Semi-Micro, Dye-Binding Assay for Rabbit Interferon

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The semi-micro method for interferon assay was based on quantitation of inhibition of cytopathic effects in 6-mm cultures. Cultures were exposed to interferon dilutions, challenged with vesicular stomatitis virus, and stained with methylrosaniline chloride. The bound dye was then eluted and measured colorimetrically. The method was more sensitive than the plaque-inhibition method and was extremely economical. Culture age was the most important factor affecting reproducibility.

Most methods of interferon assay, such as plaque inhibition and yield reduction, are expensive in terms of time and materials. Less complex methods, such as inhibition of cytopathic effects (CPE), tend to be subjective and difficult to quantitate. Finter (1) has described how cytopathology may be quantitated by the uptake and elution of a vital dye and has applied this technique to the assay of interferon. A micro-method for titration of human and chick interferon was described by Tilles and Finland (4) who based interferon activity on inhibition of CPE, as observed in the microscope or on metabolic inhibition. This paper describes the assay of rabbit interferon by a semi-micro, dye-binding method which is extremely economical, especially in respect to cell culture materials and interferon samples.

MATERIALS AND METHODS

Cell cultures. Weanling rabbit (300 to 400 g) kidney cell cultures were grown in 5-liter Povitzky bottles. Subcultures were prepared by treating the cultures with 0.25% trypsin (Difco, 1:250) and ethylenediaminetetraacetetic acid (EDTA) (0.2 g/liter) in phosphate-buffered saline. The cell suspension was centrifuged to remove trypsin and EDTA and was resuspended in medium containing 0.5% lactalbumin hydrolysate, 4% inactivated calf serum, 0.2% sodium bicarbonate, and antibiotics in Hanks balanced salt solution (BSS) at a concentration of 2 × 10^5 cells/ml. This cell suspension was then dispensed in suitable vessels and incubated at 37°C in an atmosphere of 5% CO_2 in air. For the assay described here, 0.1 ml of cell suspension was placed in each well of a Microtest II plate (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) by using a disposable, plastic, 1-ml pipette (Falcon Plastic). The microplates have eight rows of 12 flat-bottomed wells of 6-mm diameter.

Interferon. Rabbit serum interferon was prepared by injecting about 10^6 plaque-forming units (PFU) of Herts strain Newcastle disease virus intravenously into 1.0- to 2.0-kg albino rabbits. Serum was obtained 7 hr after injection and was stored frozen at −70°C. This material is stable for several years under these conditions (2).

Challenge virus. Vesicular stomatitis virus (VSV, Indiana strain) was grown in monolayer cultures of chicken embryo cells and stored frozen in BSS supplemented with 0.5% lactalbumin hydrolysate and 20% inactivated (56°C for 30 min) rabbit serum. The virus was titrated by plaque formation on rabbit kidney cell cultures, prepared as described above, in 5-cm plastic dishes.

Interferon assay: dye-binding method. Dilutions of the test samples were prepared in medium. Twofold, fourfold, or 0.5 log_{10} serial dilutions were generally used. The last named dilution series was prepared semi-serially in that a 3.2-fold dilution and a 10-fold dilution were both made from the previous 10-fold dilution to avoid propagation of the error involved in the approximation 10^{3.2} ≈ 3.2. The medium was decanted from the microplates, containing confluent monolayers, by vigorous inversion over a pan containing a moist cloth or paper towels to avoid splashing. Two to four wells were then filled with 0.1 ml of the dilutions of interferon. On each plate, 6 to 12 wells were filled with 0.1 ml of medium to serve as virus and cell controls. After overnight incubation, the interferon was decanted and 0.1 ml of VSV containing about 400 PFU was added to each well including virus controls. Wells designated cell control receive 0.1 ml of medium. It is important that the medium used in the cell controls be identical to that containing the challenge virus since small differences in serum content lead to readily observed differences in cell growth and hence dye-binding capacity. After 24 hr of incubation, the virus controls should show 50 to 90% CPE as observed microscopically, and incubation may be terminated. Occasionally a longer incubation may be needed. The plates are then decanted and rinsed twice in 0.85% NaCl, and the wells are filled with dye-fixer solution and allowed to stand for 15 min. The dye-fixer solution contains 0.5% methylrosaniline chloride, 5% Formalin (v/v), 50% ethanol (v/v), and 0.85% NaCl in distilled water. The dye-fixer solution is then decanted; the
microplates are rinsed copiously with tap water and allowed to dry. As shown in Fig. 1, a subjective estimate of interferon titer may be made at this time by visual inspection. Dry, dyed assays on microplates appear to be quite stable for several weeks under normal laboratory conditions. For quantitative assay, the dye is eluted by adding 0.2 ml of 2-methoxyethanol to each well. The elution time without agitation is 45 min. With agitation this can be reduced to 15 min. The absorbance of the eluted dye was measured in a model 6A Coleman Junior spectrophotometer at 550 nm. A properly masked 10-mm outside diameter cuvette can be used for volumes as little as 0.4 ml, the pooled volume from two wells. In the absence of automatic equipment, a plastic syringe, 2 to 3-ml size, with a 19-gauge needle is useful for handling samples. A piece of tight-fitting plastic tubing (2 cm) should be slid over the tip of the needle. Interferon titer was estimated by plotting absorbance as a function of log interferon dilution and estimating the end point graphically as that dilution of interferon which inhibited cell destruction by 50%, where 50% destruction is equal to 0.5 \( A_{550} \) (cell control) + \( A_{550} \) (virus control).

