Propagation of Lactic Dehydrogenase-Elevating Virus in Cell Culture.* (30991)

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Infection of mice with the lactic dehydrogenase-elevating virus (LDV) is recognized by a 5-10-fold increase in the plasma lactic dehydrogenase (LDH) activity of recipient mice, 3-4 days after infection(1-5). Several additional enzymes become elevated in plasma of infected mice(4,6). The elevation of plasma enzymes following infection is preceded by 1-2 days by the appearance of large quantities of virus in blood(7,8). Elevated levels of LDH and high concentrations of virus persist in the plasma of infected mice indefinitely without causing overt disease(1, 7,8).

The present investigation was undertaken to determine the ability of the virus to multiply in or alter cells in culture. The results indicate that the virus multiplies in primary cultures of mouse tissues and that infected cultures produce virus for many weeks without evidence of cellular damage. The LDV, however, does not multiply in established cell lines of murine or human origin.

Materials and methods. Animals. Female mice of strains C3H and CFW, 4-6 weeks of age, (Carworth Farms, New York) were used. Precautions were taken to prevent spontaneous infection with LDV as described previously(9).

Solutions and media. Solution 111 (S111) contained the following in mM: sodium chloride, 116; potassium chloride, 5.5;magnesium sulfate, 0.8; monosodium phosphate, 0.9; disodium phosphate, 0.2; calcium chloride, 1.8; sodium phosphate (pH 7.4), 20; glucose, 5.6; and streptomycin sulfate, 0.008. Solution 148 (S148) and medium 58 were prepared as described previously(9). The solution of trypsin contained 0.5% (W/V) trypsin (Difco 1:250) in S148.

Preparation of cell cultures. Primary cultures of normal mouse spleen, lung, liver, and embryo were prepared as follows. Mice were killed by chloroform inhalation and the appropriate tissues were removed aseptically and washed twice in S111. The tissues were finely minced, suspended in 40-50 volumes (W/V) of trypsin solution and incubated at 37°C with frequent shaking. At intervals of 20-30 minutes the larger pieces of tissue were allowed to settle, and the supernatant was removed and replaced with fresh trypsin. Usually 4-5 changes of trypsin were required to disperse all of the cells. The first supernatant was discarded and the others were diluted with an equal volume of medium 58 as soon as prepared. These were finally pooled and centrifuged at 600 *g* for 5 minutes. The cells were resuspended in medium 58 at a density of approximately 10^6 cells/ml and 5 ml aliquots were transferred to each of a series of T-30 flasks(10). The flasks were gassed with a mixture of 5% CO_2, 20% O_2, and 75% N_2 and incubated at 37°C. The medium was replaced after 1-3 days and twice weekly thereafter. When complete monolayers had formed, subcultures were prepared as described earlier(11). Cell numbers were estimated by direct count in a hemocytometer chamber.

Lactic dehydrogenase-elevating virus. The LDV was isolated from a CFW mouse bearing Sarcoma 180(9). Concentrations of virus were determined by infectivity titrations as described previously(7). Culture fluids containing virus were stored at −17°C.

Assay for lactic dehydrogenase. LDH activity was determined spectrophotometrically as described previously(4,12).

Analysis of cells for incorporation of uridine-2-C^{14} into RNA and DNA. Cultures of mouse cells were labelled with uridine-2-C^{14} (New England Nuclear Corp., Boston, Mass.)

