Immunofluorescent Cell-Counting Assay for Lymphocytic Choriomeningitis Virus

JANICE M. WEBSTER AND B. E. KIRK

Department of Microbiology, West Virginia University Medical Center, Morgantown, West Virginia 26506

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A quantitative assay for lymphocytic choriomeningitis virus was developed and standardized. The assay is based on direct immunofluorescent staining of infected L-929 cell monolayers and enumeration of cells containing fluorescent viral antigens. Maximal adsorption of virus to cells occurred within 1 h. Observations on the sequential development of viral antigens within cells showed that specific cytoplasmic fluorescence appeared within 10 h. The optimal time for enumerating fluorescent cells was from 18 to 20 h after addition of virus. A linear relationship was demonstrated between the number of infected cells and the relative virus concentration. Fluorescent cells were distributed randomly in infected cover slip cell monolayers. The immunofluorescent cell-counting assay for lymphocytic choriomeningitis virus was highly precise and reproducible.

No general agreement exists in the most suitable system for the assay of lymphocytic choriomeningitis (LCM) virus as regards maximal sensitivity and minimal variability. In the past decade, quantitative assays of virus infectivity by enumeration of cells containing immunofluorescent viral antigens have been established for agents representative of almost all major animal virus groups (3). These assays are dependent on a single cycle of infection and possess the outstanding attributes of rapidity (less than 24 h), high sensitivity, and precision. Because cells infected with LCM virus are amenable to immunofluorescent staining (2), an attempt was made to extend this technique to the assay of virus infectivity. This report describes the development and quantitative evaluation of the immunofluorescent cell-counting assay for LCM virus.

MATERIALS AND METHODS

Cell culture. Mouse fibroblast L-929 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 100 U of penicillin, and 10 μg of streptomycin per ml. For virus assays, cells were cultured on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). From 10^4 to 3 × 10^5 cells were added in 1-ml volumes to each vial and incubated at 35 C for 24 h or until a complete cell monolayer was formed. Cells were maintained in minimal essential medium with 2% fetal bovine serum and antibiotics. Media for growth and maintenance of baby hamster kidney (BHK-21) cells were the same as those described for L-929 cells.

Virus strain. The Armstrong strain of LCM virus was obtained from the American Type Culture Collection, Rockville, Md. Stock LCM virus was produced by inoculating a 10% suspension of virus-infected mouse brain onto L-929 cell monolayers. After incubation at 35 C for 4 days, cell culture fluids were harvested, distributed into vials in 1-ml amounts, and stored at −70 C. This virus pool was used for all infectivity studies. A second stock virus prepared in BHK-21 cells was used to immunize guinea pigs for the production of LCM virus antiserum.

Immunofluorescent virus assay. Determinations were made in duplicate. Serial 10-fold virus dilutions prepared in tryptose phosphate broth (pH 7.2) were introduced in 0.2-ml volumes directly into vials containing cover slip L-929 cell monolayers, and the cultures were incubated at 35 C for 1 h. Thereafter, residual inoculum was removed, cell cultures were rinsed with phosphate-buffered saline (PBS) (NaCl, 8.5 g; Na_2HPO_4, 1.136 g; KH_2PO_4, 0.272 g, per liter of distilled water) solution (pH 7.2), and 1 ml of maintenance medium was added to each vial. After a second incubation period of 17 to 19 h at 35 C, cover slip cell cultures were rinsed once with cold PBS, fixed with cold (−20 C) acetone, and stored at −20 C for subsequent immunofluorescent staining.

LCM virus antiserum conjugate and immunofluorescent staining. For the preparation of antiviral serum, guinea pigs were inoculated subcutaneously with 0.5 ml and intraperitoneally with 1.0 ml of LCM virus suspension containing 1.5 × 10^7 murine mean lethal dose. At weekly intervals thereafter, they received five similar injections. One week after the last injection, animals were exsanguinated. The globulin fraction of antiserum was precipitated with ammo-
nium sulfate at 4 C and was conjugated with fluorescein isothiocyanate at a ratio of 0.02 mg of dye per mg of protein (14). Conjugated globulin was passed through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) to remove unbound dye. To reduce nonspecific fluorescence, 5 ml of conjugated globulin was diluted with an equal volume of PBS and adsorbed twice with 200 mg of acetone-dried mouse liver powder (4).

The direct fluorescent-antibody technique was employed to demonstrate immunofluorescence of virus antigens in infected cells. Fixed cultures were rinsed twice with PBS and stained with conjugated globulin for 30 min at room temperature. Cover slip cell monolayers were rinsed in two changes of PBS and mounted in a semipermanent medium (15).

