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Vesicular Stomatitis Virus Plaque Production in Monolayer Cultures with Liquid Overlay Medium: Description and Adaptation to a One-Day, Human Interferon-Plaque Reduction Assay

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Vesicular stomatitis virus forms discrete, microscopic plaques in stationary cultures of the WISH amnion cell line. Microplaque formation is rapid, reproducible, and easily quantitated, occurs at temperatures ranging from 33 to 40°C, and does not require a semisolid overlay. WISH cells, however, are less sensitive to vesicular stomatitis virus than are chicken embryo, 3T6, or Vero cells. WISH amnion cells also are highly sensitive to the antiviral effects of human interferon, and a quantitative human interferon assay, based on vesicular stomatitis virus plaque reduction in WISH cells, is described. This interferon assay can be performed within 1 day, uses a liquid overlay medium, does not require a vital stain, is as sensitive as other methods that use diploid cell strains, and is performed in a microtiter system.

Quantitative assays of infectious virus based on plaque formation require the use of a semisolid overlay medium and a vital stain. Interferon (IF) assays based on plaque reduction are more quantitative than those dependent upon a reduction in virus cytopathic effect (CPE), but they also require special overlay media and stain (9). Microtiter techniques have been applied to virus (4, 10, 12, 14, 21, 24) and IF plaque assays (6), with a reduction in the quantity of materials used but without significant savings in time or labor.

Currently, CPE reduction is the most easily performed and, perhaps for this reason, the most comonly used method of IF determination. However, IF determination by CPE reduction is limited by the delay in obtaining the results and the variable IF sensitivity (S. E. Grossberg, personal communication) of the cell strains used. Alternative procedures (1, 2, 13, 15, 17, 20, 23) that offer some degree of rapidity and/or accuracy tend to be more difficult to perform and for this and other reasons have not gained wide acceptance. In this communication we describe the rapid production of microscopic plaques by vesicular stomatitis virus (VSV) in cultures of the WISH amnion cell line without the addition of a semisolid overlay medium. In addition, WISH amnion is as sensitive to the antiviral activity of human IF as the more commonly used diploid cell strains. The WISH cell characteristics of VSV microplaque formation and IF sensitivity have been combined in a human IF assay which is quantitative, rapid, and easily performed.

MATERIALS AND METHODS

Cell cultures. Stationary human WISH amnion cultures, a continuous line established from a normal human placenta (11), were obtained from Flow Laboratories, Rockville, Md. Cultures were maintained in Eagle minimal essential medium (MEM) prepared with Earle salt solution and supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml; achromycin, 100 μg/ml; and mycostatin, 5 μg/ml).

HFS-1, low-passage human foreskin fibroblasts obtained from Biofluids, Rockville, Md., were grown to confluency in MEM with 10% FBS and maintained in MEM with 2% FBS.

All experiments were performed in 96-well microtiter plates (no. 3040, Falcon Plastics, Oxnard, Calif.). Each well was seeded with 3×10^4 to 5×10^4 cells in 0.1 ml of MEM containing 10% FBS.

Virus. The Indiana strain of VSV was propagated in primary chicken embryo cell cultures.

VSV plaque formation. The growth medium was decanted, and $\log_{10}$ dilutions of VSV in MEM with 2% FBS were added in 0.1-ml amounts to replicate culture wells, using a Svedmer type of transfer pipette (Kontes, Vineland, N.J.). At the times indicated in the text, microplaques were counted with either an inverted microscope at a final magnification of ×78 or with a dissecting microscope equipped

with a "zoom" lens attachment. Plaque size was determined with a calibrated photographic reticle.

IF preparations. Four IF preparations were used. These preparations were: (i) induced in foreskin fibroblasts by polyinosinic-polycytidylic acid; (ii) induced in normal human peripheral leukocytes by Sendai virus; (iii) induced by Sendai virus in the Namalva continuous lymphoblast cell line derived from a Burkitt's lymphoma (C. E. Buckler and C. Zoon, personal communication); and (iv) reference preparation 69/19 induced by Sendai virus in normal human peripheral leukocytes. The first three IF preparations were adjusted to a comparable titer of approximately $10^{4.2}$ reference units/0.1 ml and stored at −70°C.

