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Rapid Sensitive Assay for Interferons Based on the Inhibition of MM Virus Nucleic Acid Synthesis

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A method for assaying mouse interferon based on the inhibition of viral ribonucleic acid (RNA) synthesis was devised. The amount of MM virus and RNA synthesized in interferon-treated L-cell cultures was determined by measuring the amount of ^{3}H-uridine converted into a trichloroacetic acid-insoluble form after treatment of the infected cultures with 2.5 μg of actinomycin D per ml. The amount of RNA synthesized was inversely related to the concentration of interferon used for treatment. A linear dose-response regression curve was obtained by plotting the log of the amount of RNA made, expressed as a percentage of the control, versus the log of the reciprocal of the interferon dilution. A unit of interferon was defined as that concentration which inhibited nucleic acid synthesis by 50% ($INAS_{50}$). The concentration of mouse interferon could be determined within 24 hr. This assay method, on the average, was approximately half as sensitive as the method which measured the 50% reduction of MM virus plaque number (PDD_{50}-MM method), but was, on the average, almost 1.7 times as sensitive as the PDD_{50}-VSV method. It averaged approximately 20 times the sensitivity of the methods which used as end points the 70% reduction in yield of MM virus or the complete inhibition of cytopathic effect by MM virus. The reproducibility of the $INAS_{50}$ technique was tested in two ways. (i) Four independent assays of an interferon specimen were performed with replicate cultures. The standard deviation was 11.2% of the mean titer. (ii) On different dates, one interferon specimen was assayed seven times and another was assayed four times. The standard deviations were 21.5 and 26.6% of the respective mean titers.

The methods presently available for the assay of interferons were comprehensively reviewed by Finter (5). Of the available techniques, those methods which are capable of detecting low concentrations of interferon require 3 to 6 days for completion, whereas the methods that may be completed more quickly are relatively insensitive. One technique, the method of quantitative inhibition of hemadsorption (QH method; 4) may be modified so that it can be completed in only 24 hr, but the time advantage obtained is offset by a substantial sacrifice in sensitivity. An assay method for mouse interferon was needed which could be rapidly performed but would be relatively sensitive. None of the available techniques satisfied both requirements. The inhibition of viral ribonucleic acid (RNA) synthesis by interferon was reported in 1962 (2, 16) and has since been confirmed by several authors (7, 10, 17). This report concerns itself with a sensitive and rapid procedure based upon the quantitative inhibition of MM virus nucleic acid synthesis by interferon and employs 50% reduction as the end point ($INAS_{50}$).

MATERIALS AND METHODS

Solutions and media. The medium used throughout these experiments was modified Eagle's medium (3) containing Hanks balanced salt solution (BSS) and twice the prescribed concentration of amino acids and vitamins. It was supplemented with 10% (v/v) fetal bovine serum when used for growth of cells or propagation of virus and with 2% (v/v) fetal bovine serum when used for radioactive labeling of cultures.

NET buffer consisted of 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate, and 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 7.4. Trichloroacetic acid was used at a concentration of 5% (w/v). Actinomycin D (Calbiochem) was dissolved in growth medium and used at a concentration of 2.5 μg/ml. ^{3}H-uridine (25.9 Ci/mmole) was used at the concentrations indicated in the text. Each liter of scintillation cocktail contained 60 g of naphthalene, 5 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)benzene in *p*-dioxane. Neutral red dye was prepared at 0.2 mg/ml in Hanks BSS.

Cells. L cells were purchased from BBL. They were propagated as monolayer cultures in Blake bottles and roller bottles. In all experiments, monolayer cultures were prepared by seeding 10^6 cells per 60-mm culture dish (Falcon Plastics) in 5 ml of growth medium and incubating the cultures for 16 to 18 hr before use. All experiments were conducted at 36 C in an atmosphere of 95% air and 5% CO_2.

Virus. The source and techniques for propagating and assaying MM virus were described previously (9). This virus is a member of the EMC group. A stock virus suspension of 2×10^8 plaque-forming units (PFU)/ml was prepared by collecting infected cells and the supernatant culture fluid 9 hr after inoculation. The virus was released by three cycles of freezing and thawing. Vesicular stomatitis (VS) virus (Indiana serotype) was kindly provided by Charles J. Gauntt, College of Medicine, University of Arizona, Tucson. A stock suspension of 6×10^8 PFU/ml was prepared in L cells by collecting the supernatant fluid 24 hr after inoculation. All virus stocks were stored at −70 C. They were diluted to the desired virus concentration with Hanks BSS just prior to inoculation.

