Uniform results were obtained with batches of albumin-I^{131} obtained from various sources. The rate of albumin degradation as determined by this method of tracer measurement was closely parallel to the degradation rate obtained by measurement of non-protein nitrogen by the micro-Kjeldahl technic.

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Assay of Sendai Virus by Immunofluorescence and Hemadsorbed Cell-Counting Procedures. (30467)

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The fluorescent cell-counting technique originally introduced by Wheelock and Tamm (1) has been successfully extended to several viruses such as adenovirus(2), human cytomegalovirus(3), and psittacosis virus(4). In this work, the technique was extended to Sendai virus by infecting L cells. Since L cells infected with egg-grown Sendai virus produce progeny infectious to egg embryos but not to L cells(5,6), the fluorescent cell-counting technique was considered to be suitable for enumerating infectious virus in the inoculum without being annoyed by the spreading of progeny virus from cell to cell through the medium. Possible use of the hemadsorbed cell-counting technique in this virus cell system was also examined, and it was shown to compare favorably with the fluorescent cell-counting technique.

Materials and methods. Virus. The Fushimi strain of Sendai virus was used. A seed virus was prepared by an allantoic inoculation of 10-day-old chick embryos with 0.2 ml of the stock virus diluted to 10^{-5} in phosphate buffered saline (PBS)(7). At the end of incubation period at 35°C for 40 hours, the allantoic fluids were harvested and stored at −25°C after centrifugation at 3,000 rpm for 15 minutes. This was used within 2 weeks after preparing. The 50% egg infectious dose (EID_{50}) of the allantoic harvests, calculated by the Reed and Muench method (8), ranged between $10^{9.7}$ and $10^{10.0}$, the hemagglutinin titer (HAU), $10^{3.3}$ and $10^{3.5}$ per ml.

Medium for cell growth and infected coverslip cultures. The medium used for cell growth contained 0.5% lactalbumin hydrolysate and 0.1% yeast extract in Earle's solution with 0.45% glucose and supplemented with 10% unheated bovine serum (growing medium). After infection, the cells were kept in a maintenance solution (MS) in which 2% inactivated horse serum was substituted for the bovine serum.

L cell cultures. A Petri dish (90 mm in diameter) with 12 coverslips (6 × 30 mm) received 20 ml of L cell suspension in growing medium at a concentration of 3×10^5 cells per ml and was incubated at 37°C in an atmosphere of 5% CO_2 in air for 3 days. By this time, a complete monolayer formed

on the coverslips and the cell number was approximately 3×10^5 per coverslip.

Virus inoculation of L cell coverslip cultures. Coverslips with complete monolayers of L cells were washed once by immersion in Petri dishes containing Hanks' solution. Each coverslip received 0.05 ml of appropriately diluted virus in Hanks' solution and the adsorption was carried out at 37°C in the CO_2 incubator. At the end of adsorption period, the coverslips were again washed 6 times by gentle immersion in Hanks' solution to remove the residual virus, returned to new Petri dishes containing 20 ml of MS, and the incubation continued.

Immune serum. Sendai virus was partially purified by differential centrifugations at 4,000 *g* for 15 minutes and at 43,000 *g* for 30 minutes. The pellet was resuspended in (PBS) to contain 104.0 HAU/ml. One ml of this material was inoculated intravenously once a week into a rabbit. A total of 7 successive injections was made and the animal was bled one week after the last injection. The antiserum used in the present work had a hemagglutination inhibition titer of 1:2048 against 8 HAU of Sendai virus and a complement fixing antibody titer of 1:1024.

Fixation and fluorescent antibody staining of coverslips. At the indicated time, the coverslips were removed from the Petri dishes, washed in PBS, dried for 5 minutes at 37°C, and fixed with acetone for 10 minutes at room temperature. The cells were stained by the immunofluorescent complement method (9).

Hemadsorption technique. The coverslips were washed in cold PBS and overlayered with 0.5% suspension of guinea pig red blood cells(10). After 60 minutes at 4°C, they were washed in cold PBS and specifically hemadsorbed cells were counted by the method used for the fluorescent cell-counting.

