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Microassay for Interferon, Using [^{3}H]Uridine, Microculture Plates, and a Multiple Automated Sample Harvester

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A microassay for interferon is described which uses target cells grown in microculture wells, [^{3}H]uridine to measure vesicular stomatitis virus replication in target cells, and a multiple automated sample harvester to collect the radioactively labeled viral ribonucleic acid onto glass fiber filter disks. The disks were placed in minivials, and radioactivity was counted in a liquid scintillation spectrophotometer. Interferon activity was calculated as the reciprocal of the highest titer which inhibited the incorporation of [^{3}H]uridine into viral ribonucleic acid by 50%. Interferon titers determined by the microassay were similar to the plaque reduction assay when 100 plaque-forming units of challenge vesicular stomatitis virus was used. However, it was found that the interferon titers decreased approximately 2-fold for each 10-fold increase in the concentration of challenge vesicular stomatitis virus when tested in the range of 10^2 to 10^5 plaque-forming units. Interferon titers determined by the microassay show a high degree of repeatability, and the assay can be used to measure small and large numbers of interferon samples.

The increasing importance of interferon as an antiviral and immunoregulatory agent in infectious disease processes has created a need for improved types of interferon assays (2, 3, 5). Measurements of interferon activity based on plaque size or number require a cytolytic virus, a narrow range of plaque-forming units (PFU) of challenge virus, and, generally, large flasks or culture plates in which to form plaques. The measurement of dye uptake by viable cells (4) provides the advantage of assaying interferon in microculture wells, but suffers the disadvantages of cytolytic assays.

Another type of interferon assay is based on the incorporation of radioactively labeled nucleotides into the ribonucleic acid (RNA) of the challenge virus as it begins its replicative process. Inhibition of viral RNA synthesis is taken as a measure of interferon titer. This method offers the advantage of being potentially applicable to both cytolytic and poorly or noncytolytic viruses; its drawbacks are that it generally requires high multiplicities of infection, large culture flasks, and mechanical removal of cells before isotope counting (1, 6, 10, 11, 13, 22). Growing the cells directly in scintillation vials reduces handling to some extent (19). Although microculture interferon assays have been used successfully, the reported harvesting procedures are rather laborious (21).

We have improved these assays by using target cells grown in wells of microculture plates, adding [^{3}H]uridine to label replicating viral RNA, and collecting the labeled RNA on glass fiber filter disks with a multiple automated sample harvester (MASH II, Microbiological Associates, Bethesda, Md.). This microassay is rapid, reproducible, easy to perform, and at least as sensitive as the plaque reduction assay.

MATERIALS AND METHODS

Cells. Mouse L 929 cells or low-passage kidney cells of bovine (BK) or guinea pig origin were grown in Eagle minimal essential medium supplemented with 5% calf serum and antibiotics. Trypsinized cells were seeded in 75-cm^2 flasks (30 ml of 7×10^4 cells per ml), 35-mm 4-well cluster plates (3 ml of 7×10^4 cells per ml), or 96-well microculture plates (see below) and incubated at 37°C in a 5% CO_2 atmosphere.

Viruses. Vesicular stomatitis virus (VSV), Indiana strain, was serially passaged five times in BK cells. The clarified infectious supernatant fluid was stored in 1-ml samples at −56°C; each sample was used only once and not refrozen. The titer of this stock virus was 3.9×10^9 PFU/ml on BK cells and 8.6×10^9 PFU/ml on L-cells. Foot-and-mouth disease virus, type O, strain Brugge, was also grown in BK cells; the virus was concentrated from the clarified supernatant fluid by two cycles of precipitation with 8% polyethylene glycol and stored at −56°C (16). The titer of this stock virus was 8.6×10^9 PFU/ml on BK cells; L-cells do not support the replication of foot-and-mouth disease virus.

Plaque assay. Procedures for plaque assays in 35-mm culture dishes with gum tragacanth overlay have been reported (14, 15). The cells were stained and

fixed 20 h later with crystal violet-Formalin solution, and the plaques were counted.

Interferons. BK cells or L-cells grown in 75-cm^2 flasks were infected with 10^9 PFU of Newcastle disease virus in 0.1 ml; after 60 min of incubation, 30 ml of medium was added. After 24 h, no cytopathic effect was observed; the supernatant fluids were collected and clarified by low-speed centrifugation. Fresh medium was added. At 48 h, no cytopathic effect was evident; the medium was collected and clarified. These individual supernatant fluids were acidified (pH 2) with 1 N HCl and stored at 4°C overnight to inactivate the virus. The pH was adjusted to neutrality with 1 N NaOH. These interferon preparations did not induce RNA synthesis in actinomycin D (Act-D)-treated L-cells or BK cells. High-titered mouse interferon (10^5 U) was also obtained commercially (Calbiochem, La Jolla, Calif.).

