Assay of Human Interferon in Vero Cells by Several Methods

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Four methods for the assay of human interferon in Vero cells were compared based on the inhibition of viral cytopathic effect (CPE) in tubes, the inhibition of CPE in microplates, the reduction of plaques, and the inhibition of quantitative hemadsorption. For inhibition of CPE, Sindbis virus, vesicular stomatitis virus, poliovirus type 2, and vaccinia virus were used for challenge. In the plaque reduction method, Sindbis virus, vesicular stomatitis virus, and poliovirus were employed, and Newcastle disease virus was used in the quantitative hemadsorption assay. Sindbis virus was most susceptible to interferon in those tests measuring inhibition of CPE, but vesicular stomatitis virus was as sensitive in the plaque reduction method. Highest titers of interferon were recorded in microplates, especially with Sindbis virus as the challenge agent, followed by the quantitative inhibition assay. The CPE inhibition method was the simplest, and the quantitative hemadsorption assay was the most rapid to perform. Reproducibilities, as shown by the coefficient of variation, were 15, 39, and 59% for plaque reduction, CPE inhibition in tubes, and CPE inhibition in microplates, respectively.

The assay of human interferon has been performed by using semiquantitative and quantitative methods (7). In the last few years, a growing interest in human interferon has led to the development of new procedures (1, 2, 12, 14, 17). However, the relative merits of newly reported techniques are difficult to judge since, with the exception of a few reports (3, 10), descriptions are not usually accompanied by comparative studies with commonly used procedures. Therefore, the many different types of assays and the diversity of cells and of challenge viruses that can be employed make it difficult to decide what method and cell-virus combination to adopt for a particular purpose.

In this report, one cell line was used (Vero cells) to ensure a better comparison between different procedures, and several of the more widely used methods for assay of interferon (7) were studied in order to provide a more uniform assessment of the methodology for titrating human interferon and to facilitate the choice of an assay for a specific need.

MATERIALS AND METHODS

Cells. Vero cells were grown in medium 199 supplemented with 5% sheep serum, penicillin, streptomycin, and amphotericin B. For experiments cells were

seeded as follows: in tubes (16 by 150 mm), 100,000 cells per ml; in 60-ml bottles, 10^6 cells per 5 ml; and in microplates, 50,000 cells per 0.15 ml per well.

Viruses. Newcastle disease virus (NDV), Victoria strain, received from Kurt Paucker, was propagated in embryonated eggs. The virus was titrated by plaque assay in Vero cells.

Vaccinia virus, vesicular stomatitis virus (VSV), Indiana strain (donated by Kurt Paucker), poliovirus type 2, Lansing strain, and Sindbis virus (kindly supplied by Norman B. Finter) were grown in Vero or HeLa cells (poliovirus). Titration of these viruses was carried out in Vero cells by determining the 50% tissue culture infective dose in tubes or wells in microtiter plates by the method of Reed and Muench (16), employing four to five tubes or six wells per dilution.

Interferon. Interferon was prepared by the method of Gresser (8) in primary human amnion cells exposed to live or ultraviolet-irradiated NDV. The fluids were collected after 24 or 72 h of incubation and held at pH 2 for 3 days before neutralization and storage at -20°C . One unit of interferon, as determined by using the inhibition of cytopathic effect (CPE) in tubes with VSV as challenge, was equivalent to 3.3 international reference units.

Interferon titrations. (i) **Inhibition of CPE in tube cultures.** The interferon preparation was diluted in twofold steps in maintenance medium (medium 199 supplemented with 1% sheep serum and antibiotics [MM1]). A 1-ml amount of each dilution was added to four tubes of Vero cells with an in vitro age of 3 to 4 days. After 24 h of incubation at 37°C , the medium was removed, and 1.0 ml of virus suspension, diluted in maintenance medium without serum (MM0) to

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contain approximately 100 50% tissue culture infective doses, was added to the cultures. The tubes were reincubated, and, when viral CPE had destroyed 75 to 100% of control cultures, the final reading was recorded. The interferon titer was expressed as the reciprocal of the highest dilution which caused an inhibition of 50% of the CPE. In each test, a parallel titration of the challenge virus was included.

