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NEW SIMPLE DYE-UPTAKE ASSAY FOR INTERFERON

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SUMMARY Using the spectrophotometer that the authors developed, the amounts of human leukocyte and mouse L cell interferons on FL cells and L_{929} cells were measured and values were compared with those measured by the cytopathogenic effect (CPE) reduction method (CPE method). The spectrophotometric method, which was simpler than the original dye-uptake method, was found to be more sensitive than the latter. When Sindbis virus was used instead of vesicular stomatitis virus (VSV), there were no significant differences in the sensitivities of the two methods or the interferon titers estimated. When FL cells or L_{929} cells were treated with interferon at the time of their dispersion, their interferon titers were almost the same as those of cells treated with interferon 2 days after dispersion. It is concluded that this new dye-uptake method is useful for assay of human and mouse interferons.

INTRODUCTION

Several methods for assay of interferon have been reported (Stewart II, 1979). However, since all these methods are based on the biological (antiviral) activity of interferon, they are not so sensitive as possible chemical or immunological methods. The dye-uptake method is fairly sensitive (McManus, 1976; Finter, 1969; Pidot, 1971), but it is complicated. The authors developed a new simple dye-uptake method, and compared it with the microassay method based on cytopathogenic effects employed up to now in this laboratory.

MATERIALS AND METHODS

1. *Cells*

The human amnion FL cell line was used for assay of human interferon and the mouse L_{929} cell line was used for assay of murine interferon. Both cell lines were subcultured and maintained in Eagle's minimum essential medium (MEN) (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 5% newborn calf serum (Microbiological Associates, Maryland).

2. *Viruses*

The New Jersey strain of vesicular stomatitis virus (VSV) and Sindbis virus were used as challenge viruses. VSV was propagated on FL cells and Sindbis virus on primary chicken embryonal fibroblasts. Virus solution was stocked at −80 C before experiments.

3. *Interferon and reference interferons*

Human leukocyte interferon produced in human peripheral leukocytes infected with Sendai virus was kindly supplied by Dr. A. Matsuo of the Central

Research Institute of Green Cross Corp., Osaka, Japan (Matsuo et al., 1974). It was concentrated with ammonium sulphate and purified by CM- and DEAE-cellulose column chromatographies and then by Sephadex G-100 gel filtration. Its specific activity was about 1×10^6 IU/mg of protein.

Mouse L cell interferon was prepared in L_{929} cell cultures infected with Newcastle disease virus (Miyadera strain: NDV). The culture supernatant was acidified to pH 2.0 with 1*N* HCl to inactivate residual NDV. Three days later, it was readjusted to pH 7.0, dialyzed against LPS-free distilled water and lyophilized. The specific activity of L cell interferon was 1.4×10^6 IU/mg of protein. Human and mouse interferons were prepared as references, based on reference interferons from NIH (Code 69/19 and Code G002-902-026).

4. *Spectrophotometer*

The spectrophotometer consisted of a light source, lens, filter, slit, photocell, amplifier and voltage meter (Fig. 1).

As a light source, a light emitting diode (LED; GL-5NG6, Sharp Electric Co., Ltd.), with a spectral response range of 530 to 610 nm was used. The emitted light was concentrated with the lens and passed through the green filter. Filtered light was then passed through the well of a microplate and slit (5 mm in diameter). The light was received by a photocell EE-D33 (OMRON Co., Ltd.) with a spectral response range of 400 to 1,200 nm, the transformed voltage was amplified, and the voltage was read (Fig. 1).

