ENUMERATION OF CELL-INFECTING PARTICLES OF NEWCASTLE DISEASE VIRUS BY THE FLUORESCENT ANTIBODY TECHNIQUE*

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The present communication reports a new procedure for measurement of infective virus, based on the counting of individual infected cells which are identified by staining of viral antigen with fluorescent antibody. In this procedure, complete monolayers of susceptible cells are used and the very cells which become primarily infected by the inoculated virus are visualized and counted.

In recent years the plaque technique has been widely adopted for the measurement of infective virus (1, 2). However, this technique, and certain modifications thereof (3, 4), are not applicable to virus-cell systems in which the spread of virus from cells infected initially to neighboring cells does not readily occur.

Some work has been reported endeavoring to base infectivity titrations on recognizable changes in single cells without requiring successive cycles of cell infection. Deibel and Hotchin (5) determined the proportion of influenza virus-infected cells in a smear of a cell suspension by means of the fluorescent antibody technique, and were thus able to calculate the infectivity titer of the virus seed. Marcus and Puck (6) used a quantitative plating technique for mammalian cells to measure Newcastle disease virus (NDV). Their virus assay procedure is based on the fact that NDV-infected HeLa cells are unable to produce macroscopic colonies.

The new quantitative procedure for measurement of infective NDV in HeLa cells is characterized by two features: the time required for assay of infective virus is short, and the experimental arrangement used is suitable for both virus assay and conduct of experiments involving observations on cells.

In this communication, the linear relationship between the concentration of virus in the inoculum and the number of fluorescent cells in the first cycle of multiplication is described. The precision and sensitivity of the procedure are also described. Results of studies on microepidemiology of NDV in HeLa cells are reported, and brought to bear on the counting procedure. The significant advantages of the new procedure are discussed.

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In a communication which follows (7), studies of the effects of multiplicity of infection on NDV-HeLa cell interaction are described. In these studies use was made of the experimental procedures described in the present report.

Materials and Methods

The experimental arrangement consisted of complete monolayer cultures of HeLa cells grown on coverslips and incubated either in Leighton tubes or in Petri dishes. The coverslip cultures were used both in virus assays and in experiments on various aspects of NDV-HeLa cell interaction.

Growth Medium for Cell Cultures.—HeLa cells were grown in Eagle's basal medium (8) supplemented with 4 per cent tryptose phosphate broth. The serum component of the medium consisted of 15 per cent human serum. The concentration of $NaHCO_3$ was 1.75 gm per liter. This medium was also used for incubation of infected cultures except during the adsorption period.

Adsorption Medium for Virus.—Virus dilutions for inoculation of coverslip cultures were made in Eagle's basal medium without serum and tryptose phosphate broth, and the cultures were held in an atmosphere of 5 per cent CO_2 in air.

Wash Medium for Cell Cultures.—Cultures were washed with phosphate buffered saline (PBS) (1).

Cell Cultures.—An uncloned line of HeLa cells was used. HeLa cells were grown in 32 ounce prescription bottles until nearly complete monolayers had formed. They were then dispersed with 0.04 per cent trypsin in a buffered salts and glucose solution lacking Ca^{++} and Mg^{++} (9), and suspended in growth medium. The yield was determined by counting cells in a hemocytometer. Leighton tubes containing 11 × 38 mm. coverslips were planted with 2×10^5 cells in 1 ml. of growth medium per tube. The tubes were gassed with 5 per cent CO_2, stoppered, and incubated in a horizontal position at 37°C. for 24 hours. Each tube then received an additional 2 ml. volume of growth medium. On the 2nd day after planting the monolayers on the coverslips were complete and each was composed of approximately 400,000 cells.

Virus.—The Hickman strain of Newcastle disease virus (NDV) was used. It had had many passages in the allantoic sac of embryonated chicken eggs. A seed virus pool was prepared by allantoic inoculation of 10-day-old chick embryos with 4×10^3 EID_{50}[1] of NDV. After incubation for 38 hours at 35°C., the infected eggs were chilled at −26°C. The allantoic fluids were collected, clarified by centrifugation, and immediately frozen and stored at −55°C. The same seed of NDV was used in all experiments. It contained 2.6×10^9 EID_{50} per ml.

