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Automated, Quantitative Cytopathic Effect Reduction Assay for Interferon

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A rapid, cytopathic effect reduction assay for human interferon (IFN) is described. Dilutions of IFN were made with an automated diluter in 96-well microtiter plates. Total incubation time was 26 h. IFN titers were calculated from optical density readings of crystal violet-stained monolayers in an automated spectrophotometer, which required less than 1 min to read each plate.

Multidisciplinary interest in the antiviral, anticancer, and immunomodulatory effects of interferon (IFN), particularly with the development of recombinant DNA-produced IFNs, has stimulated the need for an IFN bioassay that is rapid, as automated as possible, objective, quantitative, sensitive, and reproducible. The most common methods for determining IFN titers involve cytopathic effect (CPE) or plaque reduction endpoints in cell cultures (1, 2, 4, 6, 8, 9). CPE reduction assays suffer from the subjective nature of determining endpoints, which prohibits calculation of exact titers, whereas plaque reduction methods are tedious and time consuming (4). This note describes modifications in a previously reported microplaque reduction assay for human IFN (6) and the use of an automated spectrophotometer for determining objective and quantitative CPE reduction endpoints.

Cell cultures, media, encephalomyocarditis (EMC) virus, and the microplaque reduction IFN assay have been described previously (6). The IFN preparations used included: (i) Newcastle disease virus (NDV)-induced human leukocyte IFN, (ii) phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, N.C.)-induced human leukocyte IFN, (iii) a human Namalva cell lymphoblastoid (LYM) IFN (batch LN1/77/3A; obtained from the Wellcome Research Laboratories, Kent, England, with a titer of 10^6 IU/ml when assayed in V3 cells according to an accompanying product insert), (iv) a National Institutes of Health (NIH) human leukocyte IFN standard (G023-901-527; with a predicted titer of 20×10^3 U when assayed in human foreskin fibroblast cultures), and (v) supernatant fluids from human leukocyte cultures challenged with herpes simplex virus (HSV) (5).

For the photometric CPE reduction assay, IFN dilutions were made with the automated diluter in 96-well microculture plates containing preformed WISH cell monolayers as described previously (6). Two rows of wells were used per sample. After 6 h of incubation at 37°C, IFN dilutions were removed, and monolayers were washed and challenged with 5,000 PFU of EMC virus in 0.1 ml of medium. To facilitate uniform development of CPE in all microculture wells, the lids were placed at a slight angle on the top of the plate. After an additional 16 to 22 h of incubation, virus-containing supernatant fluids were removed, and monolayers were fixed and stained for 15 min with dye-fixer solution (0.5% crystal violet, 5% Formalin [vol/vol], 50% ethanol [vol/vol], and 0.85% NaCl in distilled water) (1). The dye-fixer solution was then removed, and plates were washed thoroughly under running tap water and air dried. Photometric reading of the plates (53 s for each plate) was performed as described below, using an automated spectrophotometer for microtiter plates equipped with a 600-nm filter (Titertek Multiskan; Flow Laboratories, McLean, Va.). Equations used for calculation of 50% CPE reduction endpoints are as follows: optical density (OD) for 50% CPE = (OD of cell control + OD of virus control)/2; and IFN titer = [(OD above 50% CPE OD − 50% CPE OD) × difference between higher and lower dilutions]/(difference between higher OD and lower OD) + lower dilution. These equations are a modification of the method used to determine 50% plaque reduction endpoints.

The technical brochure accompanying the automated spectrophotometer indicated that optimal reading occurs when the dye is dissolved in 0.1 ml of solution. Accordingly, comparisons were made in which endpoints were determined from stained monolayers before and after extraction of the crystal violet into a solution of 50% ethanol in water. Surprisingly, endpoints obtained with alcohol-extracted dye were quite

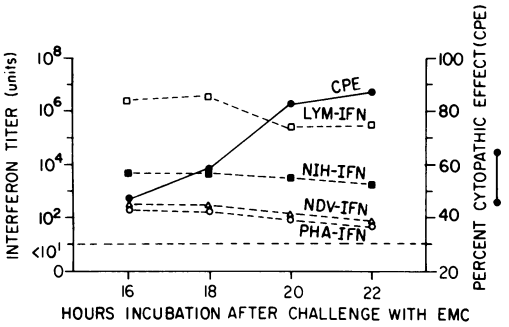

FIG. 1. Effect of incubation time (duration of viral replication) on IFN titer in the photometric CPE reduction assay.

variable and did not correlate well with the more reproducible endpoints obtained with unextracted cultures (data not shown). For subsequent experiments, we therefore used dry stained monolayers.

IFN-treated cells were exposed to EMC at an approximate multiplicity of 0.02, and the assay was terminated 16, 18, 20, and 22 hours later (Fig. 1). In virus control cultures, increased duration of viral replication was reflected in a time-dependent increase in the percentage of CPE (47 to 87%). With the exception of the unusually high-titered LYM-IFN, the length of incubation had no significant effect of the titer of IFN. Titers with NIH-IFN, NDV-IFN, and PHA-IFN were comparable irrespective of the duration of viral replication. Subsequently, assays were terminated at 16 to 18 h, at which time CPE in the virus control cultures approached 90 to 100% as determined by examination of the microculture plates with an inverted microscope. This usually corresponded to a photometric measurement of 60 to 80% CPE.

