Optimization of the Interferon Assay Using Inhibition of Semliki Forest Virus-Ribonucleic Acid Synthesis

HANS KOBLT, URSULA KOHLER, AND ROBERT WYLER
Institute of Virology, University of Zürich, CH-8006 Zürich, Switzerland

Received for publication 7 March 1972

The method for assaying chicken interferon by its inhibition of viral ribonucleic acid (RNA) synthesis was optimized for the chicken embryo fibroblast-Semliki Forest virus (SFV) system, with respect to time, multiplicity of infection, and addition of actinomycin D and 3H-uridine. Incorporation of H into viral RNA is reproducible, and amounts to values in per min per uninhibited culture per 1 µCi per 6 hr. The assay may be carried out in less than 1 day and is sensitive (0.05 units/ml and exact (±20% of the mean titers on different days); it can be used for purification procedures as well.

Allen and Giron (2), McWilliams et al. (6) and Miller et al. (7) described a technique for assaying interferon (IF) based on the inhibition of viral ribonucleic acid (RNA) synthesis because no previous method satisfied both requirements of rapidity and sensitivity. However, few methodical details were presented in the aboved mentioned articles.

This report concerns itself with the optimization of this rapid procedure in the chicken embryo fibroblasts (CEF) culture-Semliki Forest virus (SFV) system.

MATERIALS AND METHODS

Cell cultures. All experiments were performed with primary 48-hr monolayers in 50-mm Nunclon dishes; the 10-day-old White Leghorn embryos were treated with 0.125% trypsin (Nutritional Biochemicals, Cleveland, Ohio) and dispersed in 5 ml of medium 199-Hanks balanced salt solution (BSS) containing 10% calf serum (Italdiagnostic, Milan, Italy), 0.112% NaHCO₃, 100 units of penicillin (Mycofarm, Delft, Holland), and 0.1 mg of streptomycin (Mycofarm) per ml, at a concentration of 1.25 x 10⁴ cells/ml. Cultures were aerated with 7.5 liters of air and 0.4 liter of CO₂ per min at 37 C.

Virus and infection. The source of SFV (Kumba or Zürich strain, which we received originally from J. de Boer, Plum Island, N.Y.) was a suspension of brains of suckling mice, containing about 10⁶ plaque-forming units (PFU) per ml, in the 23rd passage. Stocks were diluted just prior to use with BSS-albumin, usually to a multiplicity of 0.01 or 0.1 PFU per cell in the culture. Inoculation was carried out at 37 C for 1 to 1.5 hr. BSS-albumin contained 0.05% albumin (bovine, Poviet, Amsterdam, Holland), 100 µg of kanamycin per ml and 120 µg of penbritin (Mycofarm) per ml.

Viral growth. After infection, cultures were maintained in Eagle’s minimal essential medium (MEM)-BSS (BBL, Cockeysville, Md.) supplemented with 5% calf serum, 0.112% NaHCO₃, 2 mM L-glutamine (BBL), penicillin, and streptomycin.

Plaque assays. Plaque assays in triplicate were performed according to conventional techniques with an overlay containing BSS with 5 mg of lactalbumin hydrolysate (Difco)/ml, 0.1 mg of yeast extract (Difco)/ml, 4 mg of glucose/ml, 5% calf serum, 1% Noble agar (Difco), kanamycin, and penbritin as described.

Viral RNA assay. The optimal time table was: infection for 1 hr starting at t₀; t₁, 5 ml of Eagle’s MEM added; t₂, 5 µg of actinomycin D (Calbiochem) in 100 µl of albumin BSS added; t₃, 3 µCi of uniformly labeled H-uridine (Radiochemical Center, Amersham, England); sterile, aqueous, 3.5-5 Ci/m mole, 1 mCl/ml) in 20 µl of BSS-albumin added; t₄, stopping of incubation by washing monolayers with ice-cold 0.85% NaCl and cooling to -20 C. Washings during experiments were made with prewarmed BSS-albumin. The cells were solubilized in 2 ml of cold 0.4% sodium lauryl sulfate (Serva, Heidelberg, Germany) in water and precipitated in an equal volume of 20% ice-cold trichloroacetic acid. Half an hour later the precipitates were rinsed onto Whatman GF/B filters (25 mm diameter) and washed with 10 ml of ice-cold 5% trichloroacetic acid. The filters were then dried at 70 C for 30 min and counted in a liquid scintillation spectrometer-type Tri-Carb model 33 in 10 ml of a toluene-PPO-PPOP scintillator. Counts per minute were used as such, the quenching factor being constant. All values represent means of 3 to 4 culture dishes.
IF and pretreatment of monolayers. The activity of our IF preparations is destroyed by trypsin, and their action is partially hindered by actinomycin D. They show molecular weight equivalents on Sephadex G 75 between 12,000 and 50,000. These preparations inhibit various viruses, are nontoxic to cells, withstand treatment at pH 2, are nondialyzable, and are easily water soluble. IF, the standard preparation, was prepared as follows. Fluids and embryonated hen eggs (10-11 days of incubation) infected with Newcastle disease virus (NDV) (not ultraviolet light-irradiated) were homogenized about 35 hr after infection, diluted with 0.85% NaCl, centrifuged at 2,500 rev/min, acidified to pH 2 overnight, centrifuged as above, neutralized, filtered, and lyophilized. The preparation was free from infectious NDV when assayed in 10-day-old chicken embryos. One unit is defined as that amount of protein (in micrograms) which reduces the plaque count or the counts per minute in viral RNA to 50% of the control value.