Counting of fluorescent and hemadsorbed cells. By measuring the diameter of a microscopic field in a given optical system (10 × ocular and 40 × objective), the number of fields per coverslip was calculated as 1130. The average number of cells per field was 265 with a standard deviation of 18.5. Fifty fields were usually counted per coverslip and the mean number of fluorescent or hemadsorbed cells per field was obtained. Therefore, the total number of fluorescent or hemadsorbed cells per coverslip is a product of the mean number of fluorescent cells per field and the factor of 1130.

TABLE I. Determination of Optimal Concentration of Sendai Virus for Fluorescent Cell-Counting Procedure.

Dilution of stock virus*	Mean No. of fluorescent cells/field
10^{-1}	confluent†
10^{-2}	" †
10^{-3}	" †
10^{-4}	34.5
10^{-5}	3.2

* EID_{50} was $10^{8.9}$/ml.
† All the cells were fluorescent.

Results. Determination of optimal concentration of virus for the fluorescent cell-counting procedure. In order to obtain accuracy in counting the fluorescent cells, it was first desirable to determine the optimal concentration for the virus inoculum. Serial 10-fold dilutions of Sendai virus stock were made up to 10^{-5} in Hanks' solution. A volume of 0.05 ml was layered on each coverslip. This amount was sufficient to cover the whole area of the coverslip. Four coverslips were used for each dilution. After 60 minutes adsorption at 37°C, the coverslips were washed in Hanks' solution, re-fed with MS and returned to the incubator. At 24 hours, the coverslips were collected and stained with fluorescent antibody. Fifty fields were counted on individual coverslip and the mean number of fluorescent cells per each field was calculated. The results are shown in Table I. When the concentration of inoculum was more than 10^{-3} of stock virus, all the cells fluoresced. However, with 10^{-4} dilution, the mean number of fluorescent cells was 34.5, approximately 14% of the total cells in the unit area. Thus, this dilution was set as an optimal concentration of inoculum virus for further experiments.

Determination of incubation period. The characteristics of the system used in this work have been fully studied in this laboratory(5,6,11). When an L cell is infected with egg-grown Sendai virus, it produces a progeny virus which is infectious to embryo-

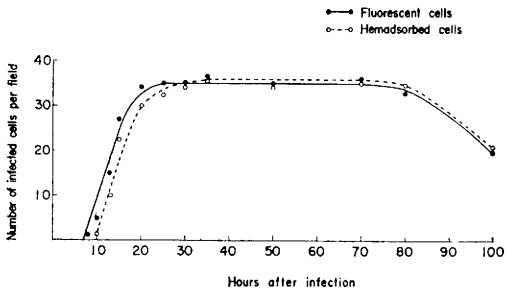


FIG. 1. Increase in fluorescing and hemadsorbing cells as a function of time.

nated eggs but not to L cells(6). Therefore, the virus produced in this system never goes to the second cycle of infection and this was thought favorable for the present work in the sense that neither addition of antiserum nor control of the time of harvest is necessary.

After infection with 0.05 ml of 10^{-4} virus dilution, the coverslips were harvested at various intervals and the appearance of fluorescent cells and hemadsorbed cells was investigated. Fig. 1 shows the increase in number of fluorescent cells and hemadsorbed cells as a function of time of incubation. As reported previously from this laboratory(12), at 8-10 hours after infection, faint fluorescence was seen in the juxtanuclear region and the number of fluorescent cells was far less so that counting was not reliable. At 13-20 hours, the intensive fluorescent masses were located mainly in the Golgi area and the number of fluorescent cells gradually increased. At 24-48 hours, the fluorescent granules filled the entire cytoplasm and the number of fluorescent cells reached a maximum and remained constant.

Following 72 hours of incubation, the intense fluorescent antigens were distributed in the peripheral cytoplasm, and the cell membrane began to disappear. From this time, it became difficult to identify a single fluorescent cell, and, therefore, to get an accurate count of fluorescent cells. As stated before, there was no sign of a second cycle of infection in this system. These results show that a 30-hour incubation period was sufficiently long enough to detect all of the fluorescent cells with great ease.

