Fluorescent Cell Counting as an Assay Method for Respiratory Syncytial Virus

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The fluorescent cell-counting technique was applied to the enumeration of cell-infecting units of respiratory syncytial (RS) virus in human fetal diploid (HFD) cover-slip cell cultures; it was a sensitive, precise, and rapid assay method. Approximately 2 hr was required for maximal adsorption of RS virus to HFD cell monolayers. However, about 15% of the infectious virus in the inoculum remained unadsorbed; this percentage was not significantly reduced even when the adsorption period was extended to 5 hr. A linear relationship between virus concentration and the number of fluorescent cells existed over a range of 1.2 $\log_{10}$ units. Variation of the mean of replicate determinations in a single experiment was approximately 7.5%. The distribution of single infected HFD cells on cover-slip cell cultures corresponded with the calculated frequencies of the Poisson distribution. The Chi square test for the extent of fit was calculated for several experiments, and the value of P was never less than 0.5. The addition of immune serum after virus adsorption effectively inhibited the development of detectable levels of viral antigen in secondarily infected cells.

The type of cytopathic change induced in cells infected with respiratory syncytial (RS) virus is influenced by the host cell and also by the composition of the medium used for maintenance of infected cells (6). Under certain conditions, the development of syncytia is the characteristic feature, whereas a cytolytic effect is the obvious response under still different cultural conditions. Recognition of newly formed or small syncytia is difficult; vital dyes have been used to aid in their detection (2). The variable, and at times subtle, cytopathic response to RS virus is an annoying impediment associated with current assay procedures.

Several improved methods for enumeration of RS virus have been reported. The plaque method of Kisch and Johnson (7) has not only the advantage of high precision, but it also allows the isolation of progeny of a single viral particle. Another assay system based upon the counting of primary syncytia was described by Taylor-Robinson and Doggett (15). Although this method does not allow for virus cloning, it is precise and reproducible. The one principal disadvantage of both of these procedures is the relatively long time required for the appearance of plaques (9 to 10 days) or of syncytia (3 days).

The purpose of this paper is to show that the fluorescent cell-counting procedure (4, 11, 14, 16)

provides a rapid and precise method for the assay of RS virus infectivity.

MATERIALS AND METHODS

Cell culture. Human fetal diploid (HFD) cells derived from embryonic lung were initiated and maintained according to the method of Hayflick and Moorhead (5). Methods and media for growth and maintenance of HFD cells on 15-mm circular cover slips have already been described (13).

Virus strain. The Balin strain of RS virus was originally isolated in this laboratory and was used throughout this study.

Immunofluorescent methods. The preparation of RS virus immune serum, the conjugation of immune globulins with fluorescein isothiocyanate, and the direct method of immunofluorescent staining of cell cultures have previously been described (13). A Zeiss fluorescence microscope was used for observation and counting infected cells. The filter system consisted of a UG5 exciter and a GG4 barrier filter. For cell counts, a 25× objective lens and 8× oculars were used. With these optics, the width of the microscopic field was found to be 0.7 mm.

Infection of cover-slip cell cultures. Cover-slip cell cultures were used for infectious virus assay after complete monolayers of HFD cells had formed. The outgrowth medium was completely removed by aspiration from petri dishes (60 mm) containing three circular cover-slip cell cultures; 0.03 ml of appropriately diluted virus was placed on each cover slip

with a 0.2-ml serological pipette. It is important that the viral inoculum be carefully placed so that it covers the entire surface of the cover slip without running off. The inoculated cultures were incubated in a humidified atmosphere at 36 C for 2 or 3 hr to allow viral adsorption. In certain experiments, the unadsorbed virus was removed by washing the cultures with serum-free maintenance medium. Approximately 5.0 ml of maintenance medium was added after the adsorption period, and incubation continued at 36 C in a humidified CO_2 incubator.

