Dye Uptake Assay: an Efficient and Sensitive Method for Human Interferon Titration

ANNE L. R. PIDOT
Department of Medicine, Dartmouth-Hitchcock Medical Center, Hanover, New Hampshire 03755

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Currently, human interferon (IF) assays are generally performed by plaque reduction or visual cytopathic effect methods. Both are time-consuming, subjective in interpretation, and, in the case of the latter, relatively insensitive. An adaption of the dye uptake method for human IF titration which uses foreskin-derived fibroblasts and vesicular stomatitis virus is described. This assay is reproducible and sensitive (1 unit = 3 international units). By direct comparison, however, it is somewhat less sensitive than the plaque reduction assay (1 unit = 1.1 international units). This assay is especially recommended for use in needed clinical investigations of human IF because of its technical simplicity, allowing efficient handling of large numbers of specimens.

Interferon (IF) must still be assayed by biological means despite considerable advances in its biochemical characterization. These assays are relatively crude, subject to considerable uncontrollable variation, and are extremely time-consuming, tedious, and frequently subjective in interpretation. These difficulties have been, in part, responsible for the limited number of observations on the role of human IF in clinical situations.

I have been interested in developing a rapid, reproducible IF assay method which will allow the screening of large numbers of samples of human IF with maximum technical ease. In this communication, I describe an adaptation of Finter's quantitative cytopathic effect, or dye uptake method (6), to a human foreskin fibroblast-vesicular stomatitis virus (VSV) assay system. Additionally, a direct comparison of conventional plaque reduction and dye uptake methods is made.

MATERIALS AND METHODS

Tissue culture. Foreskins, obtained at the time of circumcision of neonates, were placed in a flask containing basal medium (Eagle) with Earle's salts and supplemented with 20% fetal calf serum (FCS), 160 units of penicillin per ml, 80 μg of streptomycin per ml, and 30 units of mycostatin per ml (BME + 20% FCS) and were processed within 24 to 48 hr by trypsinization (14). A single foreskin yielded 10<sup>6</sup> to 10 × 10<sup>6</sup> cells and formed a confluent monolayer in a small (25 cm<sup>2</sup>) Falcon flask within 2 weeks. No significant differences were observed in IF sensitivity between strains; consequently, strains were arbitrarily chosen for extensive use in the IF assay.

Cell strains were maintained in large (75 cm<sup>2</sup>) Falcon flasks in BME + 20% FCS and were passed weekly. Because of the aging phenomenon characteristic of diploid cell cultures (12), a given strain was used for assay for only 16 to 24 weeks.

IF. Four laboratory reference human IF samples were used. Two samples (Ref-1, Ref-2) were prepared by infecting human leukocytes with Newcastle disease virus (NDV) in vitro [0.1 plaque-forming unit (PFU) of NDV per leukocyte; reference 21]. Ref-3 and Ref-4 were derived from NDV infection of foreskin fibroblasts at a multiplicity of infection of 1 PFU per fibroblast [T. C. Merigan, In B. Bloom and P. Glade (ed.), Methods for Study of Cell Mediated Immunity, Academic Press Inc., New York, in press]. After incubation, the sample was centrifuged at 2,100 × g for 10 min, and the supernatant fluid was adjusted to a pH of 2.0 for 48 hr to inactivate residual NDV before being returned to a neutral pH and frozen at −70° C in small samples. Detailed characterization of Ref-1 and Ref-2 showed that they did not directly inactivate NDV and VSV, that they failed to protect chick embryo cells against virus infection, and that they were resistant to ultracentrifugation at 150,000 × g for 1.5 hr, to repeated cycles of freeze-thawing, to heating to 56° C for 1 hr, and to ribonuclease (2.5 μg/ml for 1 hr at 37° C). Incubation with trypsin (100 μg/ml for 1 hr at 37° C) resulted in only a 70% decrease in activity, probably because of the high concentration of FCS present in the reaction mixture.