**Interferon assay: plaque reduction method.** This method has been described by Ke et al. (2). In brief,
cultures of rabbit kidney cells in 5-cm dishes were prepared as described above and treated overnight with 2 ml of suitable dilutions of interferon. The interferon was decanted, and the cultures were challenged with about 100 PFU of VSV. The plates were then overlaid with medium containing 1.5% agar and incubated for 48 hr to permit development of plaques. The dilution of interferon which reduced plaque number by 50% was estimated by plotting per cent reduction on a probit scale against log interferon dilution as suggested by Lindemann and Gifford (3).

RESULTS

Figure 1 shows a dyed, dried titration of interferon in microplate cultures of rabbit kidney cells. For demonstration purposes, 8 wells were used for each dilution with 16 virus controls and 8 cell controls at the bottom. A titer of 1:6,400 can be estimated visually. By absorbance of eluted dye, the titer was 1:4,220 or 3.625 log_{10} units/ml. The dose response curve is shown in Fig. 2.

Of various possible factors affecting response, the most important was found to be age of the cultures. As shown in Table 1, the assay becomes progressively more sensitive as the culture ages. We therefore usually use 6-day-old cultures. Reproducibility between tests is then good. Five assays of a single interferon preparation with 0.5 log_{10} dilutions and three wells per dilution were performed on five different batches of 6-day-old cultures. The mean titer was 4.5 log_{10} units/ml with a range of 4.3 to 4.7. However, the within-test variation is less than that between tests. Six assays of a single interferon preparation in the same batch of cultures had a mean titer of 3.38 log_{10} units/ml and a range of 3.30 to 3.46.

The challenge virus is also a source of variation. Eastern equine encephalitis may be used but was found to be about twofold less sensitive than VSV. Other arboviruses such as Semliki Forest virus and Sindbis virus as well as vaccinia virus and bovine vaginitis virus did not produce extensive CPE within 24 hr and were less sensitive to interferon than VSV.

The dose of challenge virus is also critical. Doses of VSV less than 200 PFU/well do not produce CPE in all wells and are therefore unusable. Doses greater than 1,000 PFU/well cause complete CPE within 24 hr in all wells in which CPE can be observed, and the assay becomes quantal in nature.

The sensitivity of the dye-binding method was compared with that of the plaque reduction assay (2). As shown in Table 2, the former method was from 16-fold (sample 7) to 100-fold (sample 2) more sensitive than the latter. The mean of the ratios in the last column of Table 2 is 1.57, indicating that the dye-binding method was on average about 37 times as sensitive as plaque reduction in this series of experiments. Comparison of repeated titrations of our internal standard interferon provides another measure of the relative sensitivity of the methods. This comparison can be made from the top line of Table 2 and indicates a 45-fold difference between the methods.

DISCUSSION

The assay described here was developed to provide an economical, sensitive, and quantitative assay for routine use. An assay takes about 1/60 the amount of cells and medium required for a plaque reduction assay and can be completed in 48 hr. The assay is about 40 times as sensitive as the plaque reduction method. The major cause of variation in the test is due to the age of the cultures used, and, since within-test variation is very low, we always include a titration of a standard interferon preparation in each assay. This procedure is also necessary when rabbit interferon is assayed by plaque reduction (2).

A colleague (M. C. Breinig) has adapted the assay for use with human interferon, using human embryonic fibroblasts and Sindbis virus, and it is doubtless applicable to other systems.

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