Infected cell counts. The number of cell-infecting units (CIU) per milliliter was determined from cell counts of a sample area of the cover slip. The specifically fluorescing cells in an area 0.7 (field width) by 15 mm (diameter of cover slip) were counted. The number of fluorescent cells in this sample area was referred to as a diameter count and was equivalent to the number of infected cells in 27.5 microscopic fields. The diameter count was multiplied by 16.8 to relate the sample area to the total area of the cover slip and hence to the number of CIU per cover slip. The number of CIU per milliliter was calculated by multiplying the number of CIU per cover slip by the reciprocal of the dilution, if necessary, and then by a volume factor for conversion to a per-milliliter basis.

RESULTS

Adsorption of RS virus to HFD cells. The rate of adsorption of infective virus to HFD cell monolayers was followed by counting fluorescent cells that developed after adsorption for various intervals of time. A replicate series of petri dishes, each containing three cover-slip cell cultures, was inoculated with sufficient virus to infect approximately 10% of the cells. The inoculated cultures were then incubated in a humidified atmosphere at 36 C, and, at the indicated intervals, a set of three cover slips was removed and washed three times. Maintenance medium was added, and the cultures were returned to the incubator. After a total of 23 hr, including the time for adsorption, the cover slips were harvested, the cells were fixed and stained, and the fluorescent cells were counted. From these counts, the amount of virus which adsorbed after each interval was determined. However, it was also of interest to know what proportion of the inoculated virus remained unadsorbed.

To measure the unadsorbed virus, the inoculum from the appropriate cultures described above was carefully removed, pooled, and diluted 1:2, and 0.03 ml of the diluted material was placed on each of three new cover-slip cell cultures. After 3 hr, the cultures were removed from the incubator, washed three times, and placed in 5.0 ml of maintenance medium; incubation continued for a total of 23 hr. The proportion of virus which remained unadsorbed after the first adsorption step was determined by counting the number of

fluorescent cells which developed after the second adsorption step. Total virus was determined as the sum of the CIU which was adsorbed in both the first and second adsorption steps.

The rate of RS virus adsorption, expressed as the percentage of total virus, is shown in Fig. 1. Adsorption occurred quite rapidly during the 1st hr and then apparently reached equilibrium at about 2 hr when maximal adsorption (85%) occurred. It is of interest, especially since a low multiplicity was used for infection, that approximately 15% of the infectious virus in the inoculum failed to adsorb during the first adsorption step. Incubation continued for up to 5 hr did not significantly increase the amount of virus adsorbed. This is similar to the adsorption of Newcastle disease virus to HeLa cells, for which approximately 12% of the inoculated virus fails to adsorb (16).

Dose-response relationship. To establish the relationship between virus concentration and the number of specifically fluorescent cells, replicate cover-slip cell cultures were inoculated with doubling dilutions of RS virus, and three cover-slip cultures were used for each dilution of virus. After incubation for a total of 23 hr, the cultures were fixed and stained, and the number of fluorescent cells was counted. The results in Fig. 2 are typical of several experiments performed; they show that there was a linear relationship and that the linearity existed over approximately 1.2 $\log_{10}$ units. Thus, each fluorescing cell represented a single cell infectious unit, not further divisible by dilution (3).

Rate of appearance of fluorescing cells. The successful application of the fluorescent cell-counting procedure for virus assay is dependent
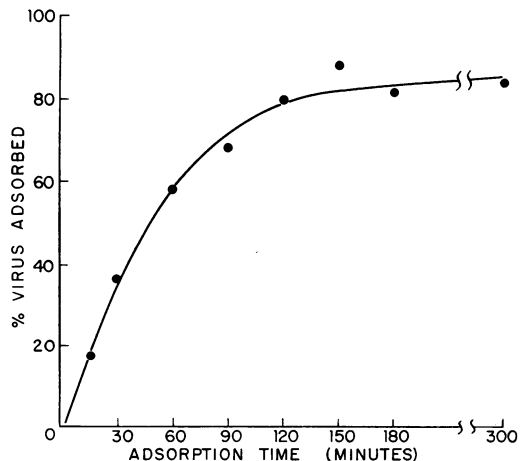