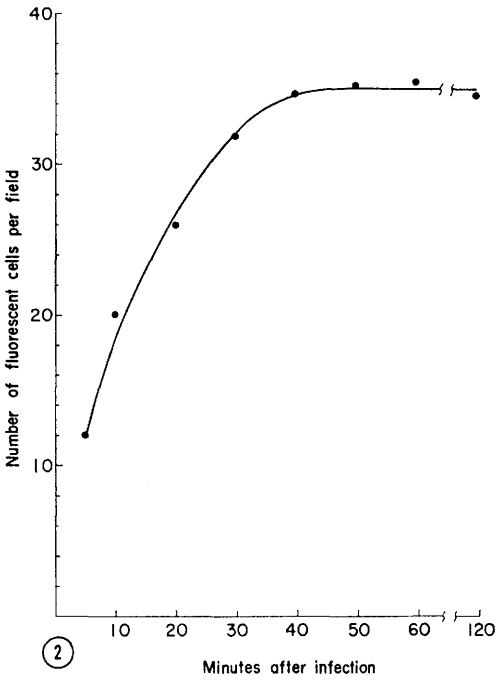
On the other hand, the appearance of hemadsorbed cells was about 2 hours behind that of fluorescent cells and the hemadsorbed cells gradually increased in number up to 30 hours, following the curve of fluorescent cells. By 30 hours, the number of hemadsorbed cells reached the maximum and the same number of infected cells was obtained in both methods.

Adsorption of Sendai virus onto L-cell monolayers. The rate of adsorption of Sendai virus onto L cells was determined previously(5). The time required for 50% adsorption was about 15 minutes and the adsorption was completed by 60 minutes. Since there was no available plaque counting method for Sendai virus at that time, rate of adsorption was calculated on the basis of hemagglutinin yields at the end of the one cycle growth assuming that each infectious center formed after an appropriate period of adsorption produced equal amounts of hemagglutinins. With the visualization of infectious centers by fluorescent cell-counting reported here, it was also possible to determine the rate of adsorption precisely.

A number of coverslips were infected with 0.05 ml of 10^{-4} dilution of Sendai virus stock and kept at 37°C. At the indicated times, they were washed, re-fed with fresh MS and incubation continued. After 30 hours the coverslips were stained with fluorescent antibody and the mean number of fluorescent cells per field was calculated. The results are shown in Fig. 2. As seen in the Figure, about 50% adsorption occurred in 10 minutes, and maximum adsorption was obtained by 40 minutes. The initial rate of adsorption in this system was about 1.2×10^{-8} cm^3 min^{-1}. In the following experiments, an adsorption period of 60 minutes was used.

Linear relationship between concentration of inoculating virus and infected cells. The relationship between concentration of stock virus and the number of infected cells was determined by enumeration of the fluorescent cells and hemadsorbed cells. Coverslips were inoculated with Sendai virus, diluted serially in 2-fold manner in Hanks' solution, and the adsorption was carried out at 37°C for 60 minutes. After 30 hours of incubation, half the coverslips were stained with fluorescent

antibody and the other half were tested for hemadsorption. The number of infected cells was counted in both cases. The results are shown in Fig. 3. In each case, a linear relationship between virus concentration and the number of infected cells was observed. These results show that each infected cell, determined either by the fluorescent cell- or hemadsorbed cell-counting techniques, can be formed by a single infectious unit.



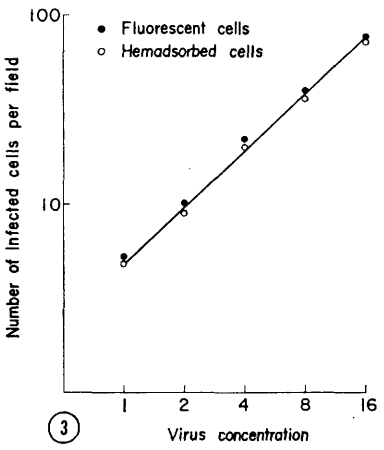


FIG. 2. Adsorption of Sendai virus to cells.
FIG. 3. Relationship of infected cells to virus concentration.

TABLE II. Reproducibility of Fluorescent Cell-Counting Technique for Determination of Sendai Virus.

