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A Sensitive Interferon Assay for Many Species of Cells: Encephalomyocarditis Virus Hemagglutinin Yield Reduction[1] (39768)

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The variety of assay methods employed in different laboratories to measure human interferon activity may account for the great variation in titers (over a 10- to 100-fold range) obtained for a single sample of interferon (unpublished results of collaborative titrations of reference standards). Several new or modified methods have been reported in recent years in an effort to fulfill the continuing need for a suitable assay which would be acceptable to many laboratories (1–5). Limitations of widely used methods (1, 2) involving vesicular stomatitis virus (VSV) led to the development of other assays, such as the hemagglutinin (HA) yield-reduction technique (5) using GDVII or Sindbis virus; however, GDVII virus has a very limited host range, and Sindbis virus produces low HA yields in many mammalian cells unless it is first adapted to grow in them. We have developed a practical, rapid, and sensitive HA yield-reduction method with encephalomyocarditis (EMC) virus that can be used with many different species of cells.

Materials and methods. Cell cultures. The continuous cell line L-132 derived from normal human embryo lung tissue (6) was obtained from Flow Laboratories. At weekly intervals cells were dispersed by scraping and subcultured at a 1:4 ratio in Eagle's basal medium (BME) supplemented with 10% fetal bovine serum (FBS). Dog kidney (DK) primary cell cultures were purchased from Flow Laboratories. The bovine cell strain, BSM, developed from skin and muscle cells, was supplied to us by Dr. George G. Jackson. Both BSM and DK cells were propagated in BME supplemented with 20 m*M* HEPES buffer at pH 7.0 and 10% FBS; cells were dispersed with trypsin-EDTA and subcultured at a ratio of 1:4 each week. A feline kidney cell line, used in the laboratory of Dr. M. Essex (Harvard University), was the clone 81 derived by Fischinger *et al.* (7) and designated CCC-81. The sources and culture conditions for other cells have been described: the BUD-8 human skin strain, human foreskin strain 7, Chang conjunctiva, FL amnion, African green monkey kidney (AGMK), RK-13 rabbit, and L cell lines (8); primary chicken embryo cells (9); primary fetal pig kidney and secondary rabbit kidney (as described for rabbit kidney cells by Gallagher [10]); and clone A_9B_1[12] of hamster-mouse hybrid cells (11).

Viruses. The EMC prototype of encephalomyocarditis virus (12) obtained from Dr. W. F. Scherer was originally maintained through several passages in mouse brain. Virus stocks for use in interferon assay were prepared in L cell cultures, and titrated by plaque assay in L-cell monolayers. Indiana strain of VSV, obtained from Dr. W. Henle, was propagated in chicken embryo cell cultures (CEC) for 13 passages, and prepared in L cell cultures for use in interferon assay. Recombinant influenza virus X7(F_1) was prepared and titrated as previously described (13).

Interferons. Crude human leukocyte interferon samples were obtained from Dr. Kari Cantell (CIF-IV) and from Smith, Kline, and French, Inc., lot 5356-136D (SKF 5356) and a lot designated SKF 100669. The reference reagent human interferons used were NIH G023-901-527 obtained from the National Institutes of Health, and MRC 69/19 and MRC 67/87 provided by the Medical Research Council, U.K.

Interferon bioassay. (a) Plaque-reduction assay was based on that described by Wagner (1); (b) yield-reduction assays were sim-

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ilar to those described by Oie *et al.* (5), using an appropriate assay for the particular challenge virus after freezing and thawing the cells to release cell-associated virus. Cultures were prepared in 16 × 125-mm tubes using 5×10^5 cells in 2 ml of growth medium appropriate for the cell type. After incubation for 24 hr in CO_2 at 36° the medium was decanted and replaced with 1 ml of interferon dilution or diluent medium, Eagle's minimum essential medium containing Earle's salts (EMEM) supplemented with 2% FBS. The optimum conditions for EMC virus infection of cells were selected from the findings of Hall and Rueckert (14). To obtain maximal virus yields, virus attachment was done at 36° for 30 min in air to maintain pH at 7.6 to 7.8 with a multiplicity of about 10 pfu/cell in a volume of 0.25 ml. Unattached virus was removed by washing three times with Earle's balanced salt solution. The initial pH of the maintenance medium was adjusted to 7.8 by addition of sodium bicarbonate to EMEM-FBS_2. After incubation in CO_2 for 18–24 hr, cultures were disrupted by freezing and thawing prior to determining HA yields.

