A More Precise and Sensitive Hemagglutination Reduction Assay for Interferon[1] (37468)

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The advantages of hemagglutination (HA) yield reduction assays for interferon (IF) are well recognized, and Sindbis virus has proven to be an excellent challenge virus for use in a number of IF assay systems because of its broad host range and high sensitivity (1). We would like to report a modification of this test that enhances its precision and sensitivity for detecting IF and makes it potentially useful in cell systems where little Sindbis virus is produced.

Materials and Methods. Cell cultures. IF assays were performed in 48 hr old primary chick embryo cell (CEC) monolayers or 6 day old monolayers of human amnion U cells (obtained from Dr. R. M. Friedman, National Institute of Health). The CEC cultures were prepared from trypsinized 10 day old embryos according to standard technique (2) and plated on 60 mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, CA). The U cells were also prepared according to standard technique (3) in T-30 cell culture flasks (Falcon Plastics). These cells were grown in medium 199 with 5% bovine fetal calf serum (FCS), and once they became confluent, they were held for at least 3 days on medium 199 with 2.5% FCS before being used in IF assays.

Viruses. A small plaque variant of Sindbis virus, cloned from strain Ar-339 (ATCC), was the challenge virus in the CEC system. Fourth passage stock suspensions of this virus titered $10^{8.5}$ plaque-forming units (PFU)/ml in CEC cultures. A human cell adapted strain of Sindbis virus (1), obtained from Dr. S. Baron, NIH, Bethesda, MD, was used as challenge virus in the U cell system. Upon arrival, this virus titered $10^{8.1}$ PFU/ml in CEC cultures and had a HA titer of 3200/0.5 ml in a routine HA test. Four serial passages of this virus in U cells were required before sufficient HA particles could be detected for IF assays, and then only after treatment with Tween 80–ether as described below.

Interferons. Human reference interferon standard 69/19 (5000 units/vial) and chick M.R.C. standard A (100 units/vial) were used in these studies. Both were diluted 1:10 in medium 199 and 10% FCS for storage at −70°, and were diluted for IF assays in medium 199 plus 2.5% FCS.

Hemagglutination assay. HA determinations were done according to the technique described by Clarke and Casals (4). A serial fivefold dilution scheme was used to dilute the hemagglutinin starting at a 1:5 dilution; the end point was read as the last tube showing HA.

Rapid extraction of hemagglutinin. To obtain the desired yields of HA particles for IF detection and assay, all samples from infected cultures were first treated with Tween 80 and anhydrous ether as described by Mussgay and Rott (5) and modified as follows: 0.8 ml of culture fluids from infected cells were added to 1.8 ml blanks of Tween 80 (5 mg Tween 80/100 ml of borate saline (pH 9.0) and 0.4% bovine serum albumin) in screw top tubes, and mixed on a Vortex Junior mixer (Scientific Laboratories, Queens Village, NY) for 5 sec. Subsequently, 2.0 ml of anhydrous ether were added to each tube and the suspensions were mixed again for 5

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sec. All tubes were then centrifuged at approximately 200*g* for 1 to 2 min to separate the ether and aqueous phases. Following centrifugation 0.4 ml of the bottom aqueous phase was quickly transferred to the first dilution tube containing 0.4 ml of borate saline (pH 9.0) and 0.2% bovine serum albumin. After this transfer, a second transfer (0.1 ml of each sample) was made before beginning overall dilutions of samples. The presence of ether and Tween 80 in the system as described did not increase or decrease the HA titer of controls placed at the first or last of several routine studies involving as many as 36 samples.

HA dilutions. The dilution of virus in the Tween 80 and ether suspension was considered 1:2.5 (0.8:2.0) although as much as 20% of the HA activity could be detected in a second Tween 80 extraction of the ether phase. The first dilution in the test after the Tween 80 and ether extraction, therefore, was considered as 1:5 and all subsequent dilutions were 1:5.

Interferon assays. Interferon assays were performed as described by Oie *et al.* (1) except that the concentration of bovine calf serum or FCS used to make the IF dilutions was 2.5%. The IF titer was determined from the dilution of IF that reduced the HA activity by $5^{0.5}$ units. No adjustments of titers were made for the volume of IF added to cultures (2.0 ml).

Results. The effect of serum on HA virus yield from U cells. It has been reported that the presence of serum during replication of Sindbis virus in human cells frequently decreased the HA yield (1). We also found this to be true. However, when virus yields were treated with Tween 80–ether, the amount of detectable HA in cultures was higher and more uniform whether serum was present or not, or even when different serum sources were used (Table I). These data suggest that U cells are rather poor producers of Sindbis virus and that treatment with Tween 80 and ether not only enhances the hemagglutinating titer but also may function to eliminate certain nonspecific inhibitors of hemagglutination present in the system.