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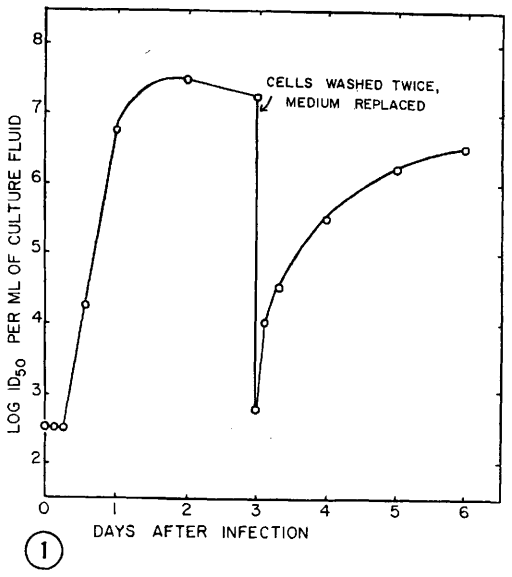
as described in the text. After removal of the medium the cell layer (2-4 $\times$ 10^6 cells/T-60 flask) was washed twice with 10 ml of S111. The cells were suspended in 10 ml of 0.5 N perchloric acid (PCA) at 0°C and centrifuged at 1500 *g* for 5 minutes. The resulting precipitate was washed twice with 5 ml of 0.5 N PCA (0°C) and once with 5 ml ether-ethanol (1:1). The pellet was dissolved in 0.9 ml of 0.4 N sodium hydroxide and incubated at 37°C for 18 hours. The sodium hydroxide was neutralized by addition of 0.1 ml of 4 N hydrochloric acid and the DNA and protein were precipitated by addition of 1 ml of 2 N PCA. The precipitate was removed by centrifugation at 1500 *g* for 5 minutes and washed once with 0.5 ml of 0.5 N PCA. The supernatants were pooled (RNA fraction). The pellet was extracted once with 1.5 ml and once with 1 ml of 0.5 N PCA at 70°C for 30 minutes and the two extracts pooled (DNA fraction). The concentrations of nucleic acid were estimated spectrophotometrically, assuming $E^{1\%}_{cm}$ = 280 at 260 mμ for hydrolyzed RNA and $E^{1\%}_{cm}$ = 260 at 260 mμ for DNA. These extinction coefficients were estimated with known samples of thymus DNA and yeast RNA. All absorbency values of the DNA fractions were corrected for haze by subtracting the absorbency at 330 mμ from that at 260 mμ. When cells grown in the presence of thymidine-2-C^{14} were fractionated according to above procedure, 97% of the incorporated radioactivity was recovered in the DNA fraction. The amount of radioactivity in each fraction was determined in a TriCarb liquid scintillation spectrometer (Packard Instrument Co., LaGrange, Ill.) as described elsewhere(13).

Incorporation of labeled valine into protein. Cultures of mouse cells were labeled with valine-U-C^{14} (New England Nuclear Corp., Boston, Mass.) as described in the text. The medium was then removed and the cell layer (1-2 $\times$ 10^6 cells/T-30) was washed three times with 5 ml of S111. The cells were dissolved in 2.5 ml of 0.2 N sodium hydroxide by incubating at 100°C for 10 minutes. The concentration of protein in the solution was determined by the method of Lowry *et al* (14).

Results. Multiplication of the LDV in cultures of mouse tissues. The LDV was found to multiply in primary cultures of lung, spleen, liver and embryo. A typical growth curve for LDV in a primary culture of spleen is presented in Fig. 1. Most of the virus was found in the medium. The continued production of virus is indicated by the fact that a second increase in the concentration of virus was observed immediately upon renewal of the medium (Fig. 1). The LDV was serially propagated through 16 passages in primary lung and spleen cultures without loss in infectivity. Experiments of the type illustrated in Fig. 1 were extended for prolonged intervals with cultures of lung and spleen. The results summarized in Fig. 2 indicate that both cultures continued to produce virus at an undiminished rate for at least 130 days. The cells proliferated at a very slow rate as indicated by the long intervals between subcultures (Fig. 2). The culture of lung continued to support virus multiplication throughout three subcultures until the cells degenerated and the culture was discarded after 150 days. The culture of spleen, on the other hand, failed to produce virus after the third subculture. Furthermore, these cells appeared to be resistant to reinfection as indicated by an absence of virus multiplication upon addition of more virus to the culture. Cultures of embryonic tissues which required subculturing at weekly intervals also lost their ability to support virus multiplication after 2-3 subcultures. Viral multiplication was not associated with alteration in the morphology of cells as judged by microscopic examination and by cytochemical studies with acridine orange. The micrographs presented in Fig. 3 show the appearance of cultures at various stages during the experiment outlined in Fig. 2. The appearance of cells in cultures of mouse lung was very similar to that of the spleen cells.