(ii) Inhibition of CPE in microplate cultures.

At 1 day after seeding, the medium was replaced with 0.075 ml of MM1 per well. Interferon was diluted in fourfold steps in MM1, and 0.075 ml of each dilution was transferred from transfer plates (Cooke Engineering Co., Alexandria, Va.) to four or five wells by capillarity. After 24 h of incubation at 37°C, the fluids were removed, and the cells were infected with 0.15 ml of challenge virus per well and diluted in MM0 to contain approximately 100 50% tissue culture infective doses. Titrations of challenge virus accompanied each test and showed that 30 to 1,000 50% tissue culture infective doses were used as inocula.

(iii) Plaque reduction test. Interferon preparations were diluted in twofold steps, and 0.5 ml of each dilution was added to a 60-ml bottle of Vero cells containing 5 ml of maintenance medium with 2% serum. Two bottles were employed per dilution. The cells were incubated at 37°C for 24 h. The medium was then discarded and replaced with 0.4 ml of Hanks saline containing about 100 plaque-forming units of virus. After virus adsorption, the cells were overlaid with medium and 1% agarose. Plaques were read after staining with crystal violet-ethanol solution.

(iv) Quantitative hemadsorption. The technique described by Finter (4), adapted for microtiter plates, was used. Interferon was diluted and added to Vero cells as for the microplate assay. After 24 h at 37°C, the cells were infected with ND in MM0, with an input multiplicity of 200. Excess virus was removed by washing the monolayers with Hanks saline solution after 1 h of adsorption; each well then received 0.15 ml of MM1, and the plates were reincubated at 37°C for 24 h. At that time the medium was removed, and the monolayers were washed with phosphate-buffered saline (0.15 M NaCl, 0.1 M sodium potassium phosphate, pH 7.2) at 4°C. To each well, 0.1 ml of 1% guinea pig erythrocytes was added, and after 25 min at 4°C, the cells were washed with phosphate-buffered saline, and the attached erythrocytes were lysed with 0.1 ml of distilled water per well. The contents of eight wells were mixed, and the optical density was determined at 410 nm with a Beckman GT spectrophotometer.

Results obtained, less values from cell controls, were plotted as optical density against the log₁₀ of the interferon dilution. The 50% reduction point was calculated from the curve, and the corresponding interferon titer could be determined.

RESULTS

Inhibition of CPE in tubes. Interferon preparation A was employed in the experiments shown in Table 1. Sindbis virus and VSV were inhibited to approximately the same degree, requiring 2.3 and 3.3 reference units, respectively, to reduce viral CPE by 50%. Poliovirus exhibited

intermediate sensitivity, and vaccinia virus was insensitive to interferon. Titers varied from 80 to 160, 20 to 160, and 30 to 60 for Sindbis virus, VSV, and poliovirus, respectively. Despite the high variation when VSV was used as challenge virus, repeated titrations ($n = 22$) in different batches of cells of the same interferon preparation showed a coefficient of variation (standard deviation/mean) of 0.39.

Inhibition of CPE microplates. Another interferon preparation (preparation B) was employed in studies for which the results are shown in Table 2. Sindbis virus required the least amount of interferon (0.07 reference units) to give a 50% reduction in CPE. Poliovirus and VSV needed, respectively, 4 and 16 times more interferon to achieve the same effect, whereas vaccinia virus was again not susceptible to inhibition by interferon.

A coefficient of variation of 0.59 was found in 25 assays with Sindbis virus.