TABLE I. The Effect of Tween 80–Ether Treatment on Sindbis Virus Hemagglutinin Produced in Human Amnion U Cells Cultured in the Presence or Absence of 2.5% Calf Serum.

Serum	Hemagglutinating titer/ml; treatment:	
	Tween 80 and ether	None
Fetal bovine	7800	300
Bovine	7800	<5
Bovine calf[a]	7800	<5
None	7800	300

[a] This serum was prepared from local calves; all other sera were purchased.

The effect of Tween 80–ether extraction of Sindbis virus yields on assays of human IF in U cells. Plaque reduction assays of IF performed in this laboratory with vesicular stomatitis virus (VSV) suggested that human amnion U cells were relatively insensitive to IF (only 450 out of 5000 units of international standard 69/19 could be detected). Attempts to utilize the more sensitive and economical HA reduction assay with these cells also were unsatisfactory because insufficient HA virus was produced, even when human adapted strains of Sindbis virus were used to infect the cultures. Extensive Tween 80–ether extraction had been shown to amplify HA yields by breaking virus particles into smaller HA components (5). Since this technique was time-consuming and relatively inconvenient for routine use in IF assays, rapid Tween 80–ether extractions were performed on sample culture fluids harvested from IF-treated cells to determine whether this technique could amplify HA yields and make HA reduction assays of IF possible in this system. The results of a sample assay are shown in Fig. 1. Each point represents the HA titer of a sample harvested from two pooled cultures (extracted and unextracted samples were taken from the same pool). A good dose–response relationship resulted when the HA titers obtained from the extracted samples were plotted against the IF dilution, whereas no HA virus was detected in any of the unextracted culture samples at a virus dilution of 1:5.

Enhanced detection of CEC IF by HA assay of Tween 80–ether extracts of Sindbis vi-

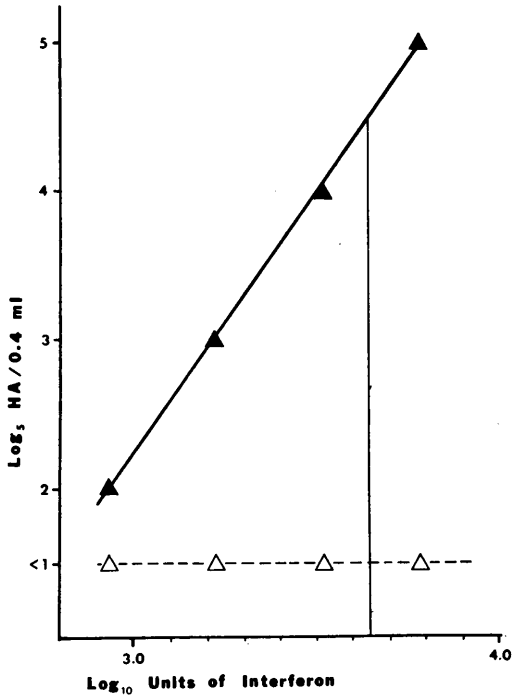

FIG. 1. Units of human interferon standard 69/19 detectable in human amnion U cells by HA assay of virus yields treated with Tween 80–ether (▲) and nontreated (△). The interferon units were calculated from the reciprocal of the dilution of interferon that decreased the HA yield by $5^{0.5}$ units.

rus. If Tween 80–ether extraction of a virus amplifies the HA yield, there should be points in the dose–response curve of an IF assay where the slope of the line changes. This change should be most obvious where the IF concentrations in the cultures are minimal and virus production is approaching maximum. Preliminary studies suggested that to observe this change, culture systems yielding more than 75,000 units/0.4 ml following Tween 80–ether extraction had to be used. The results of a sample assay in CEC cultures are shown in Fig. 2. It may be observed that an obvious change in slope occurred when the HA titer of samples harvested from pooled cultures and extracted with Tween 80–ether were plotted against the IF dilution, whereas the data obtained from samples harvested from the same pooled culture fluids that were not extracted produced a straight line. A calculation of the $5^{0.5}$ HA reduction titers from the data plotted from both methods shows that approximately a 25% increase in the IF titer was obtained using the extraction method (910 units versus 690 units). In addition, the IF titer obtained employing the extraction method was approximately 10 times the titer given for the chick M.R.C. standard A used in the test. The increased precision of the test is illustrated by the obvious increases in the slope of the dose–response curve as the IF concentration approaches the control (or zero).