IF titer determination in WISH cells. IF dilutions were prepared in MEM with 2% FBS and added in 0.1-ml volumes to duplicate confluent WISH amnion cell cultures from which the medium had been removed. IF dilutions were warmed to 37°C, and the length of time cultures were exposed to room temperature was minimized. The duration of exposure of WISH cells to IF varied as described in the text. After incubation at 37°C, the IF dilutions were decanted and 0.1 ml of VSV, diluted to contain between 10 and 30 plaque-forming units, was added per culture. At 37°C microplaque formation was first detectable between 12 and 14 h after the addition of virus, and microscopically visible plaques (including foci of rounded cells) were easily counted by 16 to 20 h after virus had been added (see above). In this system one unit of IF was defined as the reciprocal of the dilution that reduced the number of VSV plaques to 50% of those present in untreated cultures. For descriptive purposes the plaque reduction determination of IF has been abbreviated as PR_{50}, and IF determination by the CPE reduction system described above has been abbreviated as CPE-R_{50}.

RESULTS

VSV plaque development in WISH cell cultures. In cultures maintained at 37°C, areas of VSV replication first became evident at 12 to 16 h after infection as foci of a few rounded refractile cells. During the next 4 to 6 h, these foci enlarged and developed areas of central clearing (see Fig. 1). The coexistence of foci and plaques in the same culture was common. In this case foci represent the early stages of development of some plaques due to delayed absorption of virus from the relatively large (0.1 ml) volume added to the cell cultures (A. Randhawa, G. J. Stanton, J. A. Green, and S. Baron, in preparation). The results of five VSV assays performed at different times in WISH cell cultures are presented in Table 1. In these experiments, 5 to 10 replicate cultures were examined at each viral dilution and the mean number of plaques was calculated 16 to 20 h after the addition of the VSV inoculum. Reproducibility both between replicate cultures and between experiments was high.

Effect of temperature on VSV plaque formation. Plaque formation at 37°C was rapidly progressive, and plaques could only be counted during a limited time period of approximately 6 h, after which secondary plaque formation and plaque coalescence prevented accurate enumeration. The incubation temperature was varied in an attempt to prolong the period during which plaques could be reliably counted. Table 2 presents data from representative experiments in which the rate, size, and number of VSV plaques were examined inWISH amnion cells at 33, 37, and 39.5°C. Mean plaque diameters were determined at 24 h for 10 plaques at each of the indicated temperatures. Plaque development was more rapid at 37 or 39.5°C than at 33°C. Plaque diameters were approximately the same at 37 and 39.5°C. At 24 h, however, cultures incubated at 33°C had significantly smaller plaques. Small plaques, approximately 23 μm in diameter, were present in cultures after 12 h of incubation at 37°C but were not counted. The number of VSV plaques increased as the incubation temperature increased, but no plaques developed at 43°C. Although plaque counts were obtainable at all temperatures by 24 h, only at 33°C was plaque development slow enough to permit readings for extended periods of time. In general, plaques at 33°C were countable, without change in number, at 24 and 48 h, indicating that significant secondary plaque formation did not occur at this temperature. Secondary plaques did not develop during the first 24 h when cultures were incubated at 37°C. As an example, at 37°C plaque counts obtained at 24 h were 17.5 ± 5.4 at a 10^{-4} VSV dilution (mean ± standard error of the mean of six cultures at each dilution) and 1.66 ± 0.47 at a VSV dilution of 10^{-5}.

Comparative sensitivity of WISH cells to VSV replication. Infectivity assays of VSV were performed in WISH and in other cells as well. VSV with a titer of 6.5 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$)/0.1 ml in WISH amnion cells had a titer of 8.0 $\log_{10}$ $TCID_{50}$/0.1 ml in chicken embryo fibroblasts, 8.3 $\log_{10}$ $TCID_{50}$ in Vero cells, and 8.5 $\log_{10}$ $TCID_{50}$ in mouse 3T6 cells. Thus, WISH amnion was approximately 100-fold less sensitive than other randomly selected cells in which plaques do not form in the presence of a restrictive overlay medium.