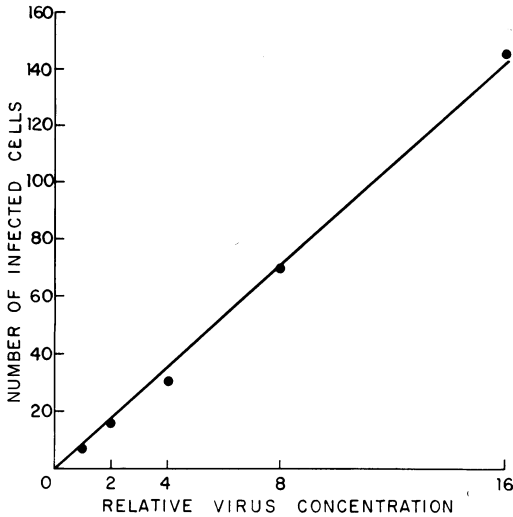FIG. 1. *Adsorption of respiratory syncytial virus to human fetal diploid lung cells.*

FIG. 2. *Dose-response relationship between the concentration of respiratory syncytial virus and the number of fluorescent cells.*

upon selection of the appropriate incubation interval between virus inoculation and the fixation of cells for staining. It is important that sufficient time be allowed for development of easily detectable concentrations of viral antigen in primarily infected cells; however, it is imperative that this interval precede the development of an approximately equal amount of viral antigen in secondarily infected cells.

If the infection of a cell culture is synchronous, or nearly so, it should be possible by observing the appearance of infected cells to detect a period when the number of infected cells remains nearly constant, a fact which indicates that essentially all primarily infected cells have developed detectable amounts of viral antigen. The rate of appearance of infected cells was followed by inoculating a number of cover-slip cell cultures with sufficient virus to infect approximately 1.0% of the cells. Three infected cover-slip cell cultures were removed from the incubator every 2 hr for 26 hr. The cultures were fixed and stained, and the number of fluorescent cells for each interval was counted. In repeated experiments, specifically fluorescent cells were first observed at 10 hr; the first appearance of infected cells was followed by a nearly linear increase in the number of fluorescing cells throughout the period of observation (Fig. 3). There was no period when the number of fluorescing cells remained constant, and, hence, the time when all primarily infected cells had developed detectable levels of viral antigen could not be distinguished from the time of first appearance of secondarily infected cells.

In another series of experiments, an attempt was made to overcome this problem by inhibiting the spread of virus from primarily infected cells by incorporating RS virus immune serum in the maintenance medium. Cover-slip cell cultures were inoculated with sufficient virus to infect approximately 1.0% of the cells. After virus adsorption, one-half of the cultures was placed in maintenance medium containing 10% normal monkey serum (preimmunization serum), and the remaining half, in medium containing 10% inactivated (56 C, 30 min) RS virus immune monkey serum (neutralizing antibody titer 1:1,024). Infected cover-slip cell cultures from both the normal and immune serum series were removed from the incubator at 2-hr intervals as indicated. The cultures were washed three times in serum-free maintenance medium, fixed, and stained, and the number of fluorescent cells was counted.

The number of fluorescent cells developing on each of three cover-slips was counted and was averaged for each interval for both the normal and immune serum series (Fig. 4). The number of fluorescing cells in the cultures with immune serum remained constant after 20 hr of incubation, in contrast to the continued increase that occurred in the control cultures; therefore, this increase represents the appearance of secondarily infected cells. On this basis, 20 hr was selected as
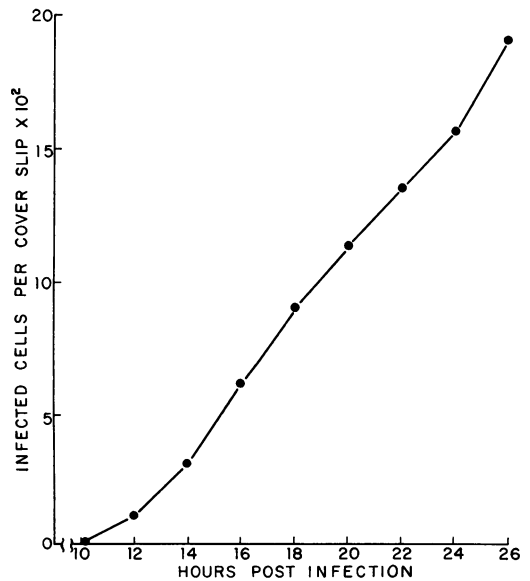