Failure of LDV to multiply in established strains of cells. The following permanent strains of cells were examined for their ability to support the multiplication of LDV: mouse embryo, strain 1, mouse lung, strain 11,



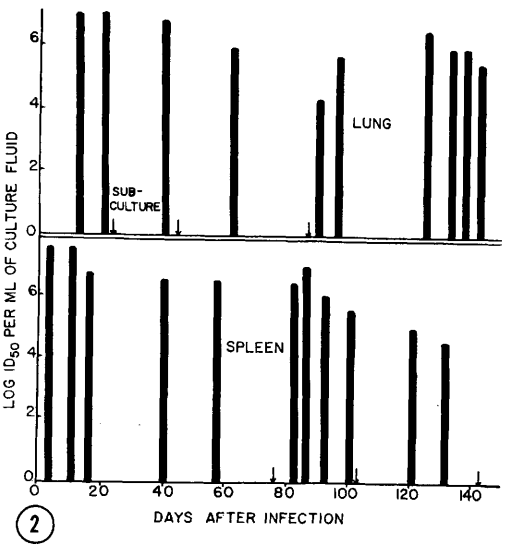


FIG. 1. Multiplication of LDV in a primary culture of mouse spleen cells. A 4-day-old culture in a T-30 flask was treated with LDV at a multiplicity of 10 for 30 minutes at 37°C. The virus was removed and the cell layer was washed twice with 5 ml S111 and 6 ml of medium 59 added. Samples of the medium were removed at indicated times and titrated for LDV. Three days after infection the medium was removed, the cell layer washed twice with 5 ml of S111 and 6 ml fresh 58 added.

FIG. 2. Continuous production of LDV by mouse spleen and lung cells. Four-day-old primary cultures were infected with LDV and the medium was replaced twice weekly thereafter. Subcultures were prepared when complete monolayers had formed as indicated by the arrows. The medium was assayed for LDV at indicated intervals.

TABLE I. Lactic Dehydrogenase Activity of Cells and Medium from Infected and Uninfected Cultures.

Days after infection	LDH activity, units/ml ± S.D. Uninfected* Medium†	Uninfected* Cells‡	Infected* Medium†	Infected* Cells‡
1	10 ±66		9 ± 6	
3	60 ±12		44 ±10	
5	66 ±17		59 ±11	
7	115 ±18	700 ±120	117 ±22	790 ±100

* Replicate cultures of mouse lung-spleen in T-30 flasks from 3 experiments containing $1\text{-}2 \times 10^6$ cells in 7.5 ml medium 58. Cells were infected with LDV at a multiplicity of 20 where indicated. Means of values of 12 cultures ± standard deviation.

† Values were corrected for 50 units of LDH/ml contributed by the horse serum of medium 58.

‡ Cells from each flask were washed twice with 5 ml S111 and lysed in 3 ml S111 by freezing and thawing several times.

mouse kidney, strain 33,§ strain L mouse cells (15) and HeLa cells(16). Multiplication of virus was not detected in any of these cultures.

DNA, RNA, and protein synthesis in cells infected with LDV. The lack of cytopathogenicity of the LDV and the continuous production of virus suggested that infection does not result in significant disturbances of the metabolism of the host cells. This conclusion is in agreement with the data presented in Fig. 4 which indicate that the rate of incorporation of precursors into DNA, RNA, and protein by a mixed culture of spleen and lung was not detectably altered by infection.

Studies of leakage of LDH from infected cells. In view of the marked elevation of LDH in the plasma of infected mice it was of interest to determine whether infection of cells *in vitro* causes either an increase in production of enzyme or acceleration of its release from cells. The data presented in Table I indicate that if such changes occur they are quantitatively too small to be detectable by the methods employed.

Discussion. The general behavior of the LDV *in vitro* is not unlike that *in vivo* with regard to the continuous production of virus