Solutions of IF, in BSS-albumin, sterilized through 0.22-μm membrane filters (Millipore Corp.), were pipetted directly into medium 199 at appropriate times before infection.

Remarks to presentation. t., in the text and 0 on the time axis in Fig. 1 and 2 always represent the start of infection; t., means 4 hr after the start of infection.

RESULTS

Results of optimization. A routine assay based on quantitative inhibition of incorporation of radioactivity into viral RNA should exhibit a large range between full inhibition and maximal uptake of label. We therefore optimized the system first; CEF and SFV were arbitrarily selected, because this combination is known to be especially sensitive to IF.

(i) Minimal actinomycin concentration blocking host RNA biosynthesis. Virtually no label is incorporated into uninfected host RNA at 1 μg of actinomycin/ml, whereas at lower concentrations uptake is measurable. In infected cells, incorporation could be observed at all actinomycin concentrations tested, but there was no release of labeled material into the culture fluid at 1 or 2 μg/ml. SFV replication, therefore, seems to be affected by actinomycin, at least during the stage of release or maturation, and it was decided to work only with cell sheets and not with supernatant culture fluid, and to add not more than 1 μg of actinomycin/ml.

(ii) Time of actinomycin addition. The regular maximum of incorporation at t, is consistently higher if actinomycin is added after infection instead of before. For this reason actinomycin (1 μg/ml) was added as late as possible—the exact time of addition depending upon the time lag between addition and activity of actinomycin, the time of the 3H-uridine addition, and this, in turn, of the incorporation activity into viral RNA in the course of the replication cycle.

(iii) Onset of actinomycin activity. 3H-Uridine (4 μCi) was added to uninfected cell cultures; some cultures were rapidly cooled at different times thereafter on an ice-CaCl₂ mixture, others received 1 μg of actinomycin per ml 2 hr after the label. Incorporation started within a few minutes and stopped immediately after addition of actinomycin. This means that actinomycin can be added as late as 0.5 hr before the label.

(iv) Time course of incorporating activity in infected cells, determination of multiplicity and choice of maintenance medium. Cells were infected at t, for 1.5 hr with SFV at multiplicities of 0.1 and 0.01. The virus suspension was replaced by 5 ml of fresh medium, either BSS-albumin or MEM-10% calf serum. Actinomycin (1 μg/ml) was added at various times after infection for a 2.5-hr period. Half an hour later, cells were exposed to 2 μCi of 3H-uridine for a period of 2 hr. Harvesting occurred hourly from t, to t, and culture fluids were titrated in a cumulative plaque assay. Figure 1 indicates that the time course of the “differential” incorporation in the cell sheets is higher with higher multiplicities (curves 1 and 3), and

![Fig. 1. Course of 3H-uridine “differential incorporating activity” into viral RNA of infected CEF cultures according to medium and multiplicity. Symbols: (●) multiplicity of infection of 0.1, MEM; (△) multiplicity of infection of 0.01, MEM; (□) multiplicity of infection of 0.1, BSS-albumin; (●) multiplicity of infection of 0.01, BSS-albumin.](image_url)
higher with the richer maintenance medium (curves 1 and 2) (4). A biphasic activity appears, representing probably a first and a second replication cycle due to low input multiplicities. The times of the maxima are, however, not clearly dependent upon multiplicity or medium. All this is reflected by the cumulative infection titers (not shown). Apparently, release of SFV into the medium is not disturbed with short exposures to actinomycin (2.5 hr) and rich media. Other experiments give evidence that the time of presence of infecting virus is not critical within the range of 0.5 to 2 hr and that the optimal serum concentration in the maintenance medium is 5%.