Interferon. Preparations of mouse interferon were induced by the infection of cultures of L-cells with MM virus. Fluids were collected 48 hr after virus inoculation and were stored at −70 C. Interference due to both viable and inactive virus particles was eliminated from these fluids by irradiating them for 5 min at a distance of 12 cm from a Westinghouse G15T8 lamp as previously described (8). The interfering agent thus obtained was previously characterized as an interferon (8) by the following criteria (14): (i) nonsedimentation at 100,000 × *g*; (ii) destruction by trypsin; (iii) prevention of activity by actinomycin D; (iv) activity against both MM virus and VS virus; (v) absence of toxicity for cell cultures.

Assay of acid-insoluble radioactivity. After exposure of monolayer cultures to ^{3}H-uridine, the radioactive medium was removed, and 1 ml of ice-cold NET buffer was added to each culture. The cells were scraped with a rubber policeman into chilled test tubes and were maintained in an ice bath until the addition of 5 ml of ice-cold 5% trichloroacetic acid to each tube. A 1- to 2-min period was allowed for the precipitation of acid-insoluble material to occur. The residue in each tube was filtered onto a glass-fiber filter (Reeve-Angel, or Gelman type E). Each residue was washed three times with 5 ml of ice-cold trichloroacetic acid and once with 5 ml of ice-cold 95% ethyl alcohol. The filters were placed in scintillation vials, with the residue side uppermost, and 5 ml of scintillation cocktail was added. Radioactivity was determined in a liquid scintillation spectrometer and recorded as counts per minute.

Assay of interferons by standard methods. In those experiments in which interferon was assayed by the method which employed as the end point the 50% reduction in plaque number, the following technique was used. Interferon specimens were serially diluted in growth medium, and duplicate cultures were each incubated overnight with 2 ml of each interferon concentration. Control cultures were incubated with growth medium. The fluids were removed from each culture, and 50 to 100 PFU of either MM virus or VS virus was added. After a 1-hr period at 37 C for virus adsorption, the cultures were overlaid with medium containing 1% agar, 1% fetal bovine serum, and 0.8 mg of protamine sulfate per ml. The monolayers were stained with 0.02% neutral red after 2 days of additional incubation, and the plaques were counted. The average number of plaques on duplicate cultures was determined. Interferon titers were estimated by plotting the per cent inhibition versus the log of the reciprocal of the interferon dilution, and interpolating to find the dilution giving 50% inhibition.

The procedures for assay of interferon in which the end point was the highest dilution giving complete inhibition of cytopathic effects in cultures inoculated with about 10 PFU of MM virus per cell (ICPE-MM) was described previously (8). The interferon assay method which employed the 70% reduction in yields of MM virus as the end point (IVY_{70}-MM) was performed as follows. Cultures of L cells were treated for 16 to 18 hr with dilutions of interferon as described above. The fluid was removed, and each culture was inoculated with 0.25 ml of fluid containing ~ 10 PFU of MM virus per cell. After 30 min at 36 C for virus adsorption, the cultures were washed three times with BSS, and 5 ml growth medium was added to each of them. Previous results (8) indicated that maximal viral yields were present in the supernatant fluid 24 hr after addition of the inoculum. Culture fluids were collected at that time and stored at −70 C until assayed for PFU.

Assay of interferons by new method. A method was devised for assaying the antiviral activity of interferon derived from L cells, based on the inhibition of viral RNA by different concentrations of interferon. One unit of interferon was defined as that concentration which inhibits viral nucleic acid synthesis by 50% in cultures infected with MM virus subsequent to exposure of the cultures to interferon for 16 to 18 hr. The technique was called the $INAS_{50}$ method. Cultures were treated with serial dilutions of interferon, and the extent of viral RNA synthesis in these cultures was determined as described above. Regression curves were fitted by sight on logarithmic graph paper. The interferon titer of each specimen was taken as the reciprocal of the dilution which would inhibit RNA synthesis by 50%. Occasionally a sample was encountered for which only one value was available in the useful range, i.e., between 5 and 80% control. When this occurred, the titer was estimated by the following procedure. The average slope for the regression curves obtained for other specimens assayed on the same date was determined, and a line having this average slope was then drawn through the single available point. The 50% end point was then estimated in the usual fashion.

