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Convenient Assay for Interferons

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A convenient assay for interferons based on reduction of cytopathic effect was developed. The number of manipulations and the lengths of the various incubation steps were reduced to a minimum. The assay is simple to perform and can be completed within 16 h. Moreover, it can be used with various types of cells and a variety of viruses.

Interferon bioassays are based on measurement of a parameter associated with either virus production or cytopathic effect (CPE) on host cells (1, 3–6, 9, 11). No single universally acceptable assay exists. In general, an assay is selected according to a given application. Thus, for example, monitoring of interferon activity during production and purification requires a rapid assay, and sensitivity can be sacrificed.

To meet the need for a rapid assay, we have developed a convenient microtiter assay based on reduction of CPE. The assay is quantitative, requires only 16 h, and can be adapted to many cell and virus combinations in common use. For simplicity, the number of technical manipulations was reduced to a minimum. Various parameters affecting both speed and sensitivity were determined and are presented in this study.

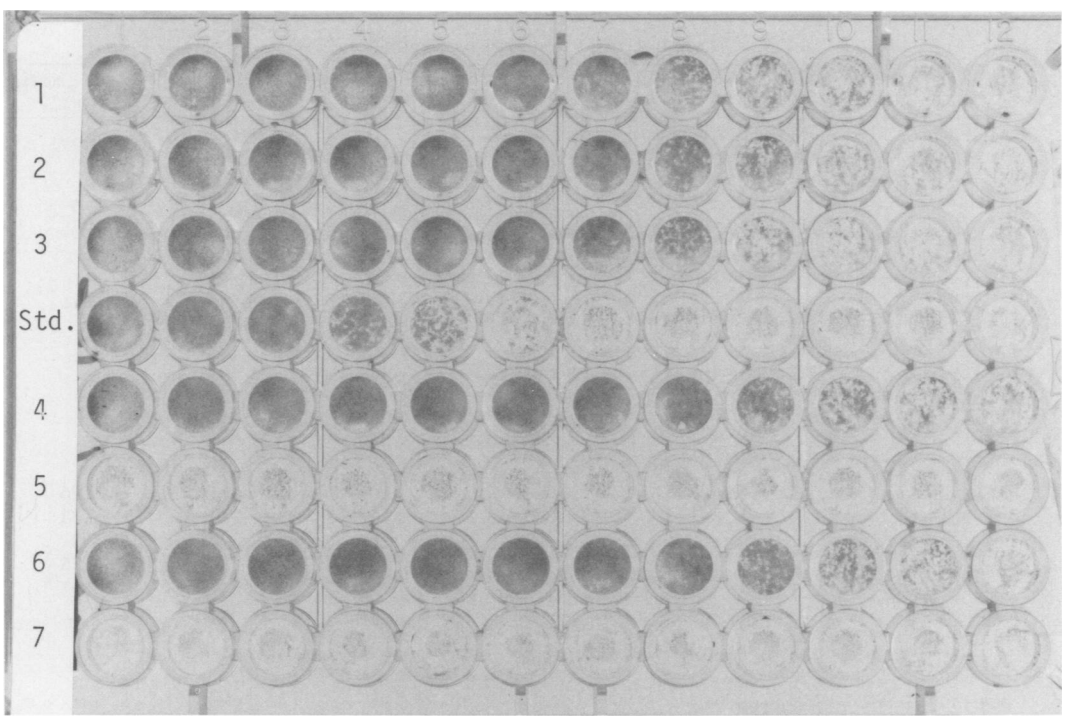

FIG. 1. *Photograph of microtiter plate. Rows 1, 2, 3, 4, and 7 contained various dilutions of human leukocyte interferon. The row designated Std. contained the laboratory standard of human leukocyte interferon with a titer of 640 U/ml. The endpoint for the standard can be seen as well 5, where there was approximately 50% protection of MDBK cells. The wells with 50% protection for the other samples were as follows: row 1, well 9; row 2, well 9; row 3, wells 8 to 9; row 4, well 10; row 6, well 10. No protection was seen in any of the wells of rows 5 and 7, which were essentially identical to virus controls. Cell control wells were similar to the early wells showing protection (all rows except 5 and 7).*

MATERIALS AND METHODS

Cell cultures. MDBK, a bovine kidney cell of epithelial origin (7) which is sensitive to human leukocyte interferon, was obtained from Jan Vilček. Two human fibroblast cell lines, GM-2504A, which is trisomic for chromosome 21, and AG-1732, were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J. GM-2504A was formerly designated GM-258. All cell lines were maintained in monolayer cultures in Eagle minimal essential medium prepared with Earle salts and supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories) and 50 μg of gentamicin per ml (MEM-10).