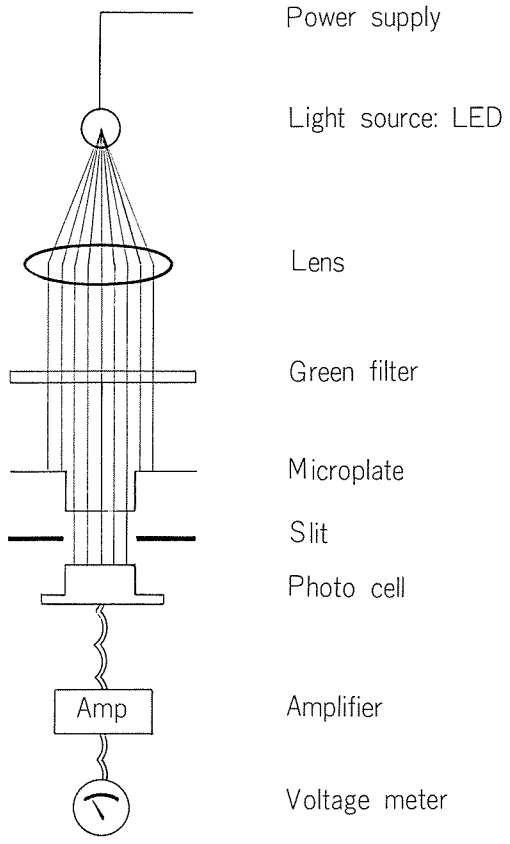

FIGURE 1. Model of the spectrophotometer apparatus.

5. *Method for interferon assay*

Interferon activity was assayed by two methods. In the first method, monolayer cells were dispersed and 2 days later were treated with interferon. In the second method, the cells were treated with interferon at the time of dispersion. The details of the two methods are described below.

1) First method

A sample of 2×10^5 /ml FL or L_{929} cells was dispersed in wells of a microplate (Nunc, Denmark) and incubated at 37 C for 2 days in an atmosphere of 5% CO_2 in air. Then the confluent monolayer cells were treated with 0.025 ml of 2-fold serially diluted interferon in quadruplicate, and incubated overnight. Then the medium was removed and the cells were challenged with about 10–50 $TCID_{50}$ /ml of VSV or Sindbis virus and 48 h later, the cytopathogenic effect (CPE) was examined and graded as positive or negative (±method), according to the extent of the CPE area (scoring method) observed under a light microscope. The reciprocal of the interferon dilution at which the CPE was reduced to 50% was calculated by the formula of Behrens-Kärber and expressed as laboratory interferon units. Then laboratory interferon units were transformed to International Units by multiplication by a factor. The factor was defined as the ratio of laboratory units of reference interferon to International Units as proposed at NIH (Imanishi et al., 1977). In the spectrophotometric method, after observation of CPE, the cells were stained with 1% neutral red solution for 30 min, carefully washed twice with distilled water and dried at room temperature. It was confirmed that the cells were not lysed during staining and washing. Then the optical density in

each well was measured in a spectrophotometer. The mean optical density was calculated for groups of four wells used for challenge with each virus concentration or treatment with each interferon concentration, and the optical density of each group of wells was calculated as a percentage of that of control wells, which were not treated with interferon or challenged by virus. The percentage in the group that was not treated with interferon, but was challenged with virus was 0%. The reciprocal of the interferon dilution at which the percentage was reduced by 50% was expressed as laboratory interferon units, and then the factor was calculated by dividing the International Units by laboratory interferon units for the reference interferon. The laboratory units of the interferon preparation were expressed in International Units by multiplication of laboratory units by this factor. The degree of CPE was proportional to the optical density in each well (Fig. 2).

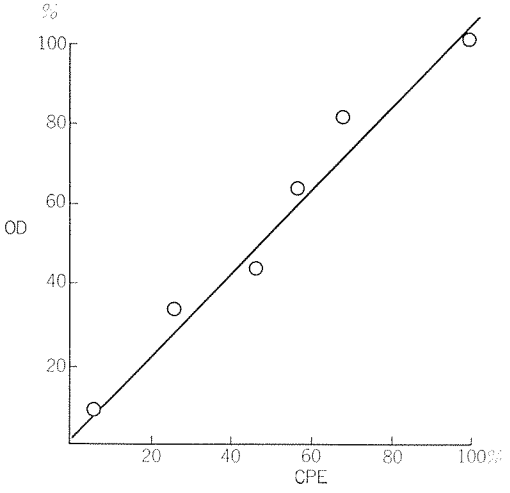

FIGURE 2. Relation between degree of CPE and optical density in each well.