Immune Serum.—Anti-NDV immune rabbit serum was prepared by two intravenous injections, 2 weeks apart, of 10 ml. of infected allantoic fluid per injection. Serum was collected 28 days after the first injection, and it had a hemagglutination-inhibition titer of 1:2048 with 8 hemagglutinating units of NDV.

Anti-NDV immune guinea pig serum was prepared in the following manner: guinea pigs were hyperimmunized with an intranasal inoculation of NDV-infected allantoic fluid followed, in the 2nd week, by one intraperitoneal injection of a saline extract of NDV-infected chorioallantoic membrane which was repeated in the 3rd week. In the 4th week an intraperitoneal injection of infected allantoic fluid was given. Serum was collected 1 week later, and it had a hemagglutination-inhibition titer of 1:25,000 with 8 hemagglutinating units of NDV.

Virus Experiments with HeLa Cell Monolayers on Coverslips.—Coverslips with complete monolayers of HeLa cells were removed from Leighton tubes, washed by gentle immersion in 6 tubes containing warm PBS, and placed in flat-bottomed Petri dishes. Virus diluted in protein-free Eagle's medium was inoculated onto the monolayers, 0.1 ml. per coverslip cul-

[1] EID_{50} signifies 50 per cent egg infective doses.

ture, and the cultures incubated at 37°C. for 2 hours except where stated otherwise. The coverslips were then washed by immersion in 6 changes of PBS, returned to the original tubes containing growth medium, and incubation at 37°C. continued. Control cultures received no virus, but in other respects were handled in a similar manner.

Fixation and Fluorescent Antibody Staining of Coverslip Cultures.—After incubation for appropriate periods, coverslip cultures were removed from Leighton tubes, washed in PBS, fixed in acetone, and permitted to dry. The indirect fluorescent antibody staining technique (10) was used. The dry cover-slip cultures were washed in PBS for 15 minutes, and a drop of undiluted anti-NDV immune rabbit serum was added to each. After 30 minutes in a humidified chamber, the coverslip cultures were washed for 30 minutes in 3 changes of PBS, and a drop of undiluted sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate was added to each culture. Thirty minutes later the coverslips were washed in 3 changes of PBS and mounted in 20 per cent glycerol in PBS. The preparations were examined and photographed with a Zeiss ultraphot II photomicroscope using an Osram 200 watt high pressure mercury burner, a BG 12 exciter filter, and an OG 4 barrier filter. For photography superanscochrome 120 daylight film was used. The colored positives were used to make black and white negatives from which prints were made.

Counting of Cells Containing Virus Antigen.—The number of specifically fluorescent HeLa cells per coverslip culture was determined as follows. A microscopic field, which was formed when 8× oculars, 1.6× optovar, and a 40× oil immersion objective were used, was selected as the unit area for counting. The area of the coverslip was equivalent to the area of 9000 microscopic fields. Each field contained 40 to 50 HeLa cells. Fifty fields were usually counted per coverslip. The mean number of fluorescent cells per field was multiplied by 9000 to give the number of fluorescent cells per coverslip. It was necessary always to use complete monolayers of cells since the number of infected cells was determined per unit area of the coverslip culture. However, the number of cells per coverslip was not important per se.

Infectivity Titrations by the Plaque Technique.—Monolayer cultures of chick embryo cells in Petri dishes (11) were used for the plaque assay of NDV. Plaques were counted at the end of a 3 day period of incubation.

Infectivity Titrations in Embryonated Eggs.—Serial half-log-fold dilutions of virus suspensions were prepared in 0.85 per cent NaCl buffered at pH 7.2 with 0.01 M phosphate, and 10-day-old embryonated chicken eggs were inoculated allantoically in groups of 4 with each dilution. All titrations were done in triplicate. After a 72 hour period of incubation at 35°C., the presence of virus in the allantoic fluids was determined by the hemagglutination procedure, and the 50 per cent infectivity end-point was calculated (12).