RESULTS

Incorporation of ^{3}H-uridine by infected cultures. A technique for incorporating ^{3}H-uridine into viral RNA and for measuring the amount of incorporation was examined as follows. Twelve

L-cell cultures were inoculated with ~ 25 PFU of MM virus per cell contained in 0.25 ml of fluid. After a 30-min period at 36 C for adsorption of virus, 2 ml of growth medium containing 2.5 μg of actinomycin D per ml was added to each culture without removal of the inoculum. At 3.5 hr after the addition of MM virus, the fluid containing actinomycin D was removed, and 2 ml of medium containing 0.1 μCi of ^{3}H-uridine per ml was added to each culture. Data which will be published separately indicated that 85 to 90% of MM virus RNA synthesis in actinomycin D-treated cells was completed by 6 hr after inoculation. Therefore, the acid-insoluble radioactivity was determined as described in Materials and Methods 6 hr after the addition of virus. The mean counts per minute per culture was 2,451, with a standard deviation of 162 or 6.6% of the average counts per minute. The small standard deviation indicates that the technique is precise and gives values clustered closely about the mean.

Effect of interferon on viral RNA synthesis. The effect of L-cell interferon on the synthesis of RNA by MM virus was examined by the following techniques. Each specimen of interferon was serially diluted in growth medium. Duplicate cultures were treated for 16 to 18 hr with 2 ml of each dilution. Control cultures were each treated with 2 ml of growth medium. All cultures, except duplicate uninfected controls, were inoculated with 10 to 25 PFU of MM virus per cell in 0.25 ml of fluid. The cultures were incubated for 30 min at 36 C for virus adsorption. All cultures were then treated for 1.5 to 3 hr with 2 ml of growth medium containing 2.5 μg of actinomycin D per ml. The medium containing actinomycin D was removed, and 2 ml of labeled medium containing 0.2 to 0.5 μCi of ^{3}H-uridine per ml was added. At 6 hr after the addition of virus, the acid-insoluble radioactivity was determined as described in Materials and Methods. We determined empirically that 0.2 μCi of ^{3}H-uridine per ml of medium resulted in the incorporation of 4,000 to 10,000 counts/min in infected control cultures which had not been treated with interferon. After doing many experiments, we observed that uninfected control cultures which were treated with actinomycin D usually incorporated 0.03 to 0.05 times as many counts per minute as the infected control cultures, though occasionally incorporation was as high as 0.1 times control. Relatively high concentrations of interferon did not reduce incorporation below that occurring in uninfected control cultures (Fig. 1A). The average number of counts per minute incorporated in uninfected control cultures was therefore considered to be the base line

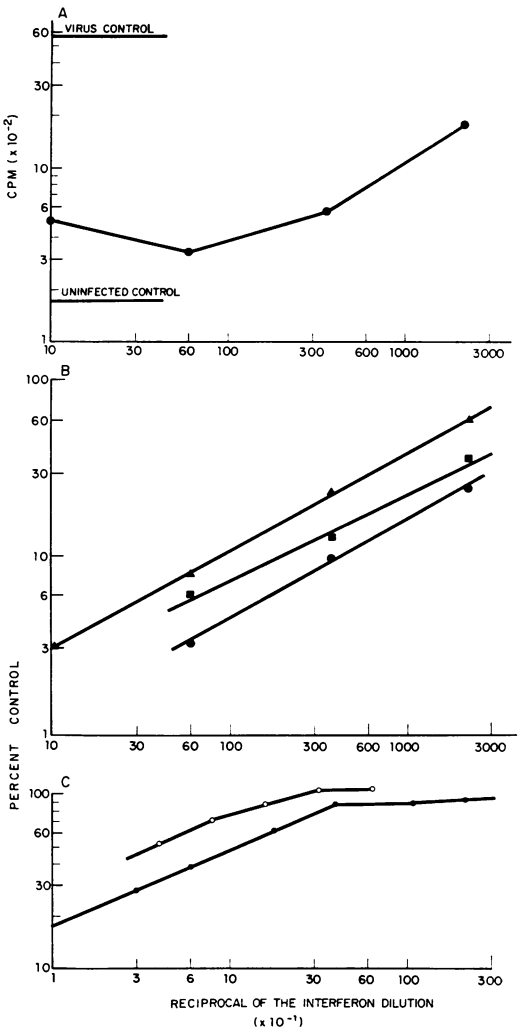

FIG. 1. *Relationship between viral RNA synthesis and interferon concentration. Each line represents a different preparation of interferon. A, B, and C demonstrate the dose-response relationships obtained with interferon preparations of high, intermediate, and low potency.*

for viral RNA, and this number was subtracted from each of the other counts per minute values before further calculations were made. Counts per minute values in interferon-treated cultures were then expressed as a percentage of the number of counts per minute incorporated into viral RNA in infected control cultures which were not treated with interferon. Reproducible results of the type shown in Fig. 1B have been obtained in numerous experiments. Linear relationships were approximated when the $\log_{10}$ per cent control was plotted versus the $\log_{10}$ reciprocal of the dilution of the interferon specimen. This linearity

was seen throughout the intermediate portion of the regression curves. Beyond 75 or 80% of the control, the curves became flattened (Fig. 1C). As the counts per minute values approached 3 to 5% of the control, the results became less reproducible. This effect could be partially overcome by using a higher concentration of ^{3}H-uridine.