Interferon assays. We compared the microassay with our routinely used plaque reduction assay. Confluent cultures in 35-mm wells were exposed to interferon dilutions (1 ml/well) during overnight incubation at 37°C. The medium was aspirated, and the cells were inoculated, without washing (3), with 0.1 ml of a virus dilution containing 50 to 70 PFU. The cells were overlayered with gum tragacanth 1 h later. The interferon titer was taken as the reciprocal of the dilution which reduced the plaque count by 50%.

For the microassay, 6×10^4 cells in 0.2 ml of medium were seeded in each of the 96 wells of the microtiter plate and incubated for 24 h at 37°C. Medium was removed from the wells by inverting and shaking the plates; all operations were carried out in a vertical laminar-flow hood. Dilutions of interferon were added to each of four wells (0.1 ml/well) with a microliter syringe (Hamilton Co., Reno, Nev.); control cells on each plate received diluent, interferon, or virus as overlay. After 20 h, the overlay medium was again removed, and virus or diluent (10 μl) was added to each well; the plates were incubated for 30 min; Act-D (50 μl containing 0.3 μg of Act-D) was then added (17). At 90 min postinfection, 1 μCi of [^{3}H]uridine (specific activity, 28 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 10 μl of medium was added to each well.

At the time of harvest (generally 20 h postinfection), the cells were lysed by adding 20 μl of 1% sodium dodecyl sulfate to each well; 50 μl of cold 10% trichloroacetic acid was then added, and the plates were kept at 4°C for at least 15 min.

The trichloroacetic acid-precipitated RNA was collected on glass filter paper strips with a multiple automatic sample harvester (8, 9, 18, 20, 23). This unit harvested and washed (0.9% NaCl) the precipitates from 24 wells simultaneously; less than 5 min was required to harvest a 96-well tray. The filter strips were air dried; each disk was removed with a forceps and placed in a minivial, to which 1 ml of scintillation fluid was added. The capped minivials were placed in transfer vials and counted. Statistically significant counts were obtained after 1 min. Control counts (Act-D treated, noninfected) were subtracted from each set. The interferon titer (per 0.1 ml) was taken as the reciprocal of the sample dilution resulting in a 50% inhibition in [^{3}H]uridine incorporation into trichloroacetic acid-precipitable viral RNA.

RESULTS

Effect of L-cell and VSV concentration on synthesis of VSV RNA in the microculture system. Initial experiments were performed to determine the optimal L-cell microculture conditions which would lead to the maximal synthesis of VSV RNA. Various amounts of L-cells (1.5×10^4 to 2.5×10^5) were seeded into microculture wells and, after incubation for 24 h to allow attachment, the microcultures were infected with various concentrations of VSV (up to 10^5 PFU/microculture). At 30 min after infection, Act-D was added to each microculture to stop host cell RNA synthesis, and at 90 min postinfection, 1.0 μCi of [^{3}H]uridine was added to each well to detect synthesis of VSV RNA. Cells were harvested at 20 h. Controls consisted of noninfected L-cells to which Act-D and [^{3}H]uridine were added to give a measure of residual L-cell RNA synthesis.

The results of this experiment are shown in Fig. 1, where VSV RNA synthesis is presented

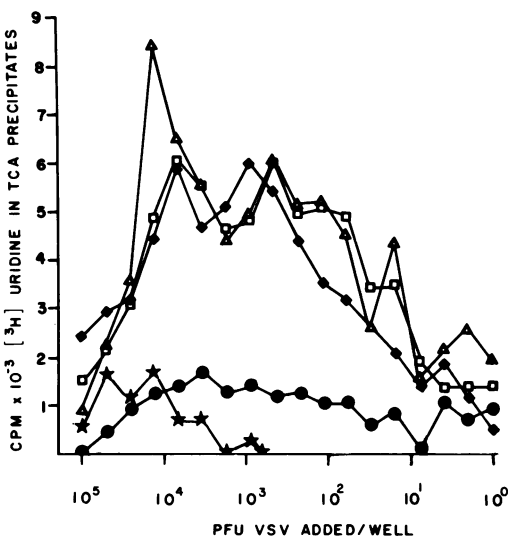

FIG. 1. *Effect of cell concentration and challenge virus dose on [^{3}H]uridine incorporation into new virus RNA. L-cells were seeded in microculture wells at the following concentrations: 2.5×10^5 (★), 1.3×10^5 (●), 6.3×10^4 (△), 3.1×10^4 (□), and 1.5×10^4 (■) cells per well. At 24 h, the cells were infected with various dilutions of VSV; after 30 min, Act-D was added. At 90 min postinfection, [^{3}H]uridine was added and the plates were reincubated. Trichloroacetic acid-precipitated RNA was collected 24 h later. Uninfected control cells treated with Act-D incorporated between 3,800 and 4,100 cpm into trichloroacetic acid-precipitated RNA.*