Comparison of the IF sensitivity of WISH and HFS-1 cells. Initial studies were performed in which the IF sensitivity of WISH cells was compared with that of the routinely used IF-sensitive HFS-1 (diploid foreskin fibroblasts) cells. Comparative IF titers were determined by VSV-CPE-R_{50} in WISH and HFS-1 cells, and

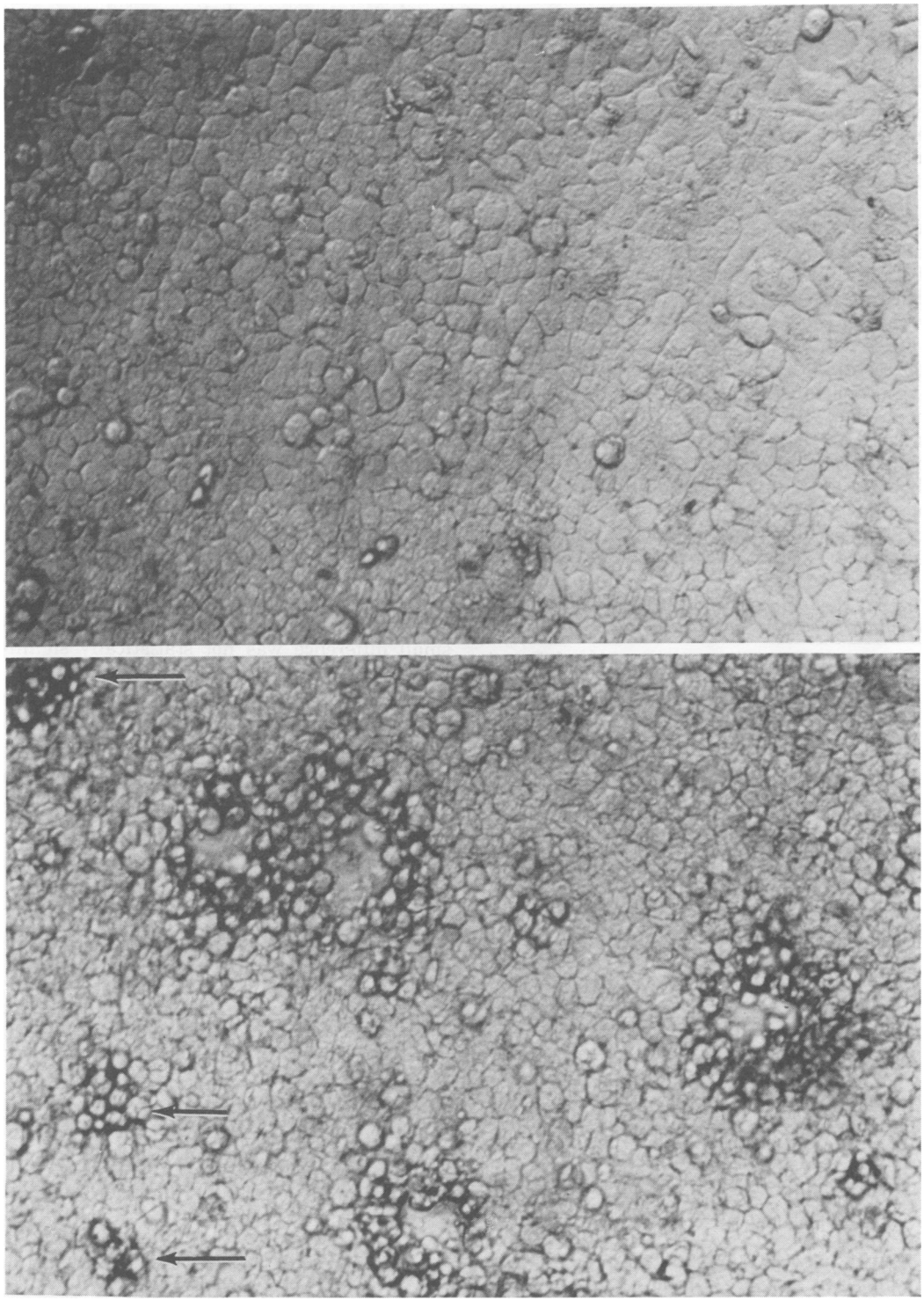

FIG. 1. *VSV plaque formation in WISH amnion cell cultures at 37°C. (Top) Control cells maintained at 37°C for 20 h without VSV. (Bottom) A culture containing four VSV plaques and three foci of refractile, rounded cells (arrows). A 5.5 $\log_{10}$ dilution of VSV was added to confluent cultures.*

by VSV-PR_{50} in WISH cultures. Agreement in IF titer was observed in all cases (Table 3).