Reproducibility of the $INAS_{50}$ interferon assay method. The reproducibility of the $INAS_{50}$ method for interferon assay was tested in 2 ways. First, four independent assays of a single interferon preparation were conducted by use of replicate cultures, all of which originated from the same cell population. The per cent control values obtained in each assay and their averages are shown in Table 1. These data were plotted, and the interferon titer indicated in each assay was determined. The mean titer was 1,450 units with a standard deviation of 162, 11.2% of the mean. The values for per cent control at each interferon concentration were clustered tightly about the means, as indicated by their standard deviations.

The reproducibility of the $INAS_{50}$ assay method was tested further by performing repeated assays on different dates of two interferon preparations (Table 2). Interferon specimen A was assayed seven times and specimen B was assayed four times. The average titer for specimen A was 2,371 units, with a standard deviation of 510, 21.5% of the mean. The average titer for specimen B was 1.462 units, with a standard deviation of 389, 26.6% of the mean.

Sensitivity of the $INAS_{50}$ interferon assay method compared with other assay methods. The $INAS_{50}$ method was compared with a method based upon 50% reduction in plaque count (PDD_{50}). MM virus and VS virus were each used in the PDD_{50} method as described in Materials

TABLE 1. *Reproducibility of $INAS_{50}$ assay with a single batch of cell cultures*[a]

Assay no.	Percentage of control incorporation with interferon diluted			Interferon titer
	1:500	1:1,500	1:3,000	
1	32.0	57.5	77.0	1,250
2	25.5	52.6	84.0	1,400
3	24.6	49.2	68.0	1,450
4	23.8	58.8	76.0	1,700
Avg	26.5	54.5	76.2	1,450
SD	±3.2	±4.2	±6.0	±162

[a] Four independent assays of a single interferon preparation were conducted by the $INAS_{50}$ technique on the same date with the use of a single batch of replicate cultures. The concentration of tritiated uridine was 0.1 μCi/ml. Uninfected control cultures incorporated 134 counts/min each and infected control cultures incorporated 2,018 counts/min each. Each value represents the average of duplicate cultures.

TABLE 2. *Reproducibility of $INAS_{50}$ interferon assay method when performed on different occasions*

Expt no.	Interferon prepn	
	A	B
1	1,900	1,450
2	2,000	900
3	2,400	2,000
4	2,000	1,500
5	2,100	
6	2,800	
7	3,400	
Avg	2,371	1,462
SD	±510	±389

TABLE 3. *Comparison of the sensitivity of the $INAS_{50}$ assay method with that of other assay methods*

Expt	Interferon specimen no.	Indicated titer of interferon/sensitivity index[a]		
		$INAS_{50}$-MM	PDD_{50}-MM	PDD_{50}-VSV
A	1	2,500	6,000/0.42	ND[b]
B	2	45,000	>60,000/<0.75	17,000/2.65
	3	16,000	15,000/1.07	7,000/2.29
C	4	3,000	15,000/0.2	ND
	5	1,000	5,000/0.2	ND
D	6	6,000	23,000/0.26	8,000/0.75
	7	1,900	6,000/0.32	2,000/0.95
Mean sensitivity index			<0.46	1.66

[a] Sensitivity index = titer indicated by $INAS_{50}$-MM method/titer indicated by alternate method.
[b] Not done.

and Methods. Four separate experiments were performed with seven different specimens of interferon. Interferon titers indicated by the different techniques are given in Table 3. Sensitivity indices were calculated by dividing the titers obtained with the $INAS_{50}$ method by the titers obtained with the alternate method. The sensitivity of the $INAS_{50}$ technique averaged <0.46 times that of the PDD_{50}-MM method and 1.66 times that of the PDD_{50}-VSV method.

Specimen 2, which was assayed in experiment B (Table 3), was also assayed by two other methods, the ICPE-MM method and the IVY_{70}-MM method, which are described in Materials and Methods. The indicated titer was 256 ICPE-MM units in each of two experiments. It was 150 and 250 IVY_{70}-MM units, respectively, in the same two experiments. This particular specimen had a titer of 45,000 $INAS_{50}$ units; thus, the $INAS_{50}$ technique was about 20 times more sensitive than either the ICPE-MM method or the IVY_{70}-MM method.

