Improved Assays for a Variety of Interferons (36636)

HERBERT K. OIE, CHARLES E. BUCKLER, CAROL P. UHLENDORF, DAVID A. HILL, AND SAMUEL BARON

National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Interferon assays are based on the inhibition of either viral function or synthesis of virus or its components. No one assay has proven to be ideal but significant improvements are constantly being introduced (1). A study was initiated to develop a widely applicable assay which would combine rapidity, simplicity and reliability and which is based on the inhibition of virus multiplication during a single cycle of growth.

Materials and Methods. Cell cultures. Mouse L cells (obtained from Dr. J. S. Youngner, University of Pittsburgh), primary and secondary mouse embryo cells (NIH Swiss), primary chicken embryo cells and 2 diploid human cell lines (MA-308 and RCTC-2) were cultured to confluency by standard procedures. For assay, cells were cultured in 16 × 150 mm tubes, held vertically in a spring rack (2) and covered loosely with a single metal lid (3).

Viruses. GD-7 virus, a mouse picornavirus, was produced and plaque-titrated [$10^{6.9}$ plaque-forming units (pfu)/ml] in BHK-21 cells as previously described (4). Sindbis virus was prepared and plaqued ($10^{8.2}$ pfu/ml) in chicken embryo monolayer cultures. Vesicular stomatitis virus (VSV), Indiana strain, was also prepared in chicken embryo cells but the plaque titration was done in mouse L cells ($10^{7.5}$ pfu/ml).

Interferons. Mouse serum interferon (MSI) (5) titered $10^{4.2}$ units/ml in mouse L cells. Human interferon was prepared from human diploid fibroblast cell cultures infected with Chikungunya virus [input multiplicity of infection (MOI) = 1] and it titered $10^{3.0}$ units/ml in MA-308 cells. Chicken interferon induced in ovo with NWS influenza virus (6) titered $10^{2.3}$ units/ml in chicken embryo cells. These titers were obtained using the methods described below.

Hemagglutination assay. Titration of GD-7 virus hemagglutinin (HA) was done by standard methods as previously reported (4). 0.06% fetal bovine serum (FBS) was added both to erythrocyte suspensions and to the virus diluent because it was found to be essential for the proper development of the hemagglutination patterns. Titration of Sindbis virus HA was done as previously reported (7) using the determined optimum pH of 6.0 and a 0.125% concentration of gander erythrocytes in the final reaction mixture.

Results. Time course of HA production. To determine the times of maximal HA production in the virus–cell systems under study, single step growth curves were determined (Fig. 1). It may be seen that maximum HA production occurred at 12 hr for Sindbis virus in chicken and mouse embryo cells, at 16 hr for Sindbis virus in human MA-308 cells and 18 hr for GD-7 virus in mouse L cells. The time course of viral production in interferon treated cells is generally not different or only slightly delayed (8). Therefore, during interferon assays fluids were harvested for HA determinations 18–24 hr after infection.

Interferon assay by inhibition of yield of viral HA. Duplicate or triplicate tube cultures of cells were each exposed to 1 ml of interferon diluted in Eagle's minimal essential medium (EMEM) plus 2% FBS. After 18–24 hr of incubation at 37°, the cultures were washed twice with 2 ml amounts of Earle's balanced salt solution (EBSS) and challenged with an input virus multiplicity of 50 or greater in 0.2 ml/tube. After 1 hr at 37° the cultures were washed three times with EBSS to remove unadsorbed virus and

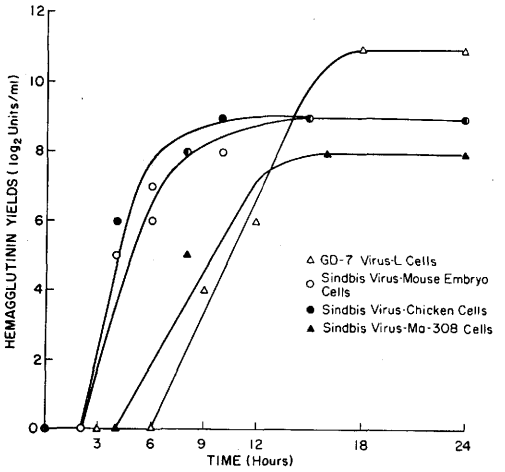

FIG. 1. Time course of production of GD-7 virus and Sindbis virus hemagglutinins.

fed with either 1 ml of EMEM plus 2% FBS in the case of GD-7 virus or with 0.5 ml of reinforced Eagle's medium (9) without serum in the case of Sindbis virus. In our experience the presence of serum during replication of Sindbis virus frequently decreases the yield of HA. After an overnight incubation at 37° the HA yields were determined immediately or after storage at —20°. The HA assay was done after replicate cultures were pooled. The titer of an interferon preparation was calculated as the reciprocal of the dilution which resulted in a $10^{0.5}$ reduction in HA yield. Figure 2 shows representative dose–response curves from which interferon titers are derived from the $10^{0.5}$ yield inhibition intercept.