(i) VSV infectivity yield. Infectious viral progeny was measured by plaque assay on either primary CEC or L cell monolayers.

(ii) EMC HA yield. The procedure, modified from Craighead and Shelokov (15), used human type O erythrocytes, washed and prepared as a 1% (v/v) suspension in 0.05 *M* boric acid–0.12 *M* potassium chloride buffer adjusted to pH 7.0 with sodium hydroxide and supplemented with 0.1% bovine serum albumin. Serial twofold dilutions of virus were made in the same buffer. An equal volume of the blood cell suspension was added to the virus suspension and the pattern was observed after incubation for 60 min at 20°.

Interferon titers for yield-reduction assays were derived graphically from the 0.5 log intercept (which corresponds to the 1.66 $\log_2$ line) on a plot of the difference between log of viral yields in control and treated cultures against the interferon dilution on semilog paper. Titers were expressed as the reciprocal of the dilution corresponding to the endpoint appropriate for the assay of viral product: median plaque reduction (PRD_{50}) (1), and 0.5 log reduction in yield (YRD_{70}) for either plaque or HA determinations (5). Geometric mean titers (GMT) are used because it was determined after the results were collected from numerous assays of a single human interferon sample that a normal distribution was obtained only with logarithmically transformed titers.

Results. Viral yield and interferon effect. EMC virus, grown in L cells, replicates well in most human cells tested. Yields of plaque-forming virus are directly proportional (slope 1.28) to the yields of HA (regression coefficient +0.93) with 10^6 pfu equivalent to 1 $\log_2$ HA unit. In cells producing 7 to 12 $\log_2$ EMC virus HA units the inhibitory effects of interferon can be measured over a 100- to 1000-fold change in viral yield within the linear portion of the dose–response curve (Fig. 1). One of the advantages of the method is the very long rectilinear portion of the curve which becomes sigmoidal only when maximum and minimum virus yields are obtained at very low doses and very high doses of interferon, respectively. Because of the length of this straight-line portion of the curve, the series of HA endpoints reinforce one another to give a reliable estimate of the interferon titer; further, dilutions tested need not fall close to the endpoint, but can be as much as

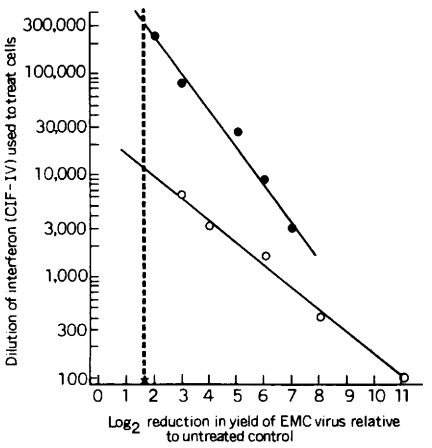

FIG. 1. Interferon dose-response curves of encephalomyocarditis (EMC) virus in a cell line and a cell strain with human interferon CIF-IV. Cell line, ○, L-132 lung; cell strain, ●, BUD-8 skin (NIH). Titers (YRD_{70}) are read from the 0.5 $\log_{10}$ intercept at 1.66 $\log_2$ (★).

10-fold lower and still satisfactorily indicate the titer. The slope of the dose-dependent response depends upon the cell used for the assay, as illustrated in this example with the BUD-8 cell strain and the L-132 cell line.

Sensitivity of the EMC HA yield-reduction assay for human interferon. A single sample of leukocyte interferon was repeatedly titrated over a period of several months by three different assay methods: EMC and VSV yield reduction, and VSV plaque reduction. The EMC HA yield-reduction assay using the L-132 cell line is at least as sensitive and precise as the two VSV methods in human cells. Geometric mean titers obtained by either the EMC or VSV yield-reduction methods or the VSV plaque-reduction test were all within a twofold range (3.21, 3.03, and 3.37 units/ml, respectively). The standard deviation for the EMC assay was 0.2 log for 15 determinations and 0.24 log for 6 titrations by the VSV plaque-reduction method. The results of two titrations by the VSV yield-reduction method could not be included in the calculations of mean and standard deviation because the titer was below 1:500, the lowest dilution tested. During this titration series, repeated measurement of the MRC 69/19 human interferon reference standard, which has an assigned value of 5000 units ($10^{3.7}$), gave a GMT of $10^{3.6\pm0.29}$ units by the EMC HA yield-reduction assay in the L-132 line.