Virus. The New Jersey strain of vesicular stomatitis virus (VSV) was grown in monolayer cultures of mouse L cells and stored frozen in MEM-10. The virus was titrated by plaque formation on mouse L cells (2). All viral dilutions used in the assay were made in MEM-10.

Interferons. Human leukocyte interferon was produced by inducing leukocyte buffy coats with Newcastle disease virus (8, 10). Human fibroblast interferon was induced in this laboratory in the human cell line GM-2504A by polyinosinic-polycytidylic acid (12). The reference standards for human leukocyte and fibroblast interferons (G-023-901-527 and G-023-902-527, respectively) were obtained from the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases. These reference interferon preparations were used to standardize the interferon assay.

Trypsinization of cells for assay. Confluent monolayers of cells in 75-cm^2 flasks were rinsed with phosphate-buffered saline (containing the following in grams per liter: NaCl, 8; KCl, 0.2; KH_2PO_4, 0.2; and $Na_2HPO_4 \cdot 7H_2O$, 2.16) and incubated at 37°C for 5 to 10 min with 2.5 ml of a solution containing 0.5 g of trypsin and 0.2 g of EDTA in 1 liter of 0.15 M NaCl (GIBCO Laboratories; no. 610-5400). The trypsin solution containing the cells was diluted in the maintenance medium to the concentration required and used directly for the assay as described below.

Interferon assay. The entire procedure, including dilutions, for the interferon assay was performed in a 96-well microtiter plate (Falcon Plastics; no. 3040). The interferon sample to be titrated was diluted in MEM-10 in the first well of a row in the microtiter plate to a final volume of 0.2 ml. Subsequent wells in the row contained 0.1 ml of MEM-10, and twofold dilutions of the samples were made by transferring 0.1 ml serially to the end of the row. On each plate, four

TABLE 1. *Effect of virus concentration*

Cell type	Interferon type	PFU per well	Time required for 100% CPE (h)	Observed titer (U/ml)	Relative sensitivity[a]
MDBK	Leukocyte	15,000	16	480	0.75
		10,000	16	640	1
		8,000	16	640	1
		6,000	16	980	1.5
		4,000	16	980	1.5
		3,000	16	1,260	2
		2,000	20	1,260	2
		1,000	>24	2,520	4
		500	>24		
GM-2504A	Leukocyte	60,000	16	2,560	4
		30,000	16	2,560	4
		15,000	24	3,840	6
		7,500	>24	5,120	8
AG-1732	Leukocyte	10,000	16	160	0.25
		8,000	16	240	0.33
		6,000	16	320	0.5
		4,000	16	460	0.75
		3,000	16	640	1
		2,000	16	640	1
		1,000	18	920	1.5
		500	20	920	1.5
AG-1732	Fibroblast	4,000	16	1,280	0.75
		2,000	16	1,920	1
		1,000	16	2,560	1.5
		500	20	5,120	3

[a] Relative sensitivity of the assay was defined as the ratio of the observed titer to the actual titer. The actual titer of interferons used was previously measured, relative to the reference standard. The assay was performed as described in the text. Each well was seeded with 2×10^4 to 3×10^4 cells. The time of challenge after the addition of interferon was 1 h for MDBK cells and 2 h for AG-1732 and GM-2504A cells. Laboratory standards of leukocyte interferon (640 U/ml) and fibroblast interferon (2,000 U/ml) were used.

to six wells were filled with 0.1 ml of medium to serve as virus and cell controls. Each well was then seeded with 1×10^4 to 4×10^4 cells in 0.1 ml of MEM-10. The plate was sealed with plastic tape (Falcon Plastics; no. 3044) and incubated at 37°C. The time of addition of VSV varied from 0 to 24 h after plating of the cells. All wells, except cell control wells, received 0.05 ml of a suspension containing VSV in concentrations ranging from 500 to 15,000 PFU per well as noted in the footnotes to tables. The final volume in the wells was 0.25 ml. The plate was resealed and incubated at 37°C until the control well displayed a CPE in 100% or close to 100% of the cells. The medium from each well was aspirated, and cell monolayers were stained with 0.1 ml of 0.5% (wt/vol) crystal violet (5 g/liter in 70% methanol) for about 1 min. After the crystal violet solution was decanted, the plate was rinsed gently with tap water and then dried in air. The interferon titer was read as the reciprocal of the dilution represented in the well in which 50% of the cell monolayer was protected. All data shown represent a minimum of two titrations. A laboratory standard of interferon was included in all assays so that the absolute titer could be determined. The laboratory standard was prepared by titration against the appropriate human interferon reference standards.