Samples of international human reference IF 67/87 were also titrated.

Virus. VSV was grown from a sample kindly provided by D. Medearis, University of Pittsburgh Medical School (17). Confluent monolayers were seeded with a small inoculum of virus (10<sup>6</sup> PFU) in 10 ml of BME + 5% FCS and harvested 30 to 44 hr later when
a 1 to 2+ cytopathic effect was evident. By direct determination of VSV multiple-cycle growth kinetics, this time corresponded to the point at which maximal virus titer was just reached. This method was adopted to avoid conditions known to promote the production of interfering particles (15). A single pool of virus, stored at \(-70^\circ\) C, was used for the entire set of experiments.

VSV titrations were performed on confluent monolayers of fibroblasts in small Falcon flasks with an agar overlay as described in a subsequent section.

Quantitative cytopathic effect assay. This assay depends upon the ability of IF to protect a monolayer against the cytopathic effects of VSV. After virus challenge, only protected monolayers contain viable cells which are able to concentrate a supravital dye. After incubation with the dye, the cells are washed and lysed, and the concentration of the released dye is determined colorimetrically.

Fibroblast monolayers were grown in flat-bottomed, disposable glass vials (surface area, 3.8 cm\(^2\)) contained in special tissue culture racks holding 24 vials each (2). Each vial was seeded 2 to 7 days before use with 1.5 \(\times\) 10\(^5\) cells (0.4 \(\times\) 10\(^5\)/cm\(^2\)) in 3.0 ml of BME + 20\% FCS and incubated at 37 \(\pm\) 1 \(\%\) CO\(_2\) atmosphere.

For IF assay, twofold dilutions of IF were made in BME + 20\% FCS, and 1 ml of each dilution was added to two to four replicate vials. After 16 to 20 hr of incubation, the layers were drained, rinsed once with Earle's salt solution, and challenged with an appropriate amount (usually 10\(^3\) PFU) of VSV in 2.0 ml of BME + 5\% FCS. The subsequent incubation time was variable but usually was 42 to 46 hr after challenge with 10\(^3\) PFU of VSV. A concentrated neutral red solution (NR) was prepared as described by Finter (6). The exact concentration of this solution was unknown because of the inability to completely dissolve all particles of NR. Serial dilutions of this stock were made in normal saline (other balanced salt solutions resulted in precipitation of the NR), and the amount necessary for adequate uptake by control monolayers was determined empirically. A 0.2-ml amount of a suitable dilution (approximately 0.05\%) was then added to each vial for 2-hr of incubation. The layers were drained and rinsed twice with phosphate-buffered saline (PBS) to remove extracellular NR. Upon the addition of 4.5 ml of an ethyl alcohol-Sorensen's citric acid buffer (1:1; pH 4.2) solution, the dye was released into the supernatant fluid and read with a 540 filter in a Klett colorimeter.

The IF titer was defined as the reciprocal of that dilution of IF which resulted in a dye uptake of 50\% of a control monolayer when the log of the final IF dilution was plotted against the per cent dye uptake on probit paper.

Plaque reduction assay. Confluent monolayers were prepared by seeding small Falcon flasks with 10\(^6\) to 2 \(\times\) 10\(^6\) cells in 4.0 ml of BME + 20\% FCS 7 to 10 days before use. In the indicated experiments, to achieve dye uptake and plaque reduction monolayers identical in cell growth activity and cell density, Falcon flasks were seeded with 9.7 \(\times\) 10\(^6\) cells (0.4 \(\times\) 10\(^6\)/cm\(^2\)) in 4.0 ml of medium at the same time as the dye uptake vials were prepared.