**Fluorescence microscopy and infected cell counting.** Cover slip cell cultures were examined with an American Optical Company microscope equipped with a Fluorolume illuminator (model 645), Corning no. 5840 and Schott BG-12 exciter filters, and an E.K. no. 2A barrier filter. With this optical system at a magnification of ×450 the number of microscope fields contained in the area of a 15-mm diameter cover slip was 1,046. For each cover slip cell monolayer, 50 microscope fields were examined for fluorescent cells. To calculate the number of cell-infecting units of virus per ml, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor for conversion to milliliter.

**RESULTS**

**Virus adsorption.** The rate of virus adsorption onto cell monolayers during stationary incubation at 35 C was determined by inoculating a group of vials containing cover slip cell cultures with an approximate virus-to-cell ratio of 0.1. At designated time intervals, vials were removed and residual inoculum from each vial was transferred to additional cell monolayers to measure unadsorbed virus. The latter were incubated for 4 h at 35 C. All cell cultures were treated in the manner described for virus assay. Maximal adsorption of virus, approximately 90%, was achieved within 1 h after inoculation (Fig. 1).

**Incubation period.** The time interval between virus inoculation and the appearance of recognizable quantities of viral antigen in cell monolayers in one infectious cycle was established from sequential counts of infected cells. The earliest sign of specific fluorescence of LCM virus antigen in cells was seen at 10 h after inoculation. Fluorescence was localized exclusively in the cell cytoplasm and was confined to individual cells (Fig. 2). When antiviral serum was added to cell monolayers 1 h after virus adsorption, the number of immunofluorescent cells reached a plateau after 20 h of incubation (Fig. 3). Infected cell counts in cell monolayers incubated with maintenance medium increased approximately fourfold between 20 and 48 h after inoculation, indicating that a secondary cycle of infection had occurred. In view of these findings, the incubation time selected for the assay was from 18 to 20 h.

**Quantitative evaluations of the assay.** A linear proportionality was demonstrated between the number of fluorescent cells and the relative virus concentration throughout the inoculum range of 1.2 log₅ units (Fig. 4). These data suggest that each fluorescent cell was the consequence of infection by a single virus particle or aggregate not divisible by dilution.

In a single experiment, 10 determinations...
were performed to estimate the precision of the fluorescent cell-counting assay for LCM virus. The number of cell-infecting units of virus per milliliter of inoculum ranged from $5.9 \times 10^4$ to $7.2 \times 10^4$ with a mean of $6.4 \times 10^4$ and a standard deviation of $\pm 0.46$. All values were within two standard deviations of the mean at the 95% confidence level.

To determine the reproducibility of the immunofluorescent cell-counting assay for LCM virus, 5 vials of virus selected randomly from approximately 200 vials of stock virus were assayed. Values ranged from $1.23 \times 10^4$ to $1.30 \times 10^4$ cell-infecting units per ml with a mean of $1.27 \times 10^4$ (Table 1). These results attest to the excellent reproducibility of the assay.

The mode of distribution of fluorescing cells on a cover slip cell monolayer was determined by examining 200 random microscope fields. The theoretical Poisson distribution was calculated and plotted with the observed distribution (Fig. 5). The frequencies of fields containing fluorescent cells corresponded closely to the theoretical frequencies. The chi-square test of goodness of fit of experimental data to the theoretical Poisson distribution showed no significant deviation ($p = 0.64$, degrees of freedom = 5). These observations indicate that the distribution of fluorescent cells on cover slip monolayers was random.

**DISCUSSION**

A fluorescent cell counting technique for quantifying LCM virus was established in the studies described here. The outstanding feature of the procedure was the ability to assess virus infectivity within 24 h after inoculation of cell monolayers. This is a marked advantage over other LCM virus assays, i.e., plaque counting and intracerebral inoculation of mice which require 4 and 14 days, respectively (9, 16).

The adsorption of virus to cell monolayers was maximal, approximately 90%, during stationary incubation at 35°C within 1 h and was comparable to that reported with different cell...
though virus may have been released from infected cells before this time. The few hours that elapsed after the liberation of virus were insufficient to permit the development of visual signs of secondary infection.

Quantitative evaluations of the assay for LCM virus, i.e., dose response, precision, reproducibility, and the random distribution of infected cells, closely paralleled the findings of fluorescent cell-counting assays for other viruses (5-8, 13, 17).

In preliminary tests, the 50% neutralizing end point of antiviral serum was determined by reacting LCM virus with specific antiserum. Over a critical range, a linear relationship was obtained between reduction percentages of immunofluorescent cell counts and dilutions of antiviral serum. This finding augurs well for the

lines and LCM virus strains (1, 12). Specific fluorescence of LCM virus antigens was visualized at 10 h after infection and was confined exclusively to the cell cytoplasm. These observations compare favorably with previous observations (11, 12). However, immunofluorescent antigen has been detected in cell nuclei of tissue preparations from virus carrier mice (10). Whether such variation in location of antigen is due to the difference between the in vitro and in vivo systems has not been established.