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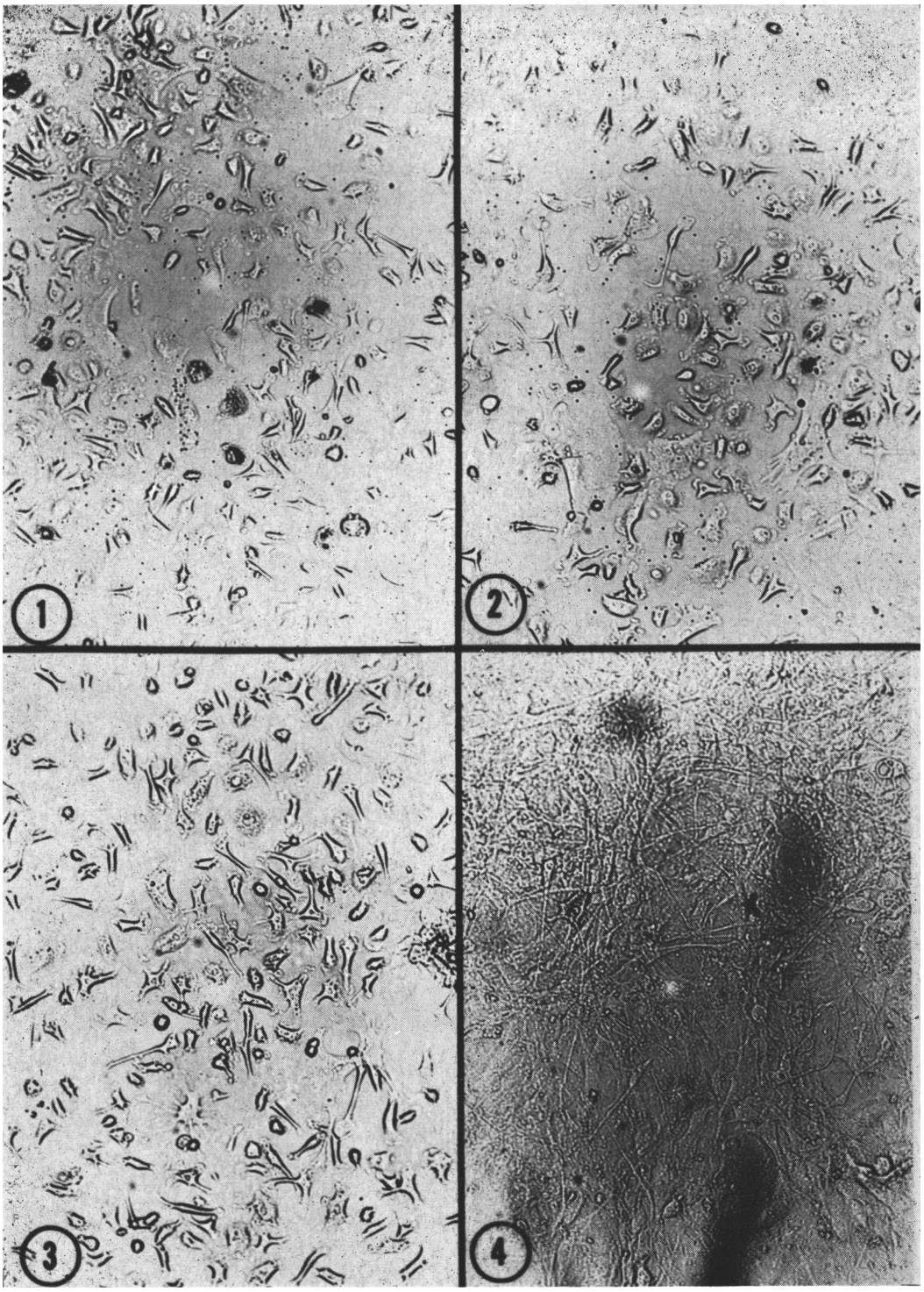

FIG. 3. Appearance of mouse spleen cells as a function of time after infection with LDV. Photographs are from cells used in the experiment outlined in Fig. 2. (1) Primary culture of spleen at 26 days; (2) 26 days after infection; (3) 53 days after infection; (4) 144 days after infection. These cells did not produce virus.

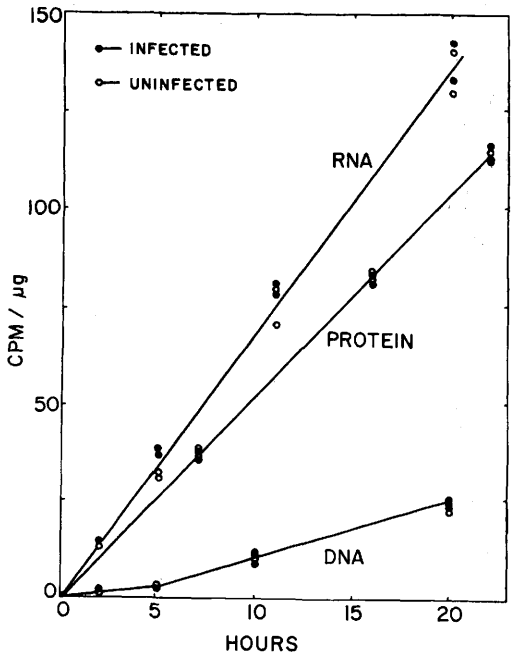

FIG. 4. Synthesis of nucleic acid and protein by infected and uninfected cells. Replicate cultures of a mixture of mouse lung and spleen were grown to a density of 2×10^6 cells per T-30 flask and the cells in one-half the flasks were infected with LDV at a multiplicity of 5. Labeled substrates were added to flasks containing 5 ml medium 58 as follows: 0.025 µmole uridine-2-C^{14} (1 µc/µmole) or 0.017 µmole valine-U-C^{14} (0.25 µc/µmole). Analyses for the incorporation of isotope into RNA, DNA, and protein were determined at intervals as described under *Methods*.