TABLE 1. Comparison of IFN titers with microplaque reduction versus photometric CPE reduction endpoints

Sample no.[a]	IFN titer (U)		Ratio[b]
	Microplaque reduction	Photometric CPE reduction	
1	650	360	0.55
2	930	1,040	1.12
3	54	48	0.89
4	17	13	0.76
5	24	18	0.75
6	25	29	1.16
7	200	160	0.80
8	7	12	1.71
9	730	390	0.53
10	2,400	2,200	0.92
11	88	70	0.80
12	64	42	0.66
13	27	15	0.56
14	14	19	1.36
15	8	21	2.63
Mean ± SD			1.01 ± 0.55

[a] IFN from leukocytes stimulated in culture with HSV, NDV, or PHA. HSV induces both nonspecific (IFN-α) and immunospecific (IFN-γ) reactions in leukocyte cultures, depending upon culture conditions and HSV serological status of the leukocyte donor (5).

[b] Quantitative CPE reduction titer/microplaque reduction titer.

The photometric CPE reduction method was compared with the microplaque reduction method which had been shown previously to be accurate and reproducible (6). Fifteen IFN samples of various potencies were examined by both methods in three separate experiments (Table 1). The 15 individual determinations had a mean ratio of 1.01, with a standard deviation of 0.55 and a range of 0.53 to 2.63, indicating a high degree of comparability between results obtained by the two methods. With 10 samples the titer was higher in the microplaque reduction assay; with 5 samples it was higher in the photometric CPE reduction assay.

Having established a basis for the quantitative photometric assay, four standard laboratory human IFN preparations were examined several times to assess the reproducibility of this system (Table 2). The mean percent CPE for 15 assays was 69, with a range of 52 to 83. With all four IFN preparations, the titer in an individual assay varied an average 47 to 63% from the mean titer for all 12 to 14 assays, with a range in variation from 0 to 175%. This variation is comparable to that observed previously with the microplaque reduction assay (6).

The current studies establish a bioassay for human IFN that is rapid, automated, quantitative, and reproducible. In contrast to previous dye-binding techniques (1, 2, 7), all steps in the current method, including sample dilution and endpoint determination, were performed in a single microculture plate. The assay procedure requires only 1.5 days to perform. Approximately 8 to 10 plates containing 40 to 50 specimens can be run in duplicate with each assay. The automated photometric machine employed in these studies reads and records the information contained in a single 96-well microtiter plate within 1 min or less. The machine can be interfaced with a microcomputer programmed to convert optical density readings directly to IFN titers. As shown in the current studies, reproducible IFN titers were obtained over a relatively wide range of virus challenge inocula and incubation periods. This provides more flexibility and less stringent technical requirements than the microplaque reduction method (6), which is affected by the size of the viral inoculum and by plaque size. The automated, photometric meth-

TABLE 2. Repetitive assay of four human IFN preparations by the photometric CPE reduction method

Expt no.	% CPE[a]	Titer (U)			
		NDV-IFN	PHA-IFN	LYM-IFN	NIH-IFN
1	52	550	720		
2	70	200	1,200		53,000
3	75	170		740,000	6,900
4	72	540	540		
5	66	76	510	940,000	35,000
6	75	110	73	2,000,000	16,000
7	80	130	80	550,000	5,400
8	54	180	690	3,700,000	41,000
9	43	230	650	6,600,000	28,000
10	81	110	230	2,000,000	11,000
11	71	210		3,600,000	18,000
12	83	150	210	2,900,000	16,000
13	75	84	90	680,000	13,000
14	63	200	300		
15	80			3,100,000	17,000
Mean	69	210	440	2,400,000	22,000
SD	±12	±150	±340	±1,800,000	±15,000

[a] Magnitude of monolayer destruction in untreated cell cultures infected with EMC, calculated from eight replicate cultures.

od therefore converts the CPE reduction assay from a qualitative procedure to one in which precise numerical endpoints can be obtained. Although the current studies report results with human IFN, the same assay system provides similar rapid and reproducible results with mouse and guinea pig IFNs (data not shown).

The methods described in the current study are for bioassay of IFN. The likely availability of high titer, high affinity, anti-IFN antibody can be anticipated to lead to the development of immunoassays for IFN. Immunoassays for IFN should complement, but not necessarily replace, bioassays. Immunoassays may overestimate the actual concentration of biologically active IFN by including nonfunctional, but antigenically recognizable molecules. On the other hand, immunoassays may underestimate biological activity by: (i) the lack of detection of IFN molecules which are antigenically different from those used to produce the anti-IFN antibody, and (ii) the failure to measure synergistic activity which can occur between several IFN species present in the same sample (3). Thus, there will be a continued need for IFN bioassays which are quantitative and reproducible. The incorporation of automated methods for dilution of samples and reading of CPE reduction results in microculture plates will enable processing of a larger number of samples and more rapid generation of results with less technical time and effort.

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