Discussion. The advantages of a rapid, simple, and reliable viral HA yield reduction assay for IF have been discussed by Oie *et al*. (1). Our findings suggest that a rapid Tween 80–ether extraction of Sindbis virus yields obtained during an HA reduction assay for IF can (a) broaden the use of this assay and (b) enhance its sensitivity and precision, by greatly amplifying the detect-

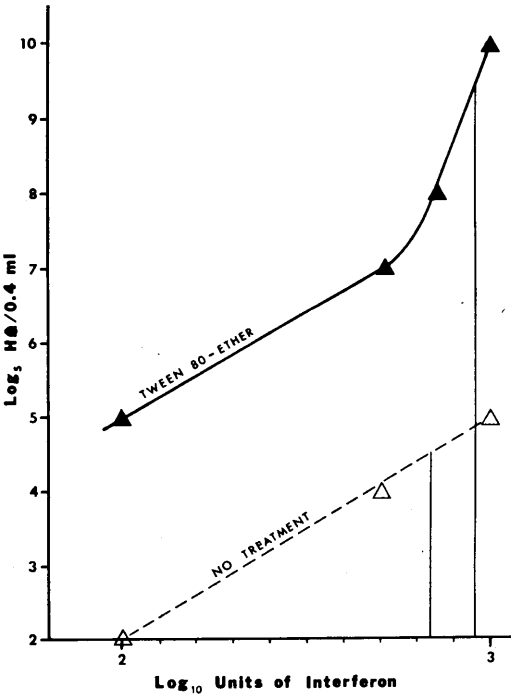

FIG. 2. Units of CEC interferon M.R.C. standard A detectable by HA assay of virus yields treated with Tween 80–ether (▲) and nontreated (△). The interferon units were calculated from the reciprocal of the dilution of interferon that decreased the HA yield by $5^{0.5}$ units.

able HA particles produced. For example, employing Tween 80–ether, we could detect and assay IF in a rapidly growing nonfastidious stable cell line of human amnion U cells (Fig. 1). These cells did not produce sufficient Sindbis virus for performing normal HA reduction IF assays, even when "adapted" human cell strains of Sindbis were used as the challenge virus. The sensitivity of the Tween 80–ether extraction assay was approximately the same as the assay used to approximate the units of IF in international standard 69/19. A plaque reduction IF assay employing VSV as the challenge virus with these cells was about one tenth as sensitive as the HA reduction assay and much more difficult to perform.

The sensitivity and precision of any assay are related to the relative positions and slopes of the dose–response curves. Therefore, for reliable HA reduction assays, at least three points should be plotted on a dose–response curve to verify its slope. Furthermore, the increase in HA titers between any two points on the line must either be significant in itself or more replicates must be obtained to make small increases significant. Therefore, in these studies, since a 25-fold increase in HA titer occurred over a narrowing range of IF values as the IF concentration approached zero (as illustrated by the position and increased slope of the solid line in Fig. 2), the Tween 80–ether extraction technique can be said to be more sensitive than the standard technique. The increased slope of the line also indicates an increase in precision, since perpendicular lines dropped from a wider range of HA values will span a narrower range of IF concentrations. Further, while working with this system it became apparent that the slope of the dose–response line continued to steepen as the concentration of IF in the system decreased. Consequently there was essentially no difference between IF titers plotted from curves drawn from several points located at the end of the titrations. Thus the line can be assumed to be straight from the last dilution to the control without making any material difference in the IF titer. This technique, therefore, appears to offer a more precise means of assaying small amounts of IF.

In the actual performance of the test we found that the additional time taken to extract the virus yields (10–15 min for 36 samples) was well justified because of the heightened sensitivity and precision of the test. In addition, since the presence of serum did not significantly affect the HA yields of this test (Table I), longer growth periods of virus could be used to obtain maximal viral yields with less danger of virus inactivation.

It was interesting that the HA yields after rapid (10 sec) Tween 80–ether extraction of Sindbis virus exceeded by approximately 13-fold those of Mussgay and Rott (5), who used extraction times of 15 to 30 min. The reason for this increase is not known. One explanation could be that relatively large amounts of unstable particles may be reacting in our test, which may have been inactivated during their more extensive extraction procedure.

In conclusion, these studies suggest that easily performed and more sensitive and precise assays for human or other interferons may be possible by either employing or developing IF-sensitive cell lines capable of producing larger numbers of virus particles that can be broken into numerous HA subunits, or by adapting IF-sensitive viruses that can be broken into subunits to grow to higher titers in presently used cell systems.

Summary. HA reduction assays for IF were performed using rapid (10 sec) Tween 80–ether extractions of Sindbis virus yields to greatly amplify measurable HA activity. Employing this technique a sensitive HA reduction assay for IF was developed for use in a cell system (human amnion U cells) that produced insufficient viral HA particles for measuring IF concentrations by normal HA reduction assays. When this extraction technique was employed to amplify HA yields in a system where large quantities of virus were produced (chick embryo cells), significant increases in sensitivity and precision of IF assays were observed. It appears possible that this technique may have wide application in IF assays employing other cell systems and viruses.

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