Dose-response relationship of IF concentration to VSV plaque reduction. Figure 2 is a representative dose-response curve for human leukocyte-IF in WISH cell cultures. Plaque reduction was related in a linear fashion to $\log_{10}$ dilutions of IF.

Development of antiviral resistance as a function of time of incubation with IF. WISH cells were exposed to prewarmed $\log_{10}$ dilutions of three IF preparations of known titer for the times indicated in Fig. 1. IF-treated cultures were challenged with VSV, and PR_{50} titers were read the next day. A composite curve representing the development of WISH cell antiviral resistance as a function of the length of incubation with IF is shown (Fig. 3). The curve is based on the results of five separate experiments (two with leukocyte IF, two with lymphoblastoid IF, and one with fibroblast IF). Incubation of IF with WISH cells for a period of 1 to 1.5 h produced 80% of maximum antiviral resistance. A 3-h exposure of WISH cells to IF was the minimum incubation period necessary for induction of maximum antiviral activity. Comparable results were obtained for the three different IF preparations. The rapid development of antiviral resistance after brief exposure to IF is consistent with results obtained using human foreskin fibroblasts (8). In subsequent experiments, IF was added to WISH cultures for at least 3 h prior to the addition of VSV.

Age of WISH cell cultures and sensitivity of the IF assay. It has been suggested that there may be a direct relationship between the age of cell cultures and their sensitivity to IF (5, 7, 16, 19). Although this relationship is still uncertain, the effect of the age of WISH cell cultures on IF sensitivity was examined (Table 4). Cell cultures were established at the indicated times prior to the addition of IF dilutions. IF assays were performed at a single time in cultures of different age, and the data were recorded either as PR_{50} at 16 h or as CPE-R_{50} at 24 h after the addition of VSV. In repeated experiments no significant age-dependent differences in IF sensitivity were observed for the PR_{50} technique. In the experiment shown in Table 4, IF titers measured by CPE-R_{50} tended to be slightly lower than those determined by PR_{50}. This small difference was not statistically signifi-

TABLE 1. *Reproducibility of VSV plaque formation in stationery cultures of WISH amnion cells at 37°C*

Expt	Dilution of VSV inoculum ($\log_{10}$)[a]	No. of cultures	No. of plaques/culture (avg ± SE)[b]
1	5.0	10	14.0 ± 2.31
2	5.0	10	16.5 ± 2.12
3	5.5	8	5.1 ± 1.88
4	5.0	6	16.6 ± 2.58
5	5.5	5	8.4 ± 1.14

[a] One-tenth-milliliter volumes of dilutions of VSV were added to replicate 24- to 48-h-old cultures of WISH amnion cells.

[b] Plaques were counted 16 to 20 h after the addition of virus. SE, Standard error.

TABLE 3. *Comparison of IF titers in WISH and HFS-1 cells*

Cell type	Assay method	IF titer $\log_{10}$ (units/0.1 ml)		
		Lymphoblastoid[a]	Fibroblast	Leukocyte
WISH	PR_{50}	4.75	4.5	3.75
	CPE-R_{50}	4.5	4.5	3.75
HFS-1	CPE-R_{50}	4.75	4.5	3.5

[a] IF preparation.

TABLE 2. *Time of appearance, mean plaque count, and plaque size of VSV in WISH cell cultures maintained at three different temperatures*

Hours after the addition of VSV	No. of plaques/culture[a]					
	Expt 1			Expt 2		
	33°C	37°C	39.5°C	33°C	37°C	39.5°C
9	0	0	0	0	0	0
12	0	NC[b]	0	0	NC	0
24	49.7 ± 4.1	74.7 ± 5.0	94.7 ± 5.6	21.7 ± 2.7	32.0 ± 3.3	50.7 ± 4.1
VSV mean plaque size (μm) at:						
12 h		23				
24 h	46	60	48			

[a] Mean ± standard error of the plaques present in three cultures at each temperature (plaque-forming units/0.1 ml).