Comparative sensitivities of different interferon assays. Four assay techniques—infectivity yield reduction of (a) VSV and (b) Sindbis virus, and HA yield reduction of (c) GD-7 virus and (d) Sindbis virus—were studied in mouse L cells, secondary mouse embryo cells, chicken embryo cells and human MA-308 cells. All assays were done in tube cultures under as nearly similar conditions as possible.

The results, summarized in Table I, show that in all cells tested, the VSV infectivity yield reduction assay was never more sensitive and usually less sensitive than the other three methods. Interferon assays using GD-7 virus in L cells consistently gave higher titers than the other three techniques employed (see representative experiments in Table I and Fig. 2). Therefore, the GD-7 virus–L cell system appears to be the assay of choice for mouse interferon. In all four cell systems, the Sindbis virus HA yield reduction assays gave similar or higher interferon titers than either the Sindbis virus or VSV infectivity yield reduction assays. Therefore, the Sindbis virus HA yield reduction procedure was suitable for assay of chicken and human interferons.

Production of Sindbis virus HA by various animal cells. GD-7 virus is suitable as a challenge virus in only a few cell types due to its limited host range (4). Sindbis virus has a broader host range as determined by HA production by cells from several different animal species (Table II). Therefore Sindbis virus is potentially usable as a challenge virus for the assay of a variety of interferons.

Reproducibility of the mouse assay and the human assay. In 32 different experiments over a period of 6 months, a laboratory reference MSI titered by the GD-7 virus HA yield reduction assay had a mean titer of $10^{4.2}$ units/ml with a standard deviation of $10^{0.3}$ units/ml. These findings were valid for a variety of mouse interferons. Similarly in 10 different titrations by the Sindbis virus HA yield reduction method, using MA-308 cells under passage 29, a reference human interferon gave a mean titer of $10^{3.6}$ units/ml and a stan-

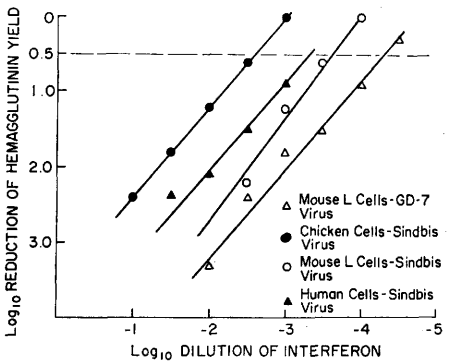

FIG. 2. Relationship of dose of mouse, chicken or human interferon to the yield of viral hemagglutinins. The interferon titer is calculated from the 0.5 $\log_{10}$ yield inhibition intercept.

TABLE I. Comparison of Different Interferon Assays.[a]

Assay method	Challenge virus	Interferon titer (log_{10} units/ml) Mouse L cells	Mouse embryo cells	Chicken embryo cells	Human MA-308 cells
Infectivity yield reduction	Sindbis	3.6	3.2	2.0	3.0
	VSV	3.2	3.0	1.9	1.8
HA yield reduction	Sindbis	3.7	3.9	2.5	3.0
	GD-7	4.1	—[b]	—	—

[a] The interferon titers are averages of titers obtained in 2 or more assays and were read directly from plots of the dose–response curves.

[b] GD-7 virus does not yield HA in mouse embryo, chicken embryo and human MA-308 cells.

dard deviation of $10^{0.3}$ units/ml.

The international mouse serum reference interferon in the GD-7 virus–mouse L cell assay titered $10^{4.5}$ units/ml. The international human reference interferon (69/19) in the Sindbis virus–MA-308 cell assay titered $10^{4.0}$ units/ml.

Discussion. The present findings demonstrate that a viral HA yield reduction assay, which is a highly modified version of Isaacs and Lindenmann's original assay (10), incorporates several desirable features. Since these assays are based on the inhibition of virus replication during a single cycle of growth, the state of resistance is measured in a short period of time following virus challenge. They are therefore highly suitable for kinetic studies. Additionally, the techniques which comprise this assay are well standardized and simplified. For example, the use of a rack with a single lid (2, 3) permits handling of multiple tube cultures as one unit. Use of tubes greatly reduces the amounts and volumes of cells, media and virus required. However, the volume of each rinse was increased to 2 ml to adequately remove unadsorbed virus from the large glass surface. Assay of each sample of interferon requires the use of as few as 6 tube cultures—i.e., 3 dilutions of interferon, using 2 tubes/dilution. The hemagglutination assay is inherently fast and simple (11) and can be easily automated (12). Finally, this interferon assay is highly sensitive as evidenced by the high titers of the reference interferons.