The L-132 cell line was about five times as sensitive as the U amnion cell line in the EMC HA yield-reduction assay; the GMT of a leukocyte interferon (SKF 100669) was 396 in 3 tests with the U line and 1640 in 15 assays with the L-132 line. Repeated independent titrations of another leukocyte interferon (CIF-IV) by the EMC HA method compared the sensitivities of the L-132 line (n = 33) with the BUD-8 strain (n = 27). The GMT in the L-132 line was $10^{3.82\pm0.40}$ units and in the cell strain was $10^{5.43\pm0.17}$ units. Thus, the BUD-8 strain was 40 times as sensitive as the L-132 cell line, and was considerably more precise, with a standard deviation range of 1.5-fold (compared with a 2.5-fold range for the cell line).

A simultaneous analysis of three freeze-dried interferon reference standards and our internal laboratory standard using BUD-8 cells established further the high interferon sensitivity of the EMC HA yield-reduction assay in comparison with the VSV plaque-reduction method (Table I). The geometric mean titers obtained by the EMC HA yield-reduction method were four to seven times the assigned values for the standards and in every case were higher than the activities measured by the VSV plaque-reduction technique.

Sensitivity of diverse animal cells to EMC virus and interferon. The wide host range of EMC virus is demonstrated by the high yields of HA obtained after infection of cell lines and strains of many different cultured human cells as well as cells from eight other species (Table II). Each of the animal cells responded to its homologous interferon as well as to a potent human leukocyte interferon. In L cells the EMC HA yield-reduction method was about as sensitive as the tests using VSV or Semliki Forest virus but less sensitive than the GDVII HA yield-reduction assay (5), depending upon the L cell subline tested.

Discussion. The EMC HA yield-reduction method offers a sensitive, suitably precise, objective means of interferon bioassay which is quite simple, convenient, and readily adaptable to handling large numbers of

TABLE I. COMPARISON OF POTENCY OF THREE FREEZE-DRIED INTERFERON STANDARDS AND AN INTERNAL LABORATORY REFERENCE MEASURED BY THREE DIFFERENT ASSAY METHODS USING THE BUD-8 CELL STRAIN.

	Mean $\log_{10}$ titer and number (N) of determinations for the indicated assay method			
	EMC HA yield reduction		VSV plaque reduction	
Human interferon sample[a]	YRD_{70}[b] Units	N	PRD_{50}[b] Units	N
NIH G023-901-527	5.07	8	4.80	6
MRC 69/19	4.34	4	4.12	3
MRC 67/87	2.82	4	2.68	3
CIF-IV	5.27	4	4.91	3

[a] Assigned values of the interferon reference standards are 20,000 units for NIH G023-901-527; 5000 for MRC 69/19; and 100 for MRC 67/87.

[b] Units of interferon are given either as the reciprocal of the dilution producing 0.5 $\log_{10}$ reduction in yield of EMC hemagglutinin (YRD_{70}) or the reciprocal of the dilution providing a median plaque-reduction (PRD_{50}) endpoint.

TABLE II. USE OF THE EMC VIRUS HEMAGGLUTININ YIELD-REDUCTION ASSAY IN CELLS OF DIVERSE SPECIES TO DETERMINE THEIR SENSITIVITIES TO HUMAN LEUKOCYTE INTERFERON (CIF-IV).

Species	Cell culture[a]	Usual HA yield (log_2)	Titer of CIF-IV[b]
Human	BUD-8 strain	8	269,000
	Foreskin-7 strain	7	203,000
	L-132	10	6,600
	FL	10	34,500
	Chang conjunctiva	12	17,500
Monkey	AGMK	7	12,000
Cow	BSM strain	6	360,000
Pig	Fetal kidney primary	11	90,000
Rabbit	RK-13	7	2,050
Cat	CCC-81	5	2,100
Hamster	Hamster–mouse hybrid, A_9B_1[12]	9	2,100[c]
Dog	Kidney primary	5	700
Mouse	L_{929}	11	340

[a] Cell cultures are continuous lines unless otherwise indicated.

[b] Units of interferon are given as the reciprocal of the dilution resulting in 0.5 log_{10} reduction in yield of EMC hemagglutinin. Arithmetic mean titers are shown for all except the human cells, in which case geometric means are given.

[c] Grossberg *et al.*, 1975 (11).

samples (>50) at a time. No expensive or unusual instruments or reagents are required. Various levels of automation can facilitate HA titrations. Both the interferon bioassay and the measurement of HA can be done with microtiter equipment, accommodating as many as 24 interferon samples per tray. Microtiter modifications do not diminish the interferon sensitivity of the EMC method.