The concomitant increase in intensity of fluorescent staining and magnitude of fluorescent cell counts from 10 to 20 h after infection established the 20-h interval as the optimal incubation period. At 20 h, comparable infected cell counts were noted in the presence or absence of immune serum. In cell cultures that had been incubated for 36 h without immune serum, infected cell counts were markedly higher. This indicated that a secondary cycle of infection had occurred at least 16 h earlier. It may be assumed that at 20 h only cells in the primary infection cycle were counted even

**Table 1. Reproducibility of immunofluorescent cell-counting assay for LCM virus**

| Assay no. | Cells exhibiting fluorescence\(^a\) | CIU/ml\(^b\) (\(\times 10^6\)) |
|-----------|-----------------------------------|-----------------------------|
| 1         | 4.76 2.12 1.08 0.54               | 1.24                        |
| 2         | 4.52 2.22 1.08 0.52               | 1.23                        |
| 3         | 4.54 2.36 1.08 0.62               | 1.30                        |
| 4         | 4.56 2.32 1.20 0.56               | 1.27                        |
| 5         | 4.80 2.36 1.10 0.52               | 1.27                        |
| Mean      | 4.64 2.28 1.10 0.56               | 1.27                        |

\(^{a}\) Average number of cells exhibiting fluorescence per 50 microscope fields.

\(^{b}\) Cell-infecting units.

\(^{c}\) Dilution of virus inoculum.
feasibility of estimating, rapidly and quantitatively, serum-neutralizing antibody against LCM virus by the immunofluorescent virus assay procedure.

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LITERATURE CITED
1. Benda, R., and J. Činátl. 1962. Multiplication of lymphocytic choriomeningitis virus in bottle cell cultures. Experimental data for the preparation of highly infectious fluids. Acta Virol. 6:159–164.
2. Benda, R., V. Hronovský, L. Červa, and J. Činátl. 1965 Demonstration of lymphocytic choriomeningitis virus in cell cultures and mouse brain by the fluorescent antibody technique. Acta Virol. 9:347–351.
3. Carter, G. B. 1973. Immunofluorescence in the rapid identification of viruses and the role of fluorescent cell counting, p. 54–65. In F. J. Baker (ed.), Microbiology of the seventies. Butterworths, London.
4. Coons, A. H., and M. H. Kaplan. 1956. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 104:1–13.
5. Fleming, W. A., and J. K. Clarke. 1970. Fluorescence assay of foamy virus. J. Gen. Virol. 6:277–284.
6. Hahon, N. 1965. Assay of variola virus by the fluorescent cell-counting technique. Appl. Microbiol. 13:865–871.
7. Hahon, N. 1966. Fluorescent cell-counting assay of yellow fever virus. J. Infect. Dis. 113:33–40.
8. Hahon, N., and W. A. Hankins. 1970. Assay of chikungunya virus in cell monolayers by immunofluorescence. Appl. Microbiol. 19:224–231.
9. Lehmann-Grube, F., R. Ackermann, K.-A. Jochheim, G. Liedtke, and W. Scheid. 1959. Über die Technik der Neutralisation des Virus der lymphozytären Choriomeningitis in der Maus. Arch. Gesamte Virushorsch. 9:64–72.
10. Mims, C. A. 1966. Immunofluorescence study of the carrier state and mechanism of vertical transmission in lymphocytic choriomeningitis virus infection in mice. J. Pathol. Bacteriol. 91:395–402.
11. Oldstone, M. B. A., and F. J. Dixon. 1968. Direct immunofluorescent tissue culture assay for lymphocytic choriomeningitis virus. J. Immunol. 100:1135–1138.
12. Pedersen, I. R., and M. Volkert. 1966. Multiplication of lymphocytic choriomeningitis virus in suspension cultures of Earle’s strain L cells. Acta Pathol. Microbiol. Scand. 67:523–536.
13. Philipson, L. 1961. Adenovirus assay by the fluorescent cell-counting procedure. Virology 18:263–268.
14. Riggs, J. L., R. J. Seiwald, J. H. Burckhalter, C. M. Downs, and T. G. Metcalf. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Amer. J. Pathol. 34:1081–1097.
15. Rodrigues, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12:316–317.
16. Sedwick, W. D., and T. J. Wiktor. 1967. Reproducible plaquing system for rabies, lymphocytic choriomeningitis, and other ribonucleic acid viruses in BHK-21/13S agarose suspensions. J. Virol. 1:1224–1226.
17. Wheelock, E. F., and I. Tamm. 1961. Enumeration of cell-infecting particles of Newcastle disease virus by the fluorescent antibody technique. J. Exp. Med. 113:301–316.