A number of factors determine the length of time required for the completion of an interferon assay. By selecting a virus with a short growth cycle and using a high MOI, maximum virus yields can be obtained as early as 12–16 hr after challenge. Determination of virus yields by the HA assay further decreases the time required for the interferon assay compared with assays which measure inhibition of infectivity yield (1). The time needed for different cells to respond to interferon is a characteristic of each cell type and is independent of the method used for determining resistance. In most assays the virus challenge is applied after the cells have been incubated with interferon long enough to develop maximum resistance. This provides for high sensitivity and reproducibility. The fixed time to develop resistance is 4 to 8 hr for mouse and chicken cells (13) and approximately 18 hr for human cells. Therefore, a 24 hr HA yield reduction assay is possible for mouse and chicken interferon, while a 48 hr interval is required for the human interferon assay.

A reliable assay is reproducible and has few failures. The HA yield reduction method has been used in this laboratory for more than 2 years to assay a variety of interferons including rat, hamster, bovine, and monkey interferons. In the various HA yield reduction assays established in our laboratory, failures have been rare and the reproducibility has been as satisfactory as that described for the mouse and human systems.

The greater sensitivity of the GD-7 virus–L cell system makes it the assay of choice for the titration of mouse interferon. However,

TABLE II. Production of Sindbis Virus Hemagglutinin in Cells from Different Species of Animals.

	HA yield (log_{10} units/ml)
Primary cells	
Rabbit kidney	<0.6
Monkey	
Owl monkey kidney	3.0
Marmoset kidney	2.5
Green monkey kidney	1.5
African green (SV5)	2.7
Mouse embryo	3.6
Cat lung	<0.6
Chicken	
Embryo	3.6
Embryonic liver	2.4
Bovine kidney	2.1
Heteroploid cells	
Mouse L	3.3
Monkey	
Vero (African green)	3.6
BSC-1 (African green)	2.7
Human (Flow Laboratories)	
A C amnion	3.9
KB	2.4
Detroit-98	<0.6
Intestine 407	3.9
Chang liver	1.8
L-132	3.3
McCoy	3.9
Diploid cells	
Human	
RCTC-2	3.6
WI-38	2.7
MA series (13 cell lines from Microbiological Ass.)	2.0–3.3
14 cell lines (B. W. Uhlendorf, NIH)	1.5–2.7
Rat	
F series (A. Freeman, Microbiological Ass.)	
19 lines	1.2–3.3
1 line (F-263)	<0.6
Hamster (A. Freeman, Microbiological Ass.) F-839	2.4

the use of GD-7 virus as a challenge virus is unfortunately limited to certain strains of L cells (4). For other types of mouse cells, the Sindbis virus provides a convenient and reasonably sensitive challenge virus. Sindbis virus produces satisfactory yields of HA in cells derived from a variety of other animals species, *e.g.*, human, chicken, rat, monkey, hamster, and bovine cells. The wide host range, the high sensitivity to the action of interferon, and the simplicity of the hemagglutination assay make Sindbis virus a particularly useful challenge agent, especially for use in comparative studies of interferons from different animal species. For small laboratories, in particular, this assay would be advantageous because it is simple, rapid, convenient and economical.

Summary. Improved assays for a variety of interferons, based on the inhibition of yield of viral hemagglutinin, have been developed. These assays are advantageous because they combine the characteristics of rapidity, simplicity, reliability, sensitivity and inhibition of viral multiplication during a single cycle of growth. Since Sindbis virus produces hemagglutinin in a wide variety of animal cells, it is applicable for comparative interferon studies.

1. Finter, N. B., *in* "Interferons" (N. B. Finter, ed.), p. 87. North-Holland, Amsterdam (1966).

2. Richter, A., and Hale, S., Appl. Microbiol. **13**, 503 (1965).

3. Baron, S., Buckler, C. E., and Takemoto, K. K., Appl. Microbiol. **14**, 1042 (1966).

4. Sturman, L. S., and Tamm, I., J. Immunol. **97**, 885 (1966).

5. Baron, S., and Buckler, C. E., Science **141**, 1061 (1963).

6. Baron, S., *in* "Fundamental Techniques in Virology" (K. Habel and N. P. Salzman, eds.), p. 402. Academic Press, New York (1969).

7. Clarke, D. H., and Casals, J., Amer. J. Trop. Med. Hyg. **7**, 561 (1958).

8. Takemoto, K. K., and Baron, S., Proc. Soc. Exp. Biol. Med. **121**, 670 (1966).

9. Bablanian, R., Eggers, H. J., and Tamm, I., Virology **26**, 100 (1965).

10. Isaacs, A., and Lindenmann, J., Proc. Roy. Soc., Ser. B. **147**, 258 (1957).

11. Hammon, W. McD., and Sather, G. E., *in* "Diagnostic Procedures for Viral and Rickettsial Infections" (E. H. Lennette, and N. J. Schmidt, eds.) p. 259. Amer. Pub. Health. Ass., New York (1969).

12. Baron, S., Burch, B. L., and Uhlendorf, B. W., Amer. J. Clin. Pathol. **36**, 555 (1961).

13. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exp. Biol. Med. **125**, 1320 (1967).

Received Apr. 4, 1972. P.S.E.B.M., 1972, Vol. 140.