The above mentioned experiments led to the following conclusions: (1) to replace the infecting virus suspension after 1 hr with MEM-5% serum (t,); (2) to use a multiplicity of 0.1, a dilution which is still economical for use in a routine assay and avoids a virus purification step; and (3) to add ³H-uridine at t, the time at which replication of SFV-RNA becomes measurable, and actinomycin at t₂₅.

(v) Dependence of incorporation upon concentration of label, logarithmic phase and end point. The aim was to determine the minimal radioactivity yielding a well measurable incorporation, an exponential, or at least a linear incorporation rate and all at a reasonable price. Cells were infected with a multiplicity of 0.1. MEM was added at t₁₅, 1 μg of actinomycin per ml at t₂₅, and 2, 5, and 10 μg/ml at t₂₅. All curves show exponential kinetics (Fig. 2) and can be transformed to straight lines on semilog paper; therefore, radioactivity is not limiting in this range. Dose and incorporation are proportional (Fig. 2 inset). Incorporation starts deviating from exponential kinetics after the ninth hour and reaches its maximum height when the label is given at t₂₅ and not later.

It was therefore decided: (i) to stop incubation at t₂₅ and (ii) to use routinely 3 μCi of ³H-uridine per culture at t₂₅, which leads to an incorporation into acid-insoluble material in the order of 30,000 to 60,000 counts per min per culture per 6 hr.

(vi) Incorporation and temperature (not shown). After an infection period of 1 hr with a multiplicity of 0.1, the incubation temperature was shifted to 25, 30, 33.5, and 37 C. No clear difference in incorporation could be detected between 33.5 and 37 C, and the latter temperature was chosen for further work.

Inhibition with additions other than IF. Experiments to find conditions for additional incorporation of ³H-uridine were unsuccessful and showed either no effect or an inhibiting effect. Simultaneous addition of cold adenine, cytidine, guanosine, and uridine between 2 and 30 μg/ml each (isotope dilution) moves the incorporation curves to the right. In other words, the rate constants remain unchanged, but the apparent time of onset of synthesis (intercept with time axis) is delayed (not shown). Addition of Cd²⁺ (10⁻⁴-10⁻⁶ M), Mn²⁺ (10⁻³-10⁻¹ M), and Zn²⁺ (10⁻⁴-10⁻⁶ M) at t₂₅ resulted in a marked and early deviation from the exponential incorporation rate, whereas the addition of Cu²⁺, Fe²⁺, Mg²⁺ had no meaningful effect. Adding a mixture of amino acids up to a concentration of three times that of MEM or various substrates of the citric acid cycle was also not clearly effective.

Results of the RNA reduction test with IF. Based upon the aforementioned results, the best experimental design for an IF assay in the CEF-SFV system can be proposed as follows: t₀, add IF; t₀, infect with SFV at a multiplicity of 0.1 in 1 ml of BSS-albumin for 1 hr at 37 C; t₁, replace with 5 ml of MEM-5% serum, 37 C; t₂₅, add 1 μg of actinomycin D/ml in BSS-albumin; t₂₅, label with 0.6 μCi of ³H-uridine.

![Fig. 2. Dependence of incorporated counts/min on input radioactivity in CEF cultures infected with SFV. Actinomycin (1 μg/ml) was added at t₂₅, ³H-uridine at t₁₅. Symbols: 1, 2 μCi; ▲, 5 μCi; and ◇, 10 μCi. Inset: plot of 8-, 8.5-, and 9-hr values against input radioactivity.](image)
dine per ml; tₙ, harvest. x is an appropriate time between 2 and 12 hr; we recommend 4 hr. Figure 3 shows the results of such a quantitative IF test with a 4-hr IF pretreatment. There exists a negative linear log concentration-incorporation relationship in the range of 5 to 80 μg of IFₓ/ml of medium 199. Under the same conditions "mock IF" exhibits no reduction of isotope incorporation, and the endogenous background incorporation in uninfected, actinomycin-treated cells amounts to 2,500 to 3,000 counts/min. Plaque assay and RNA reduction assay (4 hr treatment each) give the same results.