Exp No.	Mean No. of fluorescent cells/field Dilution of virus ($\times 10^{-4.4}$)				
	1:1	1:2	1:4	1:8	1:16
1	80	40	21	12	6
2	79	39	23	10	5
3	75	42	23	9	5
Mean	78	40	22	10	5

Reproducibility of fluorescent cell-counting technique. It was necessary to examine the reproducibility of the fluorescent cell-counting technique in this system. Three experiments were performed with the same virus stock. The coverslips were inoculated with Sendai virus diluted in 2-fold manner ranging from 2.5×10^{-4} to 1.56×10^{-5}. After 30 hours of incubation, they were washed, fixed, stained with fluorescent antibody, and the fluorescent cells were counted. The mean number of fluorescent cells was calculated in each dilution. Two more such experiments were performed on the same day but at different times. These results are summarized in Table II. With the same dilution, the number of fluorescent cells in these 3 experiments was very close to each other, and this assay technique was shown to be highly reproducible. Moreover, here again, a linear relationship was obtained between concentration of Sendai virus in the inoculum and number of fluorescent cells.

Comparison of fluorescent cell-counting technique and 50% end point titration method. Determination of infective Sendai virus in the allantoic fluid seed was made both by the fluorescent cell-counting and the 50% end point titration in eggs, both results were compared and Table III shows the results. Five independent experiments were conducted with different virus stocks. Although slightly lower titer was obtained when measured by the fluorescent cell-counting technique than EID_{50}, if one assumes that one EID_{50} is equivalent to 0.69 EID_{100}, the difference should be negligible.

Discussion. The fluorescent cell-counting technique was successfully applied to the Sendai virus-L cell system, in which only one step growth is permitted. The superiority of

TABLE III. Comparison of the 2 Assay Procedures for Determination of Infective Sendai Virus.

Sendai virus stock	Infectivity titer of stock virus determined by	
	EID_{50}/ml (log_{10})	CIU*/ml (log_{10})
X†E- 95	9.94	9.91
X E- 96	9.69	9.54
X E- 97	9.44	9.50
X E- 98	10.19	9.89
X E-100	9.94	9.94

* CIU—Cell infecting unit measured by fluorescent cell-counting procedure.
† X = $M_{12}R_1M_3$.

the fluorescent cell-counting technique for determination of infective Sendai virus in the inoculum was shown not only with respect to sensitivity but also in reproducibility. Using this technique, it was possible to determine precisely the rate of adsorption of Sendai virus onto L cells, and it was shown that the adsorption was completed by 40 minutes.

The hemadsorbed cell-counting technique gave concordant results with the fluorescent cell-counting technique with regard to the enumeration of the infected cells. This is also a convenient technique in its simplicity.

Although the hemadsorbed cell-counting technique detected the infected cells 2 hours later when compared with the fluorescent cell-counting technique, the same number of infected cells could be counted finally in either case. The linearity between concentration of Sendai virus in the inoculum and the infected cells, determined by both the fluorescent cell- and hemadsorbed cell-counting technique, shows that a single infectious unit can produce one infectious center. With the fact in mind that the only infective virus in the inoculum can produce the fluorescent cells and hemadsorbed cells, these results lead to a conclusion that both techniques detect the same infected cells by different methods.

As far as the counting of Sendai virus particles is concerned, we already have proposed the antigenic particle counting by means of immunofluorescence(13). As for the infectious particle counting, the fluorescent cell counting technique has been added to the plaque assay procedure established recently(14).

Summary. The fluorescent cell-counting technique was successfully extended to the Sendai virus and L cell system. Using this technique, the rate of adsorption of Sendai virus onto L cells was determined precisely. The hemadsorbed cell-counting technique was also devised in this system and it compared favorably with the fluorescent cell-counting technique. Because of its simplicity it is considered as a useful technique for the determination of infective virus. Evidence that a single infectious unit of this virus is sufficient to produce an infected cell, determined either by the fluorescent cell- or hemadsorbed cell-counting technique, was obtained. The fluorescent cell-counting technique was as sensitive as ordinary 50% end point titration method in eggs and its superiority was shown by its reproducibility.

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