as counts per minute in Act-D-treated virus-infected cells minus the counts per minute in Act-D-treated control cells. Optimal synthesis of VSV RNA was found to occur with L-cell concentrations of 1.5×10^4 to 6.3×10^4 cells per well. The amount of isotope incorporation increased approximately linearly as the input virus increased to 10^2 PFU/well; the counts per minute remained relatively constant in the range of 10^2 to 10^4 PFU/well. We found that peak viral RNA synthesis also occurred in cells seeded in this range but challenged 48 or 72 h later. A [^{3}H]uridine pulse of 6 h was also tested (data not shown), but maximal VSV RNA synthesis required a higher multiplicity of infection (10^5 PFU/microculture), and the amount of VSV RNA synthesis was considerably lower.

Similar results were obtained from experiments with BK and guinea pig embryo kidney cells (data not shown), which indicated that this system was versatile enough to be used with a variety of target cells. We have also measured foot-and-mouth disease virus RNA synthesis in BK cells with 6- and 20-h [^{3}H]uridine pulse-labeling, which indicates that the system is adaptable to other RNA viruses.

Comparison of the microassay with the plaque reduction assay for measuring interferon. The previous experiments established that VSV RNA synthesis could be measured over a range of cell concentrations, VSV concentrations, and time periods. To compare the microassay with the plaque reduction assay, we used 10^2 PFU/well to maintain similar conditions.

Newcastle disease virus-induced interferons collected between the 24th and 48th h postinfection from L-cell and BK cell cultures were tested on both homologous and heterologous cells. Dilutions of each interferon were added to macrocultures or microcultures and incubated for 24 h before virus challenge. The results (Fig. 2) showed that the interferon titers determined by the microassay were identical to those determined by the plaque reduction assay. Furthermore, the microassay compared favorably with the plaque assay in demonstrating the species specificity of the interferon preparations. Studies using Newcastle disease virus-induced interferons collected during the first 24 h postinfection indicated that the L-cell and BK cell interferon titers were both 1:256 (data not shown); thus, the amount of interferon produced by BK cells after Newcastle disease virus induction appeared to decrease markedly during day 2.

Effect of virus dose on interferon titer. The previous experiment measured the interferon titer in response to a fixed dose of VSV. We were therefore interested in determining whether the titer of interferon varied when the dose of challenging VSV varied. This is a difficult experiment to perform by the plaque reduction assay because at high challenge doses of virus the plaques are too numerous to count, or there is complete lysis of the monolayer. These types of experiments are generally performed by yield reduction assays, which also require large numbers of cultures to perform virus titrations (5). With the microassay system the experiment required 400 microcultures and was set up in ap-

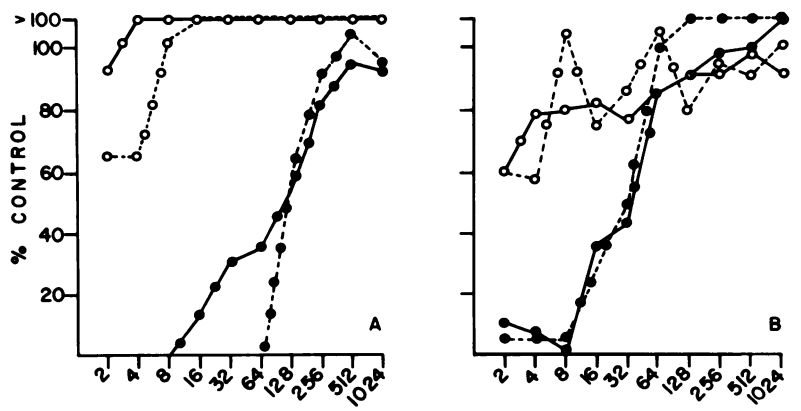

FIG. 2. *Comparison of the microassay and plaque reduction assay systems. Newcastle disease virus-induced interferons (IF), collected from BK cell and L-cell cultures, were tested in homologous and heterologous cells. A challenge dose of 100 PFU of VSV per well was used; plaques were stained, and microculture plates were harvested at 24 h postinfection. (A) L-cell interferon; (B) BK cell interferon. Symbols: (●——●) plaque reduction assay in which the interferon titer was taken as the reciprocal of that dilution reducing the plaque count by 50% (PR_{50}), homologous cells; (●- - -●) microassay, homologous cells; (○——○) PR_{50}, heterologous cells; (○- - -○) microassay, heterologous cells.*