Plaque reduction test. Titers obtained with preparation B were 800, 715, and 407 when Sindbis virus, VSV, and poliovirus were used as

TABLE 1. Assay of human interferon in Vero cells: inhibition of viral CPE in tube cultures

Virus	Interferon titer (per ml) ^a	Reference units/ml at end point ^b	Relative sensitivity ^c
Sindbis	120 (4) ^d	2.3	1
VSV	83 (6)	3.3	0.7
Polio	37 (4)	7.4	0.3
Vaccinia	<10 (3)	>27.7	<0.08

^a Reciprocal of interferon dilution inhibiting 50% of the CPE.

^b Number of reference units required to inhibit viral CPE by 50%.

^c Ratio of reference units at end-point dilution of Sindbis virus in relation to different challenge viruses.

^d The number of experiments is given in parentheses.

TABLE 2. Assay of human interferon in Vero cells: inhibition of viral CPE in microplates

Virus	Interferon titer (per 0.15 ml) ^a	Reference units/0.15 ml at end point ^b	Relative sensitivity ^c
Sindbis	1,280 ^d	0.07	1
VSV	80	1.0	0.07
Polio	320	0.3	0.25
Vaccinia	<10	>8.5	<0.008

^a Reciprocal of interferon dilution inhibiting 50% of the CPE.

^b Number of reference units required to inhibit viral CPE by 50%.

^c Ratio of reference units at end-point dilution of Sindbis virus in relation to different challenge viruses.

^d Mean titer of at least three experiments.

challenge, respectively. Graphs of the experimental results (Fig. 1) showed that there was a good linear response in the 50% range, but at the extremes of the curves the numbers of plaques varied considerably. No significant difference in sensitivity was found between VSV and Sindbis virus, and poliovirus was only slightly more resistant to interferon. When the same interferon preparation was assayed eight times with VSV or Sindbis virus, the coefficient of variation was 0.15. Plaque formation by vaccinia virus was not influenced by the greatest amount of interferon used in these tests (data not shown).

Quantitative hemadsorption. Sendai virus, as originally described by Finter (4), could not be used because the amount of hemagglutinin produced in Vero cells was too low. But infection of cells with NDV gave strong hemadsorption, and the curves obtained with this virus are shown in Fig. 2. There was a good linear response in the 25 to 75% range, and the calculated titers of preparation B were 430, 800, and 1,280, with a mean of 836, displaying high sensitivity. However, the variability in assays over a period of 1 week was large (Fig. 2, curves A and B).

Comparison of methods. Because titers depend on the 50% end point and on the volumes of interferon applied to varying amounts of me-

FIG. 1. Plaque reduction curves of different viruses in interferon-treated Vero cells. Bars represent range of findings; the points represent the mean. PFU, Plaque-forming units.

FIG. 2. Curves from quantitative hemadsorption assays of interferon in Vero cells.

dium in the different cell culture systems, a more precise manner of comparing the sensitivities is to calculate the amount of interferon that was needed in each method to reduce the effect of viral infection by half. Thus, interferon preparation B was titrated with the CPE method in tubes, and its potency in international reference units was ascertained. These data, the data in Tables 1 and 2, and the amounts in reference units for the plaque assay and quantitative hemadsorption in microplates) showed the highest sensitivities. When Sindbis virus was used for challenge, 33 and 110 times more interferon had to be available to reach a 50% end point in the CPE tube method and plaque reduction test, respectively, compared with CPE inhibition in microplates. VSV and poliovirus also behaved similarly, although the differences were less marked. NDV was highly sensitive to human interferon, since as few as 0.2 international reference units gave a 50% end point, five times less than the amount needed to inhibit the CPE of VSV (a virus commonly used for challenge in interferon assays) in microplates. The small volumes and number of cells needed (six times less than in the tube method and 60 times less than in the plaque reduction procedure) might explain these differences (5, 15).

