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# L Cell Virus: Infectivity for Type N and B Mouse Embryo Cells

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The efficiency of focus formation of four L cell virus populations was significantly higher on type N mouse embryo fibroblast cultures than on type B cells. [5-$^3$H]uridine-incorporating material sedimenting at a density of 1.158 g/cm$^3$ was observed in the supernatant fluids of type N and B cultures infected with the four viral populations.

In Dales and Howatson's experiments, no tumor-inducing activity was demonstrated when virions released by two L cell lines were injected into newborn C3H mice and Syrian hamsters (3). Kindig and Kirsten (7) have also shown that L cell virus (LCV) was unable to induce tumors in mice and rats, and that infection of primary C3H mouse or Wistar-Furth rat embryo cultures with L929 supernatant fluids did not transmit morphologically recognizable viral structures to these cultures.

More recently, Fenyo and co-workers (4, 5) have demonstrated that viral particles released by an 8-azaguanine-resistant subline (A9) of L cells are infectious and that these particles are able to induce morphological changes (foci) in mouse embryo fibroblast (MEF) cultures. Focus formation was more efficient in type N than in type B cells, indicating that the LCV released by the A9 subline was N-tropic.

In this paper we present comparative data concerning the behavior of four LCV populations on type N and B MEF cultures, using focus formation and [5-$^3$H]uridine incorporation tests.

Two lines of each L cell type (designated L-1 and L-2) and their L929 variants (designated L929-1 and L929-2) were used as virus sources. Monolayer cultures of L-1, L-2, L929-1, and L929-2 cells were seeded in Roux flasks at a density of $2 \times 10^5$ cells/ml in Eagle minimal essential medium, Joklik's modification (Grand Island Biological Co., Grand Island, N.Y.), containing 5% fetal calf serum (Grand Island Biological Co.) and 100 $\mu$g of gentamicin per ml (Schering Corp., Port Reading, N.J.), to prevent mycoplasma development (8, 10). Media were collected after 24 to 36 h, passed through a membrane filter (pore size, 0.45 $\mu$m; Millipore Corp., Bedford, Mass.) (5), and kept at $-30$°C for 48 to 72 h until the infectivity assays were performed. According to the producer cell lines, the four virus populations were designated LCV (L-1), LCV (L-2), LCV (L929-1), and LCV (L929-2).

To accomplish the focus formation test, type N (C3H and CBA) and type B (C57Bl) MEF cultures were grown in Eagle minimal essential medium, containing 10% fetal calf serum, in the presence of 100 $\mu$g of gentamicin per ml. Then, cells were seeded in 25-cm$^2$ Falcon tissue culture flasks at a concentration of $10^5$ cells/ml, and the focus formation test was performed as described by Fenyo et al. (5).

After infection with LCV released by LCV (L-1), LCV (L-2), LCV (L929-1), and LCV (L929-2), morphological alterations of type N (CBA, C3H) and type B (C57Bl) MEF cultures were observed. They were similar to those described by Fenyo et al. (5) for BALB/3T3 and MEF cultures. In our experiments, the number of foci was also higher in type N cells (CBA and C3H) than in type B cells (C57Bl), characterizing the four LCV strains studied as N-tropic (Table 1). We should note that the morphological alterations observed with high viral concentrations were less pronounced in our experiments than in those of Fenyo et al. (5).

With respect to the relationship between focus formation and oncogenicity, we noted that the four LCV populations studied did not induce tumors when inoculated to mice (unpublished data).

For the [5-$^3$H]uridine incorporation test, type N and type B MEF cultures were grown as described for focus formation. Then, cells were seeded in Roux flasks at a concentration of $10^5$ cells/ml. They were infected 24 h later, as described by Fenyo et al. (5), for focus formation.

The second day after infection new medium was added, containing [5-$^3$H]uridine (specific radioactivity, 28 Ci/mmol) to a concentration of 0.5 mCi/100 ml of medium. The same amount of

[5-$^3$H]uridine was added to the cultures on day 3 after infection. Virions were collected and purified from the medium on day 4, according to the following procedure: elimination of cell debris by centrifugation (8,000 rpm, Sorval GSA rotor, 20 min), pelleting the virions (18,000 rpm, Spinco rotor 19, 3 h), suspending the pellet in TNE (0.01 Tris-hydrochloride, 0.15 M NaCl, 0.01 M EDTA, pH 7.8), pelleting through 30% (wt/wt) sucrose in TNE (45,000 rpm, Spinco rotor SW50.1, 90 min), and suspending the pellet in TNE (25,000 rpm, Spinco rotor SW-41, 16 h). Samples of each fraction from the sucrose gradients were directly counted in a toluene-based scintillation fluid.