IF dilutions were made as previously indicated. A 1-ml amount of IF and 3.0 ml of BME + 20\% FCS were added to triplicate flasks. After 20 hr of incubation, the layers were drained. In control experiments, it was found that monolayer rinsing was not necessary, and this was eliminated because of the time involved. High concentrations of human serum or plasma demonstrate nonspecific direct antiviral activity, and the rinsing step cannot be eliminated under these circumstances. Approximately 50 PFU of VSV in a 0.45-ml volume was then absorbed for 1 hr, 5.0 ml of 0.9\% agar in BME + 5\% FCS was added, and the layers were incubated for 48 hr. Two milliliters of an approximately 0.05\% NR solution was added, the flasks were returned to 37 \(C\), and plaques were counted 4 to 6 hr later. The layers were stored overnight at 4 \(C\) and reexamined the next morning, at which time plaques were frequently more distinct. The per cent plaque reduction was plotted against the log of the final IF dilution on probit paper, and the 50\% end point was determined as before.

RESULTS

Sensitivity of cells to NR. Monolayers were incubated for 2 hr with 0.2 ml of various concentrations of NR and subjected to the usual washing and cell lysis procedures. Figure 1 shows the

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**FIG. 1. Relationship between the amount of neutral red added to the amount of dye taken up by the monolayer.**
neutral red VSV. There was a 2.8-fold difference between the reference lowest and coefficient strain relationship of leased destruction. This was evident, indicating that the uptake of dye was directly proportional to the concentration presented to the cells. In addition, the amount of dye taken up was directly proportional to the number of cells in the layer (Fig. 2) in the range employed for the assay (see also Table 4).

**VSV destruction of the monolayer.** The time required for the development of the cytotoxic effects of VSV, as measured by a decrease in dye uptake, for different doses of challenge virus was determined by infecting a set of monolayers with various doses of VSV. Maximal layer destruction was defined as the dye uptake of infected layers at 72 hr. As can be seen in Fig. 3, the rate of layer destruction was a function of the amount of challenge virus.

On the basis of this information, appropriate incubation times were chosen such that virus control vials showed 80 to 90% monolayer destruction. This was 42 to 46 hr for 10^8 PFU and was shortened for higher VSV doses.

**Characteristics of the dye uptake assay.** Table 1 lists the titer of Ref-4 over a 12-week period when titered on a 3-day-old monolayer of cell strain F34 by using a challenge of 10^4 PFU of VSV. There was a 2.8-fold difference between the lowest and the highest titers. No relationship was noted between the age of the cell strain and the the reference titer.

Six separate dilutions of a single reference sample titered within the same assay showed a coefficient of variation of 0.11.

Two titrations of 100 units of human international reference IF 67/87 by the dye uptake method yielded values of 23.8 and 23.0 dye uptake units, respectively, when assay results were normalized to the mean Ref-4 titer [corrected test IF titer = test IF titer \times (geometric mean Ref titer/test Ref titer)]. Thus, this dye uptake method of titration was 4.35 times less sensitive than the titration used to establish international unitage.

The IF titer was found to be insensitive to changes in the challenge dose of virus over a 10-fold range (500 to 5,000 PFU) and was not changed by decreasing the FCS concentration to 5%.

Cells from F34 were frozen in liquid N₂ in an attempt to extend the life time of a given strain. The mean titer of Ref-4 was 167 in nine determinations on two postfreezer monolayer growths, a decrease in sensitivity of 28% ($P < 0.05$, Wilcoxon Mann Whitney test). Two other cell strains, tested less extensively, did not demonstrate this changed IF sensitivity after storage in N₂.

As a check on the reproducibility of the assay, the ratio of one reference interferon to another was examined (Table 2). By the plaque reduction method on F4, the ratio of the means of Ref-2 to Ref-1 was found to be 0.12. This corresponded nicely to the mean of nine ratios of Ref-2 to Ref-1, each derived from a simultaneous titration of Ref-2 and Ref-1 within a single run (0.14, ranging from 0.10 to 0.22). Similar ratios were obtained by titrations on F16 by both plaque reduction and dye uptake methods.