FIG. 3. *Rate of appearance of infected cells in HFD lung cultures inoculated at zero-time with sufficient virus to infect approximately 1.0% of the cells. Each point represents the average count of three cover-slip cell cultures.*
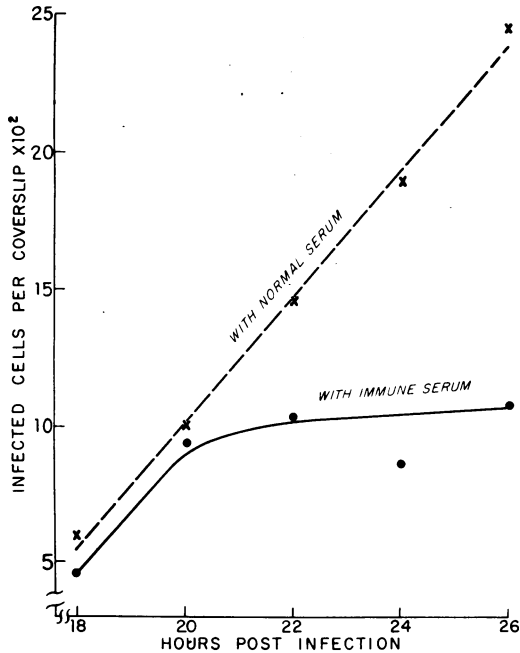
FIG. 4. *Effect of RS virus immune serum on the rate of appearance of infected cells in HFD lung cultures. The cultures were inoculated at zero-time with sufficient virus to infect 1.0% of the cells. After virus adsorption, maintenance medium with 10% normal monkey serum was added to one-half of the cultures, and maintenance medium with 10% RS virus immune monkey serum was added to the remaining cultures. Each point represents the average count of three cover-slip cell cultures.*

the appropriate time for staining of cover-slip cultures.

Mode of distribution of RS virus infected cells in cover-slip cell cultures. The validity of determining the number of fluorescent cells per cover slip by extrapolating from a count of a sample area is based upon the assumption that the distribution of infected cells over the surface of the cover slip is random. To determine whether such a distribution occurs, cover-slip cultures were inoculated with sufficient virus to infect about 1% of the cells in the monolayer. Virus was allowed to adsorb for 2 hr at 36 C, maintenance medium was added, and incubation was continued at 36 C in a CO_2 incubator. After 20 hr, infected cultures were fixed and stained, and the number of fluorescent cells in each of approximately 130 microscopic fields was determined.

In Fig. 5, the distribution of single infected HFD cells per field is compared with the expected number as calculated for a theoretical Poisson distribution. The actual counts corresponded very well with the calculated frequencies. The Chi square test for extent of fit was calculated and

gave a probability of 0.8, indicating no significant difference between the observed and calculated frequencies. The distribution can, therefore, be said to be random. The *P* values calculated for each of three similar experiments were never less than 0.5.

Precision of the fluorescent cell-counting procedure. The precision of fluorescent cell-counting was determined by infecting a series of 13 cover-slip cell cultures. After a 2-hr adsorption period, maintenance medium was added and incubation was continued at 36 C in a CO_2 incubator for a total of 20 hr. The cultures were fixed and stained, and the number of fluorescent cells per diameter count was determined. The mean number of fluorescent cells per diameter count was 167.7, and the range was 140 to 186. The standard deviation was 12.68, and, expressed as a percentage of the mean, it was 7.6%. This agrees with the precision of the plaque assay procedure (3, 10).