The incorporation of [5-$^3$H]uridine into fractions obtained after sucrose density gradient centrifugation of supernatant fluids from mock-infected (control) and LCV-infected C3H, CBA, and C57Bl MEF cultures is shown in Fig. 1. In all cases, the investigation of supernatant fluids from infected type N and B cells showed a peak of [5-$^3$H]uridine incorporation corresponding to a density of 1.158 g/cm$^3$. No peak of radioactive sedimenting material was found in the supernatant fluids of mock-infected cells (Fig. 1).

Furthermore, when type N CBA MEF cultures were infected with 10-fold dilutions of LCV (L929-2) virions (Fig. 2), no radioactivity peak was seen at low virus input multiplicities (dilutions, $10^{-4}$ and $10^{-6}$). A typical peak of radioactivity corresponding to a density of 1.158 g/cm$^3$ was observed only when the CBA MEF cultures were infected with undiluted or 100-fold diluted LCV (L929-1) virions.

The results strongly suggest that the [5-$^3$H]uridine-incorporating material with a density of 1.158 g/cm$^3$ represents progeny LCV virus particles and that once the infection is initiated, virus is produced in type N as well as in type B MEF cultures.

The uninfected and infected cultures from the same experiments were examined under the electron microscope for the presence of viral particles. The cells, gently detached from the surface of the Roux flasks, were fixed in glutaraldehyde-formol (pH 7.4) for 30 min, followed by 2% osmium tetraoxide for 1 h, dehydrated through ethanol and propylene oxide, embedded in Spurr medium (11), and sectioned with an LKB-III ultramicrotome. Four hundred cell sections of uninfected and infected C3H, CBA, and C57Bl MEF cultures were examined in a Philips EM 300 electron microscope at 60 kV. An extremely small number of type A-like par-

TABLE 1. *Focus formation test on type N and B mouse embryo fibroblast cultures*

| Virus | Days after infection | Focus-forming units/ml | | | Host range |
|---|---|---|---|---|---|
| | | type N cells | | type B cells | |
| | | C3H | CBA | C57Bl | |
| LCV (L-1) | 6 | $1.4 \times 10^4$ | $7.3 \times 10^4$ | $1.2 \times 10^1$ | N |
| LCV (L-2) | 6 | $12 \times 10^4$ | $20 \times 10^4$ | $7.0 \times 10^1$ | N |
| LCV (L929-1) | 6 | $20 \times 10^4$ | $12 \times 10^4$ | $1.2 \times 10^1$ | N |
| LCV (L929-2) | 6 | $1.8 \times 10^4$ | $9.3 \times 10^4$ | $1.2 \times 10^1$ | N |

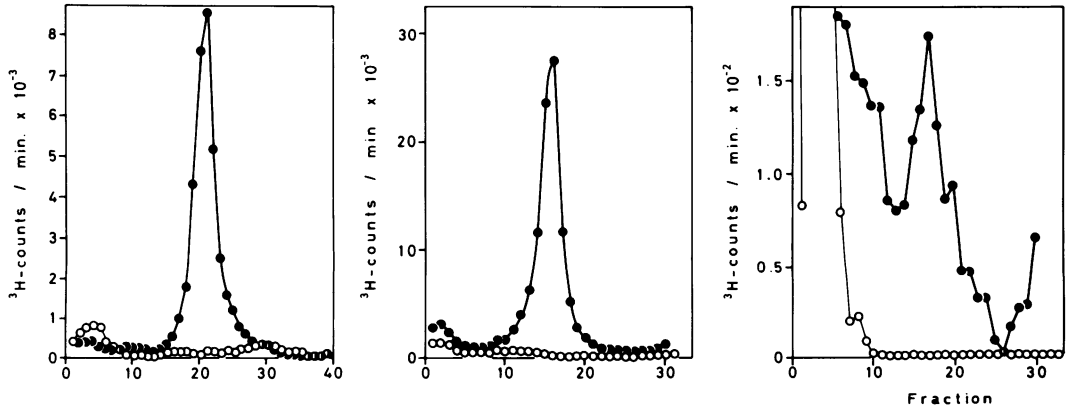


FIG. 1. *Equilibrium sedimentation in a sucrose density gradient of the [5-$^3$H]uridine-labeled progeny virions, released by C3H, CBA, and C57Bl, MEF cultures, respectively, after infection with the LCV (L929-1) population* (●); *mock-infected MEF cultures* (○).

