LDV, but did not affect the replication of VSV. Nonetheless, since primary ME cultures contain a mixture of cells, it is possible that LDV may be growing in one cell type and VSV in another. Preliminary data based on infectious center assays suggest that LDV is present in less than 2% of the ME cells. Thus, the use of VSV as a control for LDV is valid so long as the VSV-infected and LDV-infected cells in the ME cultures do not differ markedly in their sensitivity to actinomycin.

Our experiments also showed that the effect of actinomycin was most pronounced during the early phase of the infection and that inhibitors of DNA synthesis had no effect on viral replication. Moreover, virions prepared from ME cultures or from the plasma of infected mice did not contain RNA-dependent DNA polymerase activity (M. Hatanaka, S. Yamazaki, and A. Notkins, unpublished data). Further evidence that a virion-associated polymerase is not needed in the replication of LDV comes from earlier experiments which showed that it was possible to isolate an RNase-sensitive infectious RNA from LDV (18).

Although the synthesis of new DNA is not required in the replication of LDV, the inhibitory effect of actinomycin suggests that some unknown host function dependent on intact cellular

DNA is needed to initiate the replication of LDV in ME cells. Suppression of viral replication by actinomycin, but not by inhibitors of DNA synthesis, has been reported with other RNA viruses (4-6, 28, 31). The precise role of cellular DNA in the replication of these viruses is still not clear. Because of the varied effects of actinomycin on cell function (17, 25, 26), corroborative evidence obtained by independent experimental means is needed to substantiate and elucidate the possible role of cellular DNA in LDV replication.

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