EXPERIMENTAL

Determination of Virus Concentration by the Procedure of Counting Fluorescent Cells.—A procedure for determination of virus concentration was devised based on enumeration of the cells infected by virus particles introduced in the inoculum. This procedure measures virus particles in terms of cell-infecting units (CIU).

An important aspect of this procedure is the time interval between virus inoculation and collection of infected coverslip cultures for fluorescent antibody staining and cell counting. Sufficient time must elapse after infection of cells with inoculated virus to permit accumulation of amounts of virus antigen that are detectable by the fluorescent antibody staining procedure. On the other hand, it is essential to terminate incubation before cells which become

infected by new virus produced in the culture have developed detectable amounts of antigen. Preliminary experiments showed that an interval of 10 hours satisfied both requirements.

Coverslip cultures of HeLa cells were inoculated with a 1.25×10^{-4} dilution of NDV seed. The amount of virus inoculated was sufficient to infect approximately 7 per cent of cells in the monolayer. After a 2-hour adsorption period the coverslips were washed with PBS and returned to growth medium. Eight hours later they were collected, fixed, and stained with fluorescent antibody.

As can be seen in Figs. 1 and 2, infected cells could be readily visualized after a 10-hour period of incubation. Furthermore, all of the specifically fluorescent cells appeared to have been infected by virus in the inoculum, and are therefore designated primarily infected cells. Criteria are described below on the basis of which cells infected by new virus produced in the culture, *i.e.* secondarily infected cells, can be readily recognized, and distinguished from those primarily infected.

To learn whether at 10 hours all of the fluorescent cells had been primarily infected, experiments with anti-NDV immune serum were carried out. As is shown below, anti-NDV immune serum prevents cell to cell spread of NDV.

HeLa cells were inoculated with a 1.25×10^{-4} dilution of NDV seed. Two hours later the cultures were washed and then re-incubated for varying periods of time in the presence or absence of anti-NDV immune guinea pig serum.

The number of fluorescent cells at 10 or 11 hours in the absence of antiserum was closely similar to that in antibody-treated cultures at 11 or 15 hours. However, at 15 hours, the number of fluorescent cells in cultures which had not received antibody had increased to a value 5 times greater than the number at 10 or 11 hours in such cultures or that at 11 or 15 hours in treated cultures.

These results show that at 10 hours, all the fluorescent cells in cultures incubated in the absence of antibody had been primarily infected. They also show that 10 hours was a sufficiently long period to permit all primarily infected cells to develop readily detectable amounts of antigen.

It is clear from the results described above and in a later section that use of specific immune serum to prevent secondary infection obviates the necessity of using carefully selected short incubation periods. However, use of antiserum introduces an additional step into the procedure.

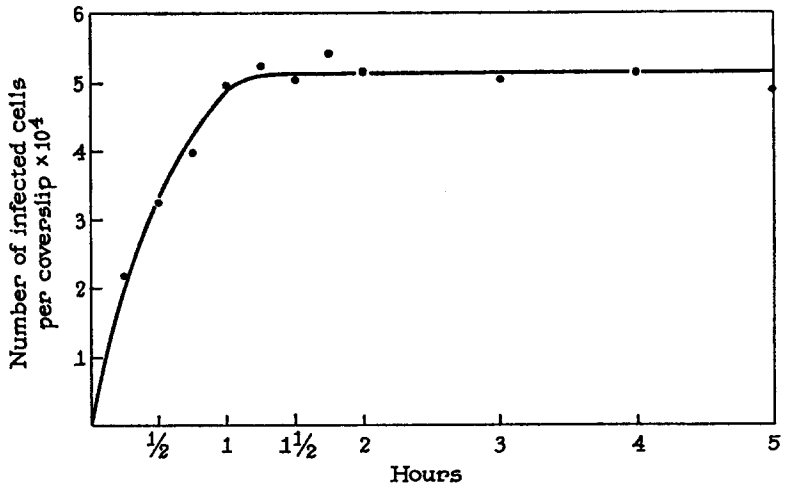
In the determinations reported in the present communication anti-NDV immune serum was not used, but in studies described in the report which follows (7) it was used.