[b] NC, Early plaques were present but were not counted.

cant. The greater disparity between PR_{50} and CPE-R_{50} in 6-day-old cultures was restricted to this experiment.

Effect of incubation temperature on the WISH cell IF assay. As noted above, a slower rate of development of VSV plaques in WISH cell cultures occured at 33°C than at 37°C. The slower rate of VSV plaque formation and spread at 33°C permits them to be read and counted between 28 and 48 h after infection rather than between 18 and 28 h at 37°C. The effect on IF titer of the lower temperature of incubation after virus challenge was examined. Dilutions of three IF preparations of varying potency were added to two duplicate 1-day-old confluent WISH cell culture plates for 3 h at 37°C, after which the dilutions were removed and the cultures were challenged with 12 plaque-forming units of VSV (Table 5). Titrations of the three IF preparations were performed in duplicate. One titration was incubated at 33°C and the other was incubated at 37°C. IF end titers were determined by PR_{50}. When examined on day 1 (20 h) no difference in titer was seen in cultures incubated at 33 or 37°C after VSV challenge. By day 2, however, cultures maintained at 37°C had confluent lysis (100% CPE), whereas plaques were still readable at 33°C. IF titers essentially were the same when determined on day 1 or 2 in cultures maintained at 33°C. Thus, incubation at 33°C after VSV challenge avoids the potential problem of confluent lysis of cultures before a plaque reading can be performed conveniently.

Additional WISH cell characteristics. WISH cell cultures were examined throughout a 12-month period, during which there were greater than 40 cell passages. No difference was observed in the ability of WISH cells to replicate VSV (form plaques or develop CPE), and

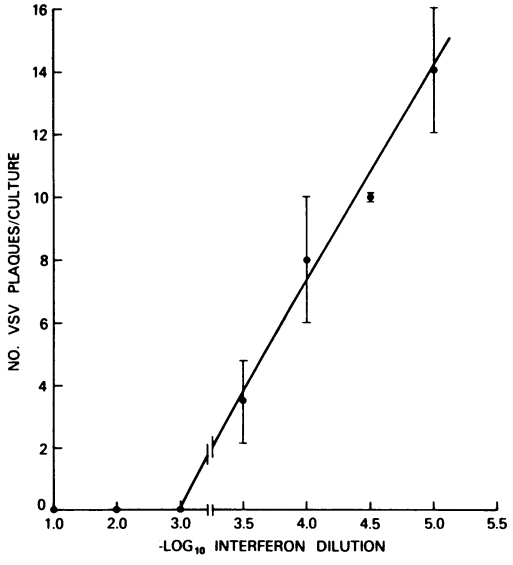

FIG. 2. *Human IF dose-response in WISH cells; number of VSV microplaques developing in WISH cell cultures exposed to 10-fold dilutions of leukocyte IF. VSV plaques were counted at 16 h. Each point represents the mean number of plaques of four cultures with standard error of the mean.*

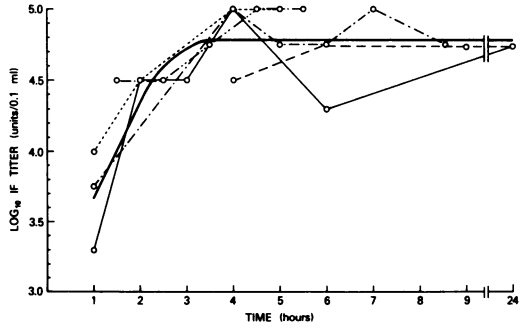

FIG. 3. *Development of viral resistance in WISH cell cultures exposed to IF for varying lengths of time. IF titer was determined by the VSV plaque reduction (PR_{50}) method. The solid line is a composite of five separate experiments represented by open circles. In each experiment VSV was added to all cultures at a single time.*

TABLE 4. *Effect of the age of WISH cell cultures on IF titer*

Age of cell cultures[b]	IF titer $\log_{10}$ (units/0.1 ml)[a]					
	PR_{50} method			CPE-R_{50} method		
	Lyb.	Fib.	Leu.	Lyb.	Fib.	Leu.
2 h	4.5	4.75	5.0	4.25	4.0	4.5
1 day	5.0	4.75	5.0	4.75	4.75	5.0
2 days	5.0	4.75	4.75	4.75	4.25	4.25
3 days	4.75	5.0	4.75	4.75	4.75	4.75
6 days	5.0	4.75	5.0	4.25	4.0	4.0

[a] Lyb., Lymphoblast IF (Namalva); Fib., fibroblast IF; Leu., peripheral leukocyte IF.
[b] Age of the cultures when IF dilutions were added.