Figure 4 may serve as an example of the usefulness of the procedure as a qualitative IF assay in the case of chromatographic purifications. We do not want to comment on the profile here. One-hundred Sephadex G 75 fractions were assayed individually and examined in 1 day. Infection was begun at 8 AM after an overnight pretreatment with 200 μl of IF, and the cells were harvested at 5 PM. Precipitation and filtering were completed at 8 PM, drying at 8:30 PM, and counting took place from 9 until 11 PM.

**DISCUSSION**

In this optimized system, the incorporation of ³H-uridine into SFV-RNA, within the same set of CEF cell cultures, is highly reproducible, resulting in a deviation of not more than 5% of the mean. The base line is given by the counts per minute in acid-precipitable material of uninfected, actinomycin-treated controls; it lies on a lower level than the values of pretreatment with the highest concentration of IF.

Linear regression curves evolve by plotting the log₁₀ of the IF concentration versus counts incorporated, the slopes of which are dependent upon time of pretreatment with IF (up to about 6 hr). With a 4-hr pretreatment, the linear range extends from about 85 to 10% of the control; this corresponds to 0.25 to 4.5 units/ml. Beyond these extremes the curve becomes flattened. Plaque tests and RNA reduction tests give the same results for the same period of pretreatment; however, the principal advantages of the latter are the relatively short duration and the ease of performance. After infection, a minimum of only 13 to 14 hr is needed. In the case of MM virus, used by Allen and Giron (1), this delay was even more favorable (8–9 hr).

Sensitivity and reproducibility are in good agreement with other assay procedures, in that 0.05 units can be detected. Standard deviation of the mean for titrations on different days of our standard preparation was about 20%. This compares well to the results of Allen and Giron (1), 21.5%, and favorably to other assay procedures (plaque test, 30% [5]; quantitative hemadsorption, 39% [2]; dye uptake method, 45% [3]; all expressed in terms of log₁₀ testₐ₀ units).

Theoretically, all pretreatment times long enough to show an activity should be allowed. However, we prefer long pretreatments for detection of small quantities or for qualitative assays in the course of fractionation.

There is no need for immediate processing; after the test period washed monolayers without medium can simply be stored at −20 C.

**ACKNOWLEDGMENTS**

This work was supported by the Swiss National Foundation, grant 3.399.70. We thank L. Rosendorf for help in preparing this manuscript.
OPTIMIZATION OF INTERFERON ASSAY

Fig. 4. RNA assay and fractionation. A Sephadex G75 column (93 by 1.8 cm) was equilibrated with 0.1 M Na₂HPO₄-NaH₂PO₄ (pH 7.65) at 4°C. Interferon 14 (30 mg; 1,500 units) was dialyzed against the same buffer, applied in 2 ml to the column, and chromatographed at a speed of 13.7 ml/hr. One hundred fractions of 2.5-ml each were collected. To reduce danger of contamination, tubes and buffer were autoclaved. Two hundred microliters of each fraction were pipetted into 35-hr CEF cultures and incubated overnight; then the routine time table was followed. The t₅₀ incorporation values are presented. Calibration was performed in a similar way. Symbols: *, *H incorporation curve with inverted scale; ○, optical density (OD) of IF-preparation; and Δ, OD of dextran blue, cytochrome c, and tyrosine.

LITERATURE CITED

1. Allen, P. T., and D. J. Giron. 1970. Rapid sensitive assay for interferons based on the inhibition of MM virus nucleic acid synthesis. Appl. Microbiol. 20:317-322.
2. Finter, N. B. 1969. Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. J. Gen. Virol. 5:419-427.
3. Finter, N. B. 1964. Quantitative hemadsorption, a new assay technique. I. Assay of interferon. Virology 24:589-597.
4. Hearn, H. J., H. R. Tribble, S. C. Nagle, and D. C. Bowersox. 1971. Replication of venezuelan equine encephalomyelitis virus in vitro. II. Viral growth response to selected nutritional additives in suspension cultures. Appl. Microbiol. 21:342-345.
5. Lindenmann, J., and G. E. Gifford. 1963. Studies on vaccinia virus plaque formation and its inhibition by interferon. Virology 19:302-309.
6. McWilliams, M., M. S. Finkelstein, P. T. Allen, and D. J. Giron. 1971. Assay of chick interferons by the inhibition of viral ribonucleic acid synthesis. Appl. Microbiol. 21:959-961.
7. Miller, P. A., H. L. Lindsay, M. Cormier, B. R. Mayberry, and P. W. Trown. 1970. Rapid semi-automated procedures for assaying antiviral activity. Ann. N.Y. Acad. Sci. 173:151-159.