The 50% endpoints were determined by visual examination of the monolayers with and without the aid of a microscope. For some cell lines, such as the MDBK cells, macroscopic and microscopic examination provided identical endpoints so that microscopic examination was unnecessary. A photograph of a representative microtiter plate is shown in Fig. 1.

RESULTS

Virus concentration. The effects of virus concentration on the bioassay were determined with VSV and several cell lines. It was found that the virus concentration affected both the sensitivity of the assay and the length of the incubation time required for 100% CPE in the virus control wells (Table 1). The sensitivity of the assay could be increased by lowering the virus concentration. The total incubation time could be shortened to 16 h by increasing the amount of virus. A further increase in virus concentration lowered the sensitivity of the assay, but failed to shorten the total incubation time to less than 16 h.

Time of challenge. The effect of time of virus challenge after the addition of interferon on the sensitivity of the assay was determined. It was found that the protective effect of interferon increased gradually during the first 6 h of incubation with the cells (Table 2). This was observed both when the interferon and the cell suspension were added consecutively, as described above, and when interferon was added to an established monolayer. The sensitivity of the assay could be increased by a factor of 5- to 16-fold by varying the time of addition of the challenge virus. Sensitivity was consistently higher in assays with established monolayers. However, when a highly sensitive assay is not required, results can be obtained more rapidly and with fewer manipulations when the cell suspension is added directly to wells containing the interferon dilutions.

Cell concentration. Table 3 shows that both

TABLE 2. *Effect of time of virus challenge after addition of interferon*[a]

Cell type	Time of challenge (h)	Assay 1		Assay 2	
		Observed titer (U/ml)	Relative sensitivity	Observed titer (U/ml)	Relative sensitivity
MDBK	0	480	0.75	960	1.5
	1	690	1	1,280	2
	2	690	1	2,560	4
	3	960	1.5	3,840	6
	4	1,280	2	5,120	8
	5	1,920	3	5,120	8
	6	2,560	4	10,240	16
AG-1732	0	240	0.25	640	1
	1	320	0.5	960	1.5
	2	640	1	1,280	2
	3	640	1	2,560	4
	4	960	1.5	2,560	4
	5	1,280	2	3,840	6
	6	2,560	4	5,120	8
	24	2,560	4	5,120	8

[a] The assay was performed as described in the text. The number of cells per well was 2×10^4 to 3×10^4. Cells were challenged with 3,000 PFU of VSV per well. A laboratory standard of leukocyte interferon of 640 U/ml was used. In assay 1, the interferon and the cell suspension were added to the wells consecutively as described in the text. In assay 2, interferon dilutions were added to established monolayers of the cells. Relative sensitivity is defined as the ratio of the observed titer to the actual titer of a standard interferon preparation. At 24 h, the MDBK cells were overgrown so that no 100% CPE could be observed at 24 h or even after 48 h.

TABLE 3. *Effect of cell concentration*[a]

Cell type	Cell concn $\times 10^{-4}$	Time required for 100% CPE (h)	Observed titer (U/ml)	Relative sensitivity
MDBK	1	16	640	1
	2	16	640	1
	3	16–20	1,280	2
	4	>24	2,560	4
AG-1732	1	16	640	1
	2	16	960	1.5
	3	16–20	1,280	2
	4	20	1,280	2

[a] The assay was performed as described in the text. Cells were challenged with 3,000 PFU of VSV per well after 1 h (for MDBK) and 2 h (for AG-1732) of incubation with the interferon. A laboratory standard for leukocyte interferon of 640 U/ml was used.

TABLE 4. *Recommended conditions for convenient interferon assay*

Assay objective	Cell type	No. of cells per well	PFU per well	Time of challenge after addition of interferon (h)	Time required for 100% CPE (h)	Relative sensitivity
Rapid	MDBK	2×10^4	10,000	0	16	0.5
Rapid	AG-1732	2×10^4 to 3×10^4	10,000	0	16	0.1
Sensitive	MDBK	3×10^4	3,000	6	20–24	4
Sensitive	AG-1732	2×10^4 to 3×10^4	500	6	16–20	8
Sensitive	AG-1732	Preformed monolayer	500	6	20–24	8
Sensitive	MDBK	Preformed monolayer	3,000	6	28	16

the sensitivity and the time required for 100% CPE increased when MDBK or AG-1732 cells were added in higher concentrations.