proximately 2 h. The experiment was performed on three separate occasions to measure the repeatability of the experiment, using a commercial source of mouse interferon. The interferon was diluted to contain 2,500 U/0.1 ml and was used to overlay L-cells for 20 h. The results (Table 1) show that the interferon titer varied approximately 2-fold with each 10-fold difference in VSV dose. This variation was consistent with previous findings that interferon titers are relatively independent of the virus challenge dose (5). At a dose of 10^4 PFU of VSV, the interferon titer as measured by the microassay (1:2,000) compared favorably with the manufacturer's titer (1:2,500). However, at a dose of 10^2 PFU of VSV, the interferon titer as measured by microassay averaged 1:7,200. Thus, for repetitive comparative assays, interferon titers should be determined with the same virus dose. In addition, the three replicate experiments demonstrate a repeatability which is equal to or superior to that which we have found with the plaque reduction assay.

DISCUSSION

The use of the multiple automated sample harvester in combination with radioisotopes and microculture plates has become the standard method of measuring lymphocyte blastogenesis in immunology in such assays as the mixed-leukocyte culture assay (20), response to mitogens (18) and antigens (23), and assays of lymphotoxins (8) and bacterial cytotoxins (9). The current studies demonstrate that interferon can likewise be assayed by using [^{3}H]uridine incorporation into viral RNA in target cells grown in microculture plates in combination with the multiple automated sample harvester. The microassay system uses small volumes of materials, culture manipulations are easy and rapid, storage and incubator space are minimal, and human error in judgment is avoided because results are expressed as counts per minute of radioactive label incorporated. The microassay was as sensitive as the plaque reduction assay (Fig. 2), and it demonstrated very good repeatability (Table 1). We have found the assay to be adaptable to other target cells (BK and guinea pig embryo kidney cells) and another RNA virus (foot-and-mouth disease virus). The microassay might have particular applications in measuring interferon activity against RNA viruses which are poor plaque producers or which take several days to develop plaques. Moreover, studies utilizing very large doses of challenge virus are possible. We have also found that it is possible to measure virus replication in this microassay when as little as 1 to 5 PFU was added per well (Fig. 1).

TABLE 1. *Effect of virus challenge dose on apparent interferon titer measured by the microassay procedure*

VSV dose (PFU/well)	Interferon titer[a] in expt:		
	1	2	3
10^2	8,200	8,300	5,200
10^3	4,400	6,400	6,800
10^4	2,000	2,100	1,900
10^5	1,400	1,500	800

[a] Expressed as the reciprocal of the interferon dilution which inhibited by 50% the [^{3}H]uridine incorporation into trichloroacetic acid-precipitable material harvested 20 h after VSV infection.

Vassef et al. (22), using a dye uptake interferon assay system, and McLaren (11), using an isotope incorporation system, reported that young cells derived from mice were less sensitive than older cells to the action of interferon or to infection by virus. We found that when 1.5×10^4 to 6.3×10^4 L-cells per well were seeded 24 h before use, the highest levels of [^{3}H]uridine incorporation into replicating viral RNA occurred (Fig. 1). These L-cells were fibroblastoid in appearance. At higher cell densities the cells appeared epitheloid, and levels of [^{3}H]uridine incorporation were drastically reduced at all multiplicities of infection.

Previous reports (12, 19) indicated a need for multiplicities of infection in the range of 5 to 10 to measure challenge virus replication by incorporation of labeled nucleotides with macrocultures. The sensitivity of the microassay was such that incorporation levels at 6 h postinfection in BK cells, L-cells, and guinea pig embryo kidney cells peaked with a multiplicity of infection of about 1 (data not shown). When virus replication was allowed to continue beyond the first cycle, measureable uptake of [^{3}H]uridine by very small amounts of input virus (e.g., a multiplicity of infection of about 0.01, or 10 PFU/microculture as shown in Fig. 1) was possible. A direct comparison of interferon titers by plaque reduction and isotope incorporation methods with similar virus challenge doses was therefore possible, and results were similar (Fig. 2).

Many samples can be handled easily with this microassay. One worker can routinely process 10 plates per work day (the equivalent of 1,000 individual RNA assays); automated dilution and sample addition devices would further increase the numbers of samples that could be processed. Variations in experimental design can be used to further reduce the time required for interferon titrations. For example, growing cells in the presence of interferon dilutions (2) shortens the assay time by 24 h without altering titers (data not

shown). This microassay is therefore useful in a variety of situations requiring a sensitive and reproducible interferon assay capable of rapidly processing a large number of samples.

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