The CPE or the optical density in groups of four wells were calculated as percentages of that of control group which was not treated with interferon or challenged with virus. When the cells were not treated with interferon, but challenged with virus, the value was 0%.

2) Second method

Fifty microliters of interferon solution was serially diluted two-fold in microplates and then 0.05 ml of FL or L_{929} cells (8×10^5 /ml) were dispersed in the wells and incubated overnight at 37 C in an atmosphere of 5% CO_2 in air. Subsequent processes were the same as in the first method.

6. *Statistical evaluation*

The interferon titer was expressed as the geometric mean and standard error of the mean (SEM) with the coefficient of variation (Pidot, 1971).

RESULTS

1. *Comparison of CPE methods with the first spectrophotometric method using FL cells and VSV*

In 16 observations, the geometric mean was 4873.19 ± 0.33 with a coefficient of variation of 0.00027 when human leukocyte interferon was titrated using FL cells and VSV, and CPE was judged as positive or negative ($\pm$method). When CPE was scored as 0 to 4 (scoring method), the geometric mean was 4772.30 ± 0.32 with a coefficient of variation of 0.00026. In the spectrophotometric method, the geometric mean was 4316.54 ± 0.40 with a coefficient of variation of 0.00036. There were no significant differences in the geometric means, standard errors or coefficients of variation of interferon titers determined by these methods (Table 1). However, the factor varied according to the method; namely, the spectrophotometric method was more sensitive than the CPE methods (Table 1).

2. *Comparison of CPE methods with the second spectrophotometric method using FL cells and VSV*

When FL cells were treated with human leukocyte interferon at the time of dispersion, the geometric means of interferon titers were respectively 5107.34 ± 0.38, 4418.70 ± 0.41 and 4422.02 ± 0.41 in the $\pm$ method, the scoring method and the spectrophotometric method. The interferon titer assayed by CPE methods tended to be higher than that assayed by the spectrophotometric method, although there were no significant differences in interferon

titers in these methods. The spectrophotometric method was more sensitive than the CPE methods (Table 1).

3. *Comparison of CPE methods with the first spectrophotometric method using FL cells and Sindbis virus*

In 17 observations, the geometric mean was 4931.59±0.38 with a coefficient of variation of 0.00031 when human leukocyte interferon was assayed using FL cells and Sindbis virus and CPE was judged as positive or negative (±method). When CPE was scored, the geometric mean was 4762.82±0.34 with a coef-

TABLE 1. *Comparison of CPE methods with the spectrophotometric method using FL cells and VSV*

Method for IFN assay	CPE method		Spect. Method	Time of IFN Treatment	No. of Experiments
	±method	scoring method			
Factor[a]	22.83±0.57	4.68±0.59	2.43±0.62	1st method	16
	(0.043)[b]	(0.49)	(0.98)		
Titer	4873.19±0.33	4772.30±0.32	4316.54±0.40		
(IU/ml)	(0.00027)	(0.00026)	(0.00036)		
Factor	35.66±0.75	6.74±0.50	4.94±0.54		
	(0.075)	(0.27)	(0.40)	2nd method	14
Titer	5107.34±0.38	4418.70±0.41	4422.04±0.41		
(IU/ml)	(0.00027)	(0.00033)	(0.000333)		

[a] Factor = $\frac{\text{reference interferon activity}}{\text{laboratory interferon activity}}$

[b] Numbers in parentheses indicate coefficients of variation.

TABLE 2. *Comparison of CPE methods with the spectrophotometric method using FL cells and Sindbis virus*

Method for IFN assay	CPE method		Spect. Method	Time of IFN Treatment	No. of Experiments
	±method	scoring method			
Factor[a]	22.93±0.49	5.98±0.56	2.72±0.75		
	(0.085)[b]	(0.38)	(1.10)	1st method	17
Titer	4931.59±0.38	4762.82±0.34	4104.82±0.38		
(IU/ml)	(0.00031)	(0.00028)	(0.00037)		
Factor	37.79±0.42	9.66±0.40	4.43±0.41		
	(0.041)	(0.16)	(0.35)	2nd method	15
Titer	5512.48±0.36	5052.43±0.34	4370.93±0.35		
	(0.00024)	(0.00025)	(0.00030)		

[a] Factor = $\frac{\text{reference interferon activity}}{\text{laboratory interferon activity}}$

[b] Numbers in parentheses indicate coefficients of variation.