To determine whether the monolayer is sensitive to the total quantity of IF or to the concentration of IF, replicate monolayers were treated with 1.0 ml of IF with or without added plain media (Table 3). Cell and virus control layers had

**FIG. 2. Relationship between the cell number and neutral red (NR) uptake.** Five replicate vials were prepared with various cell inocula. Twenty-four hours later, two vials were counted and three received NR.

### Table 1. Week-to-week variability of a reference interferon titer

| Age of cell strain (weeks) | Titer of Ref-4* | Titer international reference 67/87 (100 units) |
|---------------------------|----------------|---------------------------------------------|
| 6                         | 182            | 17.2                                        |
| 7                         | 160            | 17.2                                        |
| 8                         | 230            | 17.2                                        |
| 9                         | 168            | 22.1                                        |
| 10                        | 307            |                                              |
| 11                        | 164            |                                              |
| 12                        | 440            |                                              |
| 13                        | 260            |                                              |
| 14                        | 270            |                                              |
| 15                        | 182            |                                              |
| 16                        | 400            |                                              |
| 17                        | 186            |                                              |

* Geometric mean, 232 (2.36 ± 0.15 log).
have traction cells assayed. Consistently media plaque should be added to samples. Virus titer can be determined from the dye uptake at the time dye was made to improve the product sensitivity of interferon (IF). Reduction of the dye uptake was measured by multiplying the IF titer of added IF by the total volume in a vial.

**Table 2. Ratio of two reference interferons**

| Cell strain | Method of assay         | No. of observations | Titer (geometric mean) | Overall (b/a) | Determined same run |
|-------------|-------------------------|---------------------|------------------------|---------------|---------------------|
| F 4         | Plaque reduction        | 16                  | 5,692                  | 0.12          | 9                   | 0.14                |
| F 16        | Plaque reduction        | 10                  | 704                    |               |                     |
|             | Dye uptake              | 2                   | 1,010                  |               |                     |

**Table 3. Effects of interferon (IF) concentration on IF titer**

| IF sample | IF titer\(a\) |
|-----------|----------------|
| Ref-1     | 906 \( \times 1 = 906 \) |
| Ref-2     | 133 \( \times 1 = 133 \) |
| Ref-3     | 299 \( \times 1 = 299 \) |
| Ref-4     | 207 \( \times 1 = 207 \) |

\(a\) IF titer determined by multiplying IF titer/1 ml of added IF (b) by total volume in vial (c).

identical dye uptakes irrespective of the volume of media used in the IF treatment period. If the cells protected by IF are sensitive to the concentration of IF, the product of the IF titer per milliliter of added IF times the total volume should be constant. This is precisely what is observed for Ref-1, Ref-3, and Ref-4. Ref-2 has consistently yielded anomalous results which have not been explained.

**Comparison of plaque reduction and dye uptake assays.** Finter found that in L cells challenged with Semliki Forest virus and in mouse embryonic monolayers challenged with VSV, the dye uptake method of assay was, respectively, 25 and 400 times more sensitive than the corresponding plaque reduction assay (6). As shown for F16 (Table 2), this was not observed in our experiments. However, since the assay monolayers had been prepared with different seeding densities and were not the same age, other factors could be involved. Consequently, dye uptake and plaque reduction monolayers were prepared in identical fashion, and assays were run completely in parallel on the third day after seeding. In all six runs (Fig. 4), the plaque reduction assay gave a median IF titer 2.8 times higher than the corresponding dye uptake titer (\(P < 0.05\), sign test for paired samples).