Adsorption of NDV to HeLa Cells.—The rate of adsorption of NDV to HeLa cells was determined.

Coverslip cultures of HeLa cells were inoculated with 0.1 ml of a 2.5×10^{-4} dilution of NDV seed and incubated at 37°C. The inoculum contained sufficient virus to infect ap-

proximately 14 per cent of cells in the monolayer. At appropriate intervals, two coverslips were removed, washed in PBS, and placed in growth medium. Ten hours after inoculation, all coverslips were collected and stained and the specifically fluorescent cells were counted.

As can be seen in Text-fig. 1 adsorption was nearly completed during the 1st hour after virus inoculation. Similar rates of virus adsorption have been reported for herpes simplex virus and rabbit kidney (13), HeLa (14) or human amnion cells (15), and for mouse encephalomyelitis virus and Earle's L cells (16). The rate of adsorption of NDV virus to cultured cells of chick embryo lung epithelium was faster (17), and of vaccinia virus to chick embryo fibroblasts was slower (18).



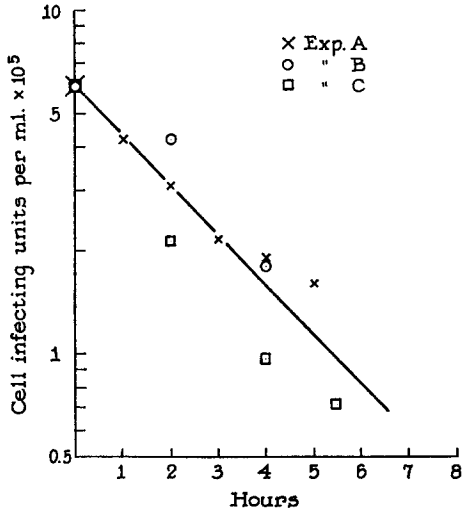
TEXT-FIG. 1. Adsorption of NDV to HeLa cell monolayers. The amount of virus inoculated was sufficient to infect 14 per cent of cells after maximum adsorption.

The experiments summarized in Text-fig. 1 reveal only the fraction of infective virus introduced in the inoculum which succeeded in adsorbing to and multiplying in cells of the monolayer. It was therefore of interest to determine how much virus remained in the supernatant after the adsorption process had reached an apparent equilibrium. This was investigated in experiments in which the supernatant from the first set of coverslip cultures was collected and transferred to a fresh set of cultures. The number of infective units which adsorbed to each of the two sets of cultures was related to the sum of both of them.

Coverslip cultures of HeLa cells were inoculated with a 2.5×10^{-4} dilution of NDV seed, 0.1 ml. per culture. The amount of virus inoculated was sufficient to infect approximately 14 per cent of the cells. At the end of a 2 hour adsorption period, 0.1 ml. of medium was added to each culture. After mixing the medium on coverslips, 0.1 ml. was removed from each and

placed on fresh coverslip cultures. The first set of coverslips was washed in PBS and then returned to growth medium for further incubation at 37°C. Two hours later, the second set of coverslips was washed in PBS and also placed in growth medium. Each set of coverslips was incubated for a total of 10 hours, including the 2 hour adsorption period. After incubation the cultures were fixed and stained and the number of infected cells was determined by counting.

It was found that of all cell-infecting units that were adsorbed in the two-step experiment 88 per cent adsorbed in the first step and 12 per cent in the second. Thus, in determinations of virus concentration by the usual one-step



TEXT-FIG. 2. Thermal inactivation of NDV in protein-free Eagle's medium at 37°C. NDV seed was used at a dilution of 1:4000.