TABLE 3. *Comparison of CPE methods with the spectrophotometric method using L_{929} cells and VSV*

Method for IFN assay	CPE method		Spect. Method	Time of IFN Treatment	No. of Experiments
	±method	scoring method			
Factor[a]	22.83±0.57	4.68±0.59	2.43±0.62		
	(1.02)[b]	(2.28)	(3.22)	1st method	18
Titer	2402.27±0.33	2442.91±0.32	2233.53±0.31		
(IU/ml)	(0.00056)	(0.00054)	(0.00058)		
Factor	2.38±0.35	1.00±0.42	0.74±0.4		
	(0.61)	(1.75)	(2.22)	2nd method	18
Titer	2515.97±0.30	1981.05±0.31	1759.06±1.05		
(IU/ml)	(0.00050)	(0.00065)	(0.00074)		

[a] Factor = $\frac{\text{reference interferon activity}}{\text{laboratory interferon activity}}$

[b] Numbers in parentheses indicate coefficients of variation.

ficient of variation of 0.00028. In the spectrophotometric method, the geometric mean was 4104.82±0.38 with a coefficient of variation of 0.00037. There were no significant differences in the interferon activities determined by the CPE methods and spectrophotometric method, but the latter method was about 4.0- to 8.5-fold more sensitive than CPE methods (Table 2).

4. *Comparison of the CPE methods with the second spectrophotometric method using FL cells and Sindbis virus*

In 15 observations, the geometric means were respectively 5512.48±0.36, 5052.43±0.34 and 4370.93±0.35 in the ± method, the scoring method and the spectrophotometric method using FL cells and Sindbis virus (Table 2). There were no significant differences in the interferon titers or factors with VSV and Sindbis virus in the last method. There were no differences in the interferon titers with VSV and Sindbis virus between the first and the second methods.

5. *Comparison of the CPE methods with the first spectrophotometric method using L_{929} cells and VSV*

In 18 observations, the geometric mean was 2402.77±0.33 in the ± method using L_{929} cells and VSV. There were no significant differences in interferon titers in the ± method, the scoring method and the spectrophotometric method. However, the spectrophotometric method was more sensitive than the CPE methods. Results showed mouse interferon could be assayed by the spectrophotometric method using L_{929} cells and VSV (Table 3).

6. *Comparison of CPE methods with the second spectrophotometric method using L_{929} cells and VSV*

Similar results were obtained when L_{929} cells were treated with mouse interferon at the time of dispersion, the geometric means of interferon activities being respectively 2512.97 ±0.30, 1981.05±0.31 and 1759.06±1.05 by the ± method, the scoring method and the spectrophotometric method. There were no significant differences in the interferon titers in the first and the second methods (Table 3).

DISCUSSION

Many methods for interferon assay have been reported (Stewart II., 1979). Because all

methods are based on biological (antiviral) activity, they are time-consuming and laborious, and results are variable. Dye-uptake methods are more sensitive and reproducible (McManus, 1976; Finter, 1969; Pidot, 1971), but the procedures are complicated. We developed a new spectrophotometer by which the dye-taken into the cell is measured without dissolving in appropriate solvent. This spectrophotometric method is more sensitive than CPE methods for assay of human and mouse interferons. The interferon titer determined by the spectrophotometric method tended to be lower than that measured by CPE methods, although there were no significant differences in the titers determined by these methods. The reason for this tendency is unknown. The spectrophotometric method was as precise as the CPE methods, because the coefficients of variation in the CPE methods and the spectrophotometric method were almost the same. When FL or L_{929} cells were treated with interferon at the time of dispersion in microplates, their interferon titers were almost the same as those of cells pretreated with interferon. This means that time required for interferon assay can be shortened. Since this spectrophotometric method is simpler than the conventional dye-uptake method, it is useful for assay of human and mouse interferon.

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