DISCUSSION

We investigated the relationship between the concentration of mouse interferon used to treat cultures of L cells and the amount of ^{3}H-uridine labeled viral RNA synthesized when the cultures were subsequently inoculated with MM virus, and treated with actinomycin D. The amount of RNA synthesized was expressed as a percentage of that made in control cultures which were not treated with interferon. Linear regression curves were obtained by plotting the logarithm of the percentage of control values versus the logarithms of the interferon concentrations. A similar relationship has been reported between the logarithms of percentage of control virus yield and the logarithms of interferon concentration by others (1, 12). The region of linearity in the regression curves we have obtained extended from approximately 5% of the control to approximately 80% of the control. Beyond these extremes, the curves became more flat. The linear portion of the dose-response curves corresponded to a range in interferon concentrations of about 10 units to about 0.3 units. In assaying an interferon specimen, if serial threefold dilutions of interferon were made, three or four points in the linear portion of the dose-response curve were usually obtained. Thus, each estimate of interferon concentration was based on several data points, thereby enhancing the precision of the technique. Occasionally it was necessary to sacrifice precision to assay a large number of specimens. We found that 6-fold or even 10-fold serial dilutions could be used without missing the end point.

Reproducibility of the $INAS_{50}$ interferon assay method was compared with that of other methods for which such information is available. We assayed one interferon preparation on four occasions and one on seven occasions, obtaining standard deviations of 26.6 and 21.5% of the respective titers. In comparison, Lindenmann and Gifford (13) assayed a specimen of chick interferon on six occasions. Analysis of their data indicated an average titer of 90 units, with a standard deviation of 29, or about 32% of the mean titer. Finter (4) reported data on the reproducibility of the quantitative hemadsorption (QH_{50}) method for assaying interferon. A preparation of mouse interferon assayed 15 times in L-cell cultures had a mean titer of 3.22 $\log_{10}$ QH_{50} units, with a standard deviation of 0.127 $\log_{10}$. A dye uptake (DU_{50}) method for interferon assay was recently described by Finter (6). The mean activity and standard deviation for a single interferon preparation assayed 79 successive times was 3.97 ± 0.18 $\log_{10}$ DU_{50} units. Standard deviation values are minimized if titers are expressed in $\log_{10}$ units. Expression of our results in $\log_{10}$ $INAS_{50}$ units and subsequent calculation of standard deviations indicates the average titers of the two interferon preparations of Table 2 to be 3.334 ± 0.092 and 3.148 ± 0.125 $\log_{10}$ $INAS_{50}$ units. These comparisons indicate that the reproducibility of the $INAS_{50}$ assay technique is equal to or better than that of other methods for which information on reproducibility is available.

A principal advantage of the $INAS_{50}$ assay technique was that a relatively short time was required for its performance. Once the interferon-treated cultures were inoculated, only about 8 or 9 hr were needed for completion, including the time required to process the radioactive samples and to make the necessary calculations for estimating interferon titers. We did not investigate the length of time required for the development of maximal interferon effect, but others have reported that maximal protection of cells was achieved within 10 to 16 hr after the addition of interferon to cultures (11, 15). In our experiments, we routinely exposed L-cell cultures to interferon for 16 to 18 hr before challenging them with MM virus; thus, the entire procedure took 24 to 28 hr. However, Finter (5) reported that 3 or 4 hr of treatment with mouse interferon afforded about one-third of the maximal interfering activity in L-cell cultures. It is probable, therefore, that with some sacrifice in sensitivity the $INAS_{50}$ technique could be shortened so that the entire procedure, including addition of interferon, development of protection, and the viral infection, could be completed in 12 or 14 hr.

We have found the $INAS_{50}$ method to be a relatively sensitive technique for assaying mouse interferon. Although it averaged <0.46 times the sensitivity of the PDD_{50}-MM method, it was on the average 1.66 times more sensitive than the PDD_{50}-VSV method. It was about 20 times more sensitive than either the ICPE-MM method or the IVY_{70}-MM method. We observed a considerable amount of variability in the sensitivity ratios when the two plaque reduction techniques were compared with the $INAS_{50}$ method. We did not conduct a study of the reproducibilities of the PDD_{50} methods, but the results of our studies on the reproducibility of the $INAS_{50}$ method suggest that the variability observed was probably due to low reproducibility of the PDD_{50} techniques.

In addition to rapid accomplishment, good reproducibility, and high sensitivity, the $INAS_{50}$ interferon assay method was also flexible. When large numbers of specimens were being assayed, it was sometimes inconvenient to process the samples on the day they were collected. We have found that the samples may be collected in the usual fashion and then stored at -40 C until assayed for acid-insoluble radioactivity, with no significant effect on the indicated interferon titers.

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