DISCUSSION

Sindbis virus is very sensitive to human interferon (Tables 1 and 2), as has been pointed out by others (1, 18). However, the time of reading

TABLE 3. Assay of human interferon in Vero cells: comparison of interferon reference units needed for 50% inhibition in several methods

Assay measuring inhibition of:	Total interferon reference units needed for 50% inhibition of: ^a			
	Sindbis virus	VSV	Poliovirus	NDV
CPE (tubes)	2.3 (33) ^b	3.3 (47)	7.4 (106)	
CPE (microplates)	0.07 (1)	1.0 (14)	0.3 (4)	
Plaques	7.7 (110)	8.7 (124)	15.4 (220)	
Quantitative hemadsorption				0.2 (3)

^a Recorded per milliliter for the tube assay, per 5.5 ml for the plaque assay, and per 0.75 ml for the assay in microplates and quantitative hemadsorption.

^b Figures in parentheses denote relative sensitivity of method in relation to the microplate assay with Sindbis virus.

the CPE in Vero cells infected with Sindbis virus or VSV is critical, because viral inhibition by interferon could be overcome rapidly by prolonged incubation. The coefficient of variation of 0.39 for this type of assay is in the same range as shown by Siewers *et al.* (18).

Moreover, the comparative sensitivities to interferon of two viruses in one system was not necessarily paralleled in another system. For instance, 16 times more interferon was needed to reduce the CPE of VSV than that of Sindbis virus in microplates (Table 2), whereas in the macrotechnique the ratio was only 1.4 (Table 1). Poliovirus was more inhibited by interferon than was VSV in the microtechnique, and the opposite occurred in the tube method. These findings can best be explained by differences in the experimental conditions under which these tests are carried out (2, 12). However, multiplicity does not appear to be a factor since 30-fold differences in the dosage of VSV did not alter the interferon titer in the microplate assay, as has also been reported for the tube method (7).

VSV and Sindbis virus were about equally susceptible to interferon in the plaque reduction assay. VSV plaques appeared earlier (36 instead of 48 h for Sindbis virus), and they were easier to read. The coefficients of variation in the assay of human interferon of 0.25 (13), 0.26 (19), and 0.15 (see above) show a high degree of reproducibility.

In the quantitative hemadsorption method, high titers of interferon were revealed when NDV was used as challenge virus. NDV is considered to be a virus resistant to interferon (11), but, under the experimental conditions employed, the growth of NDV was probably restricted to one cycle, and it has been shown (9) that the inhibition of multiplication of NDV and VSV in a single cycle were in the same range. Curves similar to those shown in Fig. 2 were obtained in the macrosystem by Finter (4).

From the data obtained and following the criteria described by Finter (7), a comparison of the characteristics of the various methods can be made. A method is considered simple when a minimum of preparation, technical expertise, and equipment are needed, and CPE inhibition measured in tubes or microplates, as well as the plaque reduction method, were the simplest. The CPE inhibition method in microplates was very convenient, taking little time to perform and to read and allowing a larger number of samples to be handled compared with the other methods studied. Because the presence of NDV hemadsorption could be demonstrated after 24 h of infection of Vero cells and the hemoglobin content measurement took about 1 h, this method was the most rapid.

The coefficient of variation demonstrated that the plaque reduction method was the most reproducible procedure, with a coefficient of variation of only 0.15, compared with 0.39 and 0.59 for the CPE method in tubes and microplates, respectively. The poor reproducibility of semi-quantitative methods when visual reading of CPE is used is well documented (7).

When many samples are tested and a high sensitivity is required, the CPE inhibition in microplates with Sindbis virus as the challenge should be the method of choice. If relative potencies of standard preparations of interferon are to be compared, or when there are other conditions when small differences in titer must be ascertained, the plaque reduction assay with VSV should be preferred.

The major disadvantage of CPE inhibition in microplates, as compared with other methods, is its poor reproducibility. The inhibition of growth of Sindbis virus in Vero cells can be quantitated by the dye uptake method (6, 12) or by the hemagglutinin yield-reduction assay (14), and a modification of the latter method to a quantitative assay might lead to improved results. Com-

parative studies including these modifications will be required to determine whether this is in fact the case.

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