**Modification of the dye uptake assay.** An attempt was made to improve the sensitivity of the dye uptake assay system by mimicking a single cycle virus yield reduction assay which has been...
found to be the most sensitive assay in some systems (4, 11) and has the advantage of measuring the effects of IF close to the time of virus challenge. It was found that a single cycle of virus growth was completed in less than 12 hr, although 18 hr was required for 80% monolayer destruction as measured by dye uptake (Fig. 3). IF-pretreated monolayers were infected with 10^4 PFU of VSV (~1 PFU/fibroblast) and incubated for 16 to 20 hr. The IF titers obtained were compared with those obtained on replicate vials challenged with 10^5 PFU of VSV and incubated for 42 hr. In 7 of 18 runs, a twofold or greater increase in IF titer was seen at the higher virus dose. In the remaining 11 runs, this modification resulted in no increase in sensitivity. Two IF assays were run with 10^6 PFU of VSV with similar results. Although these findings suggest that the use of a higher virus challenge dose and a shorter incubation period may result in a more sensitive dye uptake assay, the variability led us to abandon this modification.

In vitro aging of the assay monolayer. Some workers have reported that in vitro monolayer aging results in an increased interferon sensitivity (3, 10). In five parallel experiments (Table 4), we found that an additional 2 days of aging was associated with a 90% increase in the IF titer (P < 0.05, sign test for paired samples) and a 33% increase in the initial numbers of fibroblasts per monolayer. Further aging did not yield higher interferon titers. The final number of fibroblasts per monolayer in control vials at the end of the assay was the same for all groups; thus, all of the fibroblasts in 3-day-old monolayers divided once, whereas only 60% in older layers underwent mitosis between the first and last day of the assay.

A 5-day-old monolayer was then used routinely for IF assays. Over a 3-month period, the geometric mean of 13 determinations of Ref-4 yielded a titer of 337 units in comparison to the previous value of 232 units on a 3-day-old monolayer (Table 1; P < 0.01, Wilcoxon Mann Whitney test). Since 1 dye uptake unit on a 3-day-old monolayer was shown to be equivalent to 4.35 international units, the sensitivity of the assay on a 5-day-old layer can be calculated by the use of the formula already given. Thus, 1 unit of a dye uptake assay on an aged monolayer is equivalent to 3.0 international units [4.35 x (232/337)].

### Table 4. Effect of in vitro aging on interferon (IF) sensitivity

| Age of monolayer (days) | IF titer of Ref-4 | Cells/vial | Doublings |
|-------------------------|-------------------|------------|-----------|
|                         |                   | Start      | End       |           |
| 3                       | 152               | 6.6 ± 2.0^a | 13.3 ± 4.7| 1.0       |
| 5                       | 294               | 8.8 ± 1.6  | 14.2 ± 2.7| 0.6       |
| 7                       | 688               | 9.2 ± 1.5  | 14.9 ± 2.6| 0.6       |

* Values are expressed x10^2.

^ Cell counts are expressed as mean ± one standard deviation and represent the counts of two or three vials in each of 5 to 24 separate experiments.

DISCUSSION

An interferon assay is a highly complex system which takes advantage of the unique biological capability of IF to interrupt the normal sequence of a virus infection. The exact mechanism of this interruption is unknown, but the most widely accepted working hypothesis is that IF, or some substance stimulated by IF, acts to allow transcription of a specific messenger ribonucleic acid (mRNA), resulting in the synthesis of a hypothetical new antiviral protein which acts by blocking the translation of virus-specific mRNA (8).

In an IF assay, this translation block is meas-
ured by one of several methods (1, 5, 19): a decrease in single or multiple cycle virus yield as determined in a two-step assay, a decrease in virus RNA production, an inhibition of a virus-induced cytopathic effect either determined visually or by a quantitative technique, or a decrease in the number of plaques formed by a known amount of virus. These methods differ significantly in their technical complexity and IF sensitivity. An ideal IF assay attempts to measure the effect of IF on virus multiplication at a time as close to the point of virus application as possible, while minimizing technical complexity and maximizing sensitivity.