with a minimum of cell damage. The characteristics of LDV are not entirely unique since certain leukemic viruses multiply in primary cultures of mouse spleen for several weeks without evidence of cell damage(17). It is estimated that each cell in cultures of adult mouse spleen or lung (Fig. 2) produced a minimum of 10^3 ID_{50} of virus/day. This calculation is based on the assumption that every cell produced virus and on the fact that the LDV has a half-life of approximately 3 hours in medium 58 at 37°C. The failure of cultures to produce virus after 2-3 passages *in vitro* suggests that a relatively small proportion of them is involved in virus production and that such cells multiply at a slow rate relative to the majority and as a consequence are gradually lost by dilution on continued serial propagation. This probably accounts for the observed differences between cells from embryonic tissues and from adult tissues with respect to the period of virus production. For example, cultures of adult lung were subcultured 3 times over a period of 130 days (Fig. 2) and continued to produce virus during this period whereas corresponding cultures from embryonic tissues required subculturing at weekly intervals and thereby lost their capacity to produce virus during the first 2-3 weeks *in vitro*. The loss of susceptible cells may also explain observations of other investigators which indicate that cultures of mouse embryo, spleen, and peritoneal macrophages lose their capacity to support the replication of LDV within 2-3 weeks *in vitro*(18,19). Similarly this may account for the unsuccessful attempt to propagate LDV in mouse embryo cultures(20). The failure of several permanent lines of mouse cells to support viral replication in the present experiments also indicates that the requirements of the LDV may be highly specific.

The observation that LDV does not affect the rate of nucleic acid or protein synthesis in infected cell cultures (Fig. 4) is in keeping with the observation that cells *in vitro* produce virus for long periods (Fig. 2) without evidence of cytologic changes (Fig. 3). Failure to detect metabolic changes in cell cultures cannot be evaluated with certainty in the absence of definitive information on the relative number of cells infected. Similarly, the significance of the data in Table I is difficult to assess. It remains possible that such a small proportion of the cells were infected that changes in the production or release of LDH would not be detected by the methods employed. It is of interest in this connection that data have been presented recently which indicate that the infection of mice with LDV results in impaired clearance of LDH from the peripheral circulation(21-23). The question of whether this is solely responsible for the observed elevation of plasma-LDH or whether increased production and/or release of enzyme by infected cells also contributes significantly remains to be answered.

Summary. Primary cultures of adult mouse

lung, spleen, and liver and of mouse embryo support the multiplication of the lactic dehydrogenase-elevating virus. Such cultures produced virus continuously until they had been subcultured 2-3 times. This corresponded to 20 weeks in the case of lung and spleen and to 2-3 weeks with cultures of embryo. Viral multiplication was not accompanied by cytologic alterations in the cells or by changes in their rate of synthesis of nucleic acids or protein. Infection did not cause detectable changes in either the production of LDH or in its release from cells.

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Effect of Renin, Angiotensin II and Aldosterone on Erythropoiesis. (30992)

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Although ten years have elapsed since the initial demonstration of the relationship of the kidney to erythropoietin(1), a specific renal site of erythropoietin production has not been found. Diminished juxtaglomerular cell granularity in bled anemic mice(2) focused attention on the juxtaglomerular apparatus. In contrast, further investigation(3-5) has shown hyperplasia of the juxtaglomerular apparatus or an increase in the granularity of the juxtaglomerular cells in experimental situations wherein erythropoietin production is augmented.

Increased juxtaglomerular granularity has also been noted when there is augmented production of renin, a substance known to be elaborated by the juxtaglomerular cells(6-8). The interrelationship of renin, angiotensin II, and aldosterone in regulation of arterial pressure and fluid balance has been established (6-8). Thus a possible explanation for the coincidence of increased juxtaglomerular granularity and augmented erythropoietic stimulating activity is that renin, angiotensin or aldosterone exert direct or indirect erythropoietic effects. Fisher and Crook(9) and