DISCUSSION

The procedure described was developed to provide a rapid, simple, and sensitive interferon assay for routine use. The entire assay can be completed within 16 h. The procedure consists of the following steps: samples of interferon are diluted; then cells are added and subsequently challenged with virus. There is no need to prepare monolayers in advance or to remove the interferon before challenge. Since virus can be added immediately after cells are added, samples can be assayed late in the afternoon, and results can be obtained on the next morning.

Various parameters can be modulated to provide both speed and sensitivity. The sensitivity can be increased by decreasing the amount of the challenge virus, increasing the time of incubation of cells with interferon before challenge, or using established monolayers. Appropriate assay conditions for specific objectives are summarized in Table 4.

The procedure is not limited to a single cell type, interferon species, or challenge virus. Other viruses, such as Sindbis, and additional cell lines, such as FS-7, WISH, and mouse L cells, provided similar results.

Quantitation of results may be affected by other factors, such as growth conditions before use or age of stock cells at the time of use. These factors are eliminated by comparison of titers of samples with standard interferon preparations. This rapid assay has proven to be a powerful tool for the purification of human leukocyte interferon (10). The availability of the results of chromatographic steps by the next morning enabled chromatographic procedures to be performed on successive days, rather than days apart. In addition, the use of this interferon assay for determination of interferon titers in human serum is being evaluated.

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LITERATURE CITED

1. **Armstrong, J. A.** 1971. Semi-micro, dye-binding assay for rabbit interferon. Appl. Microbiol. **21**:723–725.
2. **Cooper, P. D.** 1967. The plaque assay of animal viruses, p. 243–311. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 3. Academic Press, Inc., New York.
3. **Finter, N. B.** 1973. Assay and standardization of interferon and interferon inducers, p. 135–169. *In* N. B. Finter (ed.), Interferons and interferon inducers, 2nd rev. ed. Elsevier/North Holland Publishing Co., Amsterdam.
4. **Green, J. A., G. J. Stanton, J. Goode, and S. Baron.** 1976. Vesicular stomatitis virus plaque production in monolayer cultures with liquid overlay medium: description and adaptation to a one-day human interferon-plaque reduction assay. J. Clin. Microbiol. **4**:479–485.
5. **Jordan, G. W.** 1972. Quantitative aspects of interferon-induced plaque reduction: kinetics of interferon action. Virology **48**:425–432.
6. **Lindenmann, J., and G. E. Gifford.** 1963. Studies on vaccinia virus plaque formation and its inhibition by interferon. III. A simplified plaque inhibition assay of interferon. Virology **19**:302–309.
7. **Madin, S. H., and N. B. Darby, Jr.** 1958. Established kidney cell lines of normal adult bovine and ovine origin (24111). Proc. Soc. Exp. Biol. Med. **98**:574–576.
8. **Mogensen, K. E., and K. Cantell.** 1977. Production and preparation of human leukocyte interferon. Pharmacol. Ther. Part A Chemother. Toxicol. Metab. Inhibitors **1**: 369–381.
9. **Richmond, J. Y., J. Polatnick, and R. C. Knudsen.** 1980. Microassay for interferon, using [^{3}H]uridine, microculture plates, and a multiple automated sample harvester. Appl. Environ. Microbiol. **39**:823–827.
10. **Rubinstein, M., S. Rubinstein, P. C. Familletti, R. S. Miller, A. A. Waldman, and S. Pestka.** 1979. Human leukocyte interferon: production, purification to homogeneity, and initial characterization. Proc. Natl. Acad. Sci. U.S.A. **76**:640–644.
11. **Sedmak, J. J., S. E. Grossberg, and P. Jameson.** 1975. The neuraminidase yield-reduction bioassay of human and other interferson (38822). Proc. Soc. Exp. Biol. Med. **149**:433–438.
12. **Vilček, J., and E. A. Havell.** 1973. Stabilization of interferon messenger RNA activity by treatment of cells with metabolic inhibitors and lowering of the incubation temperature. Proc. Natl. Acad. Sci. U.S.A. **70**:3903–3913.