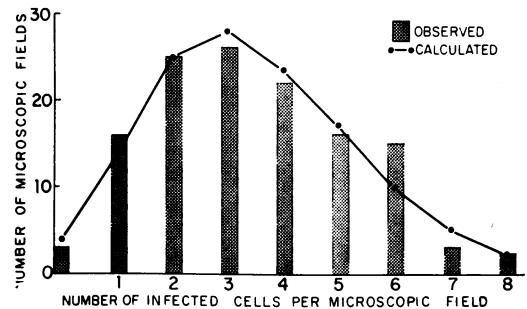*Comparison of the fluorescent cell-counting and dilution end point methods for the assay of RS*

FIG. 5. *Frequency distribution of infected cells per microscopic field is compared with the calculated frequencies of the Poisson distribution.*

TABLE 1. *Comparison of the fluorescent cell-counting and dilution end point methods for the enumeration of respiratory syncytial virus*

Test no.[a]	Fluorescent cell-counting method[b]	Dilution end point method[c]
1	6.2	3.2
2	6.5	1.9
3	5.9	2.1
4	6.6	2.4
5	6.0	1.7

[a] In all tests, the virus lot used was no. 7130.

[b] Expressed as 10^5 times the number of cell infecting units per milliliter.

[c] Expressed as 10^5 times the median tissue culture infectious dose per milliliter. Four KB cell culture tubes per virus dilution were used, and the test was observed for cytopathic effects for 7 to 10 days.

virus. For the dilution end point method, KB cells were grown in Eagle's minimal essential medium with 10% fetal bovine serum and maintained in Leibovitz Medium no. 15 (9) with 2% fetal bovine serum. Each virus dilution was inoculated in quadruplicate; the cultures were refed every 2 or 3 days and were observed for cytopathic effect without the aid of vital dyes for 7 to 10 days. The same virus pool was tested five times, on different days, with the dilution end point and fluorescent cell-counting methods; the results (Table 1) obtained with the latter were less variable, and, assuming comparable cell sensitivity, the latter was also a more sensitive method.

DISCUSSION

The flourescent cell-counting procedure has been used for the assay of several viruses (4, 11, 14, 16), and, although it is applicable to the assay of cytolytic viruses, it is more useful with those viruses which grow slowly or induce little or no cytopathic effect. It is because the cytopathic effect of RS virus, at or near the limits of infectivity, is often difficult to detect that fluorescent cell counting is uniquely advantageous.

The validity of the fluorescent cell-counting technique is demonstrated by the linear relationship between virus concentration and the number of specifically fluorescent cells. This relationship existed over approximately 1.2 $\log_{10}$ units. The precision of this method of assay had the statistical advantage of the plaque assay and was more sensitive than the dilution end point technique. Furthermore, fluorescent cell counting provides for rapid assay, less than 24 hr, of RS virus; this is the principal advantage of the method.

RS virus adsorption to HFD cells reached an apparent equilibrium, and was essentially complete after 2 hr. Approximately 85% of the inoculated virus was adsorbed during this period, and continued incubation did not appreciably increase virus adsorption. RS virus adsorption as reported herein was somewhat slower than that of Newcastle disease virus to HeLa cells (16), but faster than that of vaccinia virus to chick embryo fibroblasts (12). The rate of RS virus adsorption to Hep II cells as reported by Bennett and Hamre (1) was significantly slower, requiring 10 hr. Although this difference may be attributable to the different host cells and virus strains used, it is more likely that the variation in the volume of the viral inoculum in relation to the surface area used for adsorption has a greater effect on the rate of adsorption and that it is responsible for the observed differences.

Experiments in which the appearance of secondarily infected cells was inhibited by addition of immune serum after virus adsorption suggest that one of the principal methods of the spread of virus, at least during the initial cycles of virus synthesis, is via the fluid phase of the culture and that cell-to-cell spread through cytoplasmic bridges or cellular processes does not occur at a significant rate. In similar experiments, Taylor-Robinson and Doggett (15) have shown that fewer and smaller syncytia are formed when immune serum is added after virus adsorption, indicating that syncytium formation also results, in part at least, by spread of the virus via a route in which the virus is accessible to the action of antibodies.

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