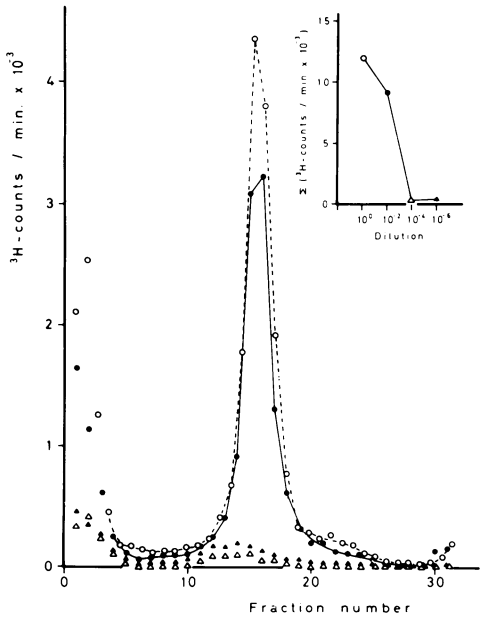


FIG. 2. *Equilibrium sedimentation in a sucrose density gradient of the [5-$^3$H]uridine-labeled progeny virions, released by CBA MEF cultures after infection with different LCV (L929-1) input multiplicities:* (○) *LCV (L929-1) undiluted;* (●) *LCV (L929-1), dilution $10^{-2}$;* (△) *LCV (L929-1), dilution $10^{-4}$;* (▲) *LCV (L929-1), dilution $10^{-6}$.*

ticles was seen in uninfected and infected cultures. No viral particles morphologically identical to LCV were seen budding from the cell membrane or extracellularly, confirming the results of Kindig and Kirsten (7).

This observation, compared with the results obtained with the [5-$^3$H]uridine incorporation test, suggest that under our experimental conditions the number of virus-producing cells in infected cultures was very low, below the minimum detectable number by electron microscopy.

Comparing the results of the infectivity assays of the virions released from L-1, L-2, L929-1, and L929-2 cell lines, a great similarity is observed among the characters of the four viral populations. Therefore, we agree with Hall et al. (6) on the possible common origin of virions found in different lines and clones of L cells. Probably the LCV was present in the original pools of tissue from C3H mice and vertically transmitted to all derivative lines and clones (6). The spontaneous (after long-term in vitro cultivation) or induced appearance of type C viral particles in no-producer cultures of mouse fibroblasts was described for other murine systems (1, 2, 9) and may represent a satisfying explanation for the LCV origin. But until more comparative data is available on LCV from different cell lines, we cannot definitely exclude that the virus may have been introduced into the cultures from an extraneous source. If this is the case, the event probably occurred in the very early history of the L cell line.

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## LITERATURE CITED

1. **Aaronson, S. A., J. W. Hartley, and G. J. Todaro.** 1969. Mouse leukemia virus: "spontaneous" release by mouse embryo cells after long-term in vitro cultivation. Proc. Natl. Acad. Sci. U.S.A. **64**:87-94.
2. **Aaronson, S. A., and J. R. Stephenson.** 1974. Differential cellular regulation of three distinct classes of type C RNA viruses endogenous to mouse cells. Cold Spring Harbor Symp. Quant. Biol. **XXXIX**:1129-1137.
3. **Dales, S., and A. F. Howatson.** 1961. Virus-like particles in association with L-strain cells. Cancer Res. **21**:193-197.
4. **Fenyo, E. M., and G. Grundner.** 1973. Characteristics of murine C-type viruses. I. Independent assortment of infectivity in one in vivo and four in vitro assays. Int. J. Cancer. **12**:452-462.
5. **Fenyo, E. M., K. Nazerian, and E. Klein.** 1974. Characteristics of murine C-type viruses. II. The behaviour of viruses resident in various cell lines and their hybrids on BALB/3T3 and mouse embryo fibroblast cultures. Virology **59**:574-579.
6. **Hall, W. T., W. F. Andresen, K. K. Sanford, and V. J. Evans.** 1967. Virus particles and murine leukemia virus complement-fixing antigen in neoplastic and non neoplastic cell lines. Science **156**:85-88.
7. **Kindig, D. A., and W. H. Kirsten.** 1967. Virus-like particles on established murine cell lines: electron microscopic observations. Science **155**:1543-1545.
8. **Leonard, A., and S. Botis.** 1975. Chromosome damage induced gentamicin in mouse L-cells. Experientia **31**:341-343.
9. **Lowy, D. R., W. P. Rowe, N. Teich, and J. W. Hartley.** 1971. Murine leukemia virus: high-frequency activation in vitro by 5-iododeoxyuridine and 5-bromo-deoxyuridine Science **174**:155-156.
10. **McClain, K., and W. H. Kirsten.** 1974. Mouse leukemia virus growth in mouse cells contaminated with mycoplasma. Cancer Res. **34**:281-285.
11. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31-43.