TABLE 5. *Effect of incubation temperature, after virus challenge, on the titer of human IF*

Day of reading	IF titer (log_{10})[a]					
	69/19 IF		Leukocyte IF		Fibroblast IF	
	33°C[b]	37°C[c]	33°C	37°C	33°C	37°C
1	3.5	3.5	3.0	3.0	5.0	5.0
2	3.0	CL[d]	3.0	CL	4.8	CL

[a] Units of IF per 0.1 ml as determined by the PR_{50} method (reciprocal of the IF dilution producing 50% reduction in VSV-CPE).

[b] IF was incubated with WISH cell cultures for 3 h at 37°C prior to being transferred to 33°C for the remainder of the experiment.

[c] WISH cell cultures maintained at 37°C throughout the experiment.

[d] CL, Confluent lysis.

no change in sensitivity to IF was noted. Other samples of frozen WISH cells from the same supplier gave comparable IF and VSV titers.

The effect of serum concentrations on maintaining WISH cells was also examined, and WISH cell cultures, maintained for 5 days with medium containing 2, 4, 5, 10, 15, or 20% FBS, showed no overgrowth or degeneration. Stability of this nature facilitates routine maintenance and use of these cells.

DISCUSSION

The current studies describe VSV plaque formation in WISH amnion cells maintained with liquid medium. At 37°C plaques develop rapidly and can be counted within 16 to 20 h, but cannot be counted after 28 h due to secondary plaque formation and plaque coalescence. When the incubation temperature is lowered to 33°C, plaques develop slowly, maintain their morphology, and can be counted without change in number for periods up to 48 h. The formation of microscopic as opposed to macroscopic plaques makes this system well suited to microtiter techniques, which provide a savings in material and permit the routine assay of large numbers of samples. The foregoing characteristics of VSV plaque formation in WISH cell cultures provide a basis for the rapid quantitative assay of VSV without the use of special overlay media or vital cell stain.

Plaque formation in liquid medium has also been demonstrated for encephalomyocarditis (3) and picornaviruses (18). It has been suggested that the production of plaques in liquid medium by cytolytic viruses may represent more efficient transmission of virus to adjoining cells during the first several cycles of replication before the general dissemination of virus throughout the culture (3). The current data indirectly support this hypothesis by the demonstration of a decreased sensitivity of WISH to VSV infection, which indicates that normally cytolytic virus may spread more efficiently by direct transfer to adjacent cells in cultures of low sensitivity. The magnitude of WISH insensitivity to VSV replication (approximately 2 to 2.5 log_{10} less sensitive than randomly selected cell types) is, however, a potential limiting factor in the use of this assay for VSV preparations of low titer.

The plaque reduction IF assay described here has several advantages which should recommend it for routine use in the assay of human IF. It is rapid, with less than 24 h being required (3 to 4 h of incubation with IF and an additional 16 to 20 h for microplaque development at 37°C). The short period of time needed to complete an assay permits samples to be added to cells throughout most of the average work day. Provided virus is added before 5 p.m., plaques can be read during the early part of the following day. If the virus is allowed to replicate at 33°C, the assay period can be prolonged to 48 h with no loss of sensitivity. The method is simple, since microplaques are produced without the addition of a semisolid overlay or stain. Thus, it is similar to the materials and methods used in the popular CPE reduction microtiter assay for IF (22). The method may be more reproducible since it uses a continuous cell line that appears to be stable with respect to VSV replication and IF sensitivity through a number of passages. Also, WISH cells are unaffected by storage in the frozen state and are commercially available. The method using WISH cells is as sensitive as methods that use the sensitive HFS-1 cells. Finally, the potential exists for the use of a cell stain such as crystal violet (2) and low-power (hand lens) magnification to facilitate counting plaques without a microscope and for the assay of non-human primate IF in WISH cells.

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