There has been a considerable amount of conflicting data on the relative sensitivity of different IF assay methods even for the same species. The reasons are self-evident when one considers how many variables are involved. In addition to the obvious variables of choice of challenge virus, strain of virus, cell line, and assay method, it has been shown that the rate of decay of the IF-induced antiviral activity once IF is removed (11), the conditions of virus growth (i.e., multiple versus single cycle, agar-limited versus liquid media; reference 11), the age and confluency of the monolayer (3, 9, 10), the species tested (18), the concentration of FCS (20), pH (16), and the induction of new IF by the challenge virus (7) are all important variables in determining assay sensitivity. We conclude that, at our present state of knowledge, it is impossible to predict the sensitivity of any given assay method and that sensitivity can be determined only by the direct assay of the appropriate international reference. Unfortunately, this information is not generally available.

In the reported experiments, many of the variables necessary for characterization of a dye uptake assay for human IF have been investigated. The demonstration of a direct relationship between the number of cells in the monolayer and the amount of NR uptake is a prerequisite for any quantitative measure of a cytotoxic effect. The week-to-week variation in the assay is similar to that reported for other systems and illustrates the necessity for correcting each assay by including a laboratory standard. However, such a correction may introduce a twofold variation in test IF titers, since we have found a twofold range in the ratio of two reference IF to each other when determined within the same assay.

Few workers have performed adequately controlled experiments which allow direct comparison of different assay methods. Our experiments show that, under all conditions tested, the dye uptake assay is somewhat less sensitive than a plaque reduction assay. When conditions of the dye uptake assay are altered to mimic a single cycle virus growth yield reduction assay, increased sensitivity is sometimes seen. The failure of this modification may be related to the observation that IF pretreatment cannot protect against cytopathicity mediated directly by information contained within the infecting virion, when adequate numbers of virions are supplied (22).

The increased IF sensitivity of a 5-day-old monolayer may either result from a decrease in the number of cell divisions occurring after IF was removed with less loss of antiviral protection or from the absence of an inhibitor substance elaborated by young, growing cells (10).

Technical and economic considerations are major advantages of the dye uptake assay method. Since well dispersed plaques are not required, smaller monolayers may be used. Suitable vials are inexpensive, disposable, and are contained in racks, allowing efficient preparation and processing and eliminating individual handling. Dye uptake results in an integration of the cytopathic effect over the entire monolayer, in contrast to the evaluation of a limited number of virus-cell interaction sites in the plaque reduction method or to the several areas examined in a visual cytopathic effect assay. The end point is entirely objective and is not subject to bias induced by observer fatigue. In this laboratory, the dye uptake assay allows handling of twice as many IF samples in less time than a plaque reduction assay.

The above considerations are valid only if the dye uptake assay is reproducible and sensitive. The experiments demonstrate that a dye uptake assay for human IF is indeed as reproducible as other currently used assays. The increased sensitivity seen when monolayers are aged in vitro results in an assay in which 1 unit of IF is equivalent to 3 international units. In contrast, by direct determination under identical conditions, the plaque reduction assay is 2.8 times more sensitive than the dye uptake assay. This is similar to the sensitivity described by Mergan (13).

In conclusion, the small improvement in IF sensitivity yielded by plaque reduction assay of human IF does not justify the time and expense required. The described dye uptake assay method is convenient, simple, and well suited for clinical investigations of human IF.

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ADDITION IN PROOF

Since submission of the manuscript, this laboratory has received samples of the new international human IF reference 69/19 which, at an assigned unitage of 5,000 units per ampoule, has officially replaced 67/87 (100 units) as the human standard. Five titrations of this reference have yielded a geometric mean titer of 1,090 at a time when the titer of Ref-4 was 336. By calculation, the ratio of the titers of 69/19 to 67/87 is 32.7:1. This value is lower than the arbitrarily assigned ratio of 50:1 but is in good agreement with that reported by personal communication from the Medical Research Council at Mill Hill, London, England (35.97 with 95% confidence limits of 16.75 to 77.24). The data already presented concerning assay sensitivity can easily be recalculated in terms of the new international unit.

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