The sensitivity of any interferon bioassay is a function of the sensitivities of both virus and cell type (3). Titers obtained with the EMC HA yield-reduction method in BUD-8 cells were within 0.25 log of those measured simultaneously by the X7 (F_1) recombinant influenza virus neuraminidase yield-reduction assay (7), the most sensitive of any bioassay we have tested (17). For any given cell we have tested, the EMC assay compares well with the VSV yield- or plaque-reduction methods, as indicated by direct assay comparisons and by titrations of reference standard interferons. Campbell and Colter (16) found that Mengo virus in L cells was similar to VSV in sensitivity to interferon; and Oie *et al.* (5) showed that GDVII virus was more sensitive than VSV. Since precision as well as sensitivity of interferon assays unaccountably seems to depend upon the cell used for the test (3), more sensitive and more consistently responsive cell lines will improve the assay.

Hemagglutination of EMC virus is more easily measured than that of GDVII virus or the arboviruses: A single buffer solution is used for both the virus dilution and erythrocyte suspension, and the endpoint is easily determined from the very clear patterns which form within 1 to 2 hr at room temperature. The slight variation that may result from differences in visual appreciation of individual HA patterns is diminished by plotting the responses observed with several interferon dilutions, as in most bioassays, to obtain a graph for the determination of interferon titer.

The EMC yield-reduction assay may offer a practical alternative to the plaque- and yield-reduction methods using VSV which others have described as laborious, tedious, and time-consuming (3); further, VSV is prohibited in some countries (3). The time and effort required for processing samples and counting VSV plaques restricts the number of samples that can be tested at one time; and both the number and size of plaques in a plaque-reduction assay tend to increase as time elapses after the removal of interferon, thereby altering the apparent titer of the sample as a function of time and the ability to detect very small plaques. The HA yield-reduction method further may be preferable to the cpe-inhibition assays because (i) viral growth is evaluated quantitatively by an objective titration method

rather than semiquantitatively by a subjective visual estimation, and (ii) HA endpoints can be easily determined without the need for a highly experienced observer such as may be needed to interpret the extent of cpe.

Relative interferon activity can be easily estimated by the HA yield-reduction method for very large numbers of samples, e.g., eluates from column chromatography. For example, a single dilution of all samples can be tested by appropriately calculating from the known activity of the original sample; relative activity in the eluate can be expressed as a proportion of the yield in the untreated virus control ($\log_2$ differences) or given in interferon units from a standard dose–response curve determined simultaneously on the original sample. Because the slope of the linear dose–response curve essentially does not vary for a given system, titrations can be done using wide dilution intervals in which only a few points fall within the linear portion of the graph, and endpoints are rarely missed if the lowest dilution is conservatively selected. The HA yield-reduction assay may also be preferable to the dye-uptake methods of quantitating cpe effects, because the latter require the use of closely spaced dilution intervals very near the endpoint of interferon activity. Radiochemical assay methods are rapid and expensive but no more precise or convenient. The neuraminidase yield-reduction assay is sensitive and very precise (7). Evaluations of the variety of other interferon bioassay methods available have been reviewed elsewhere (3, 17).

Because of its wide host range EMC virus can be used to measure interferon in many species of cells. An occasional human or animal cell strain or line did not support EMC virus growth well, for unknown reasons. At least one cell type of each of the nine species tested produced adequate yields of EMC virus and responded to the homologous species interferon prepared *in vivo* or *in vitro*. Because reference standards are not available for most animal species, responses to a high-titered human leukocyte interferon were measured to provide some point of comparison. Heterologous species activity of human leukocyte interferon was observed with bovine, porcine, rabbit, hamster, mouse, and simian cells as reported previously (4, 11, 18), and, in addition, with dog and cat cells.

Summary. A new, highly sensitive assay for human interferon was developed in which the reduction in HA yield of a single-cycle infection with EMC virus is measured. The sensitivity and precision of the assay are comparable to or better than the other assays tested, depending most of all upon the cells used in the test. This simple method requires no sophisticated instrumentation, can be used on a microtiter scale, provides objective numerical results, and is readily applicable to testing large numbers of samples. The assay can be used in human lines and diploid cell strains as well as in monkey, rabbit, cat, mouse, pig, cow, dog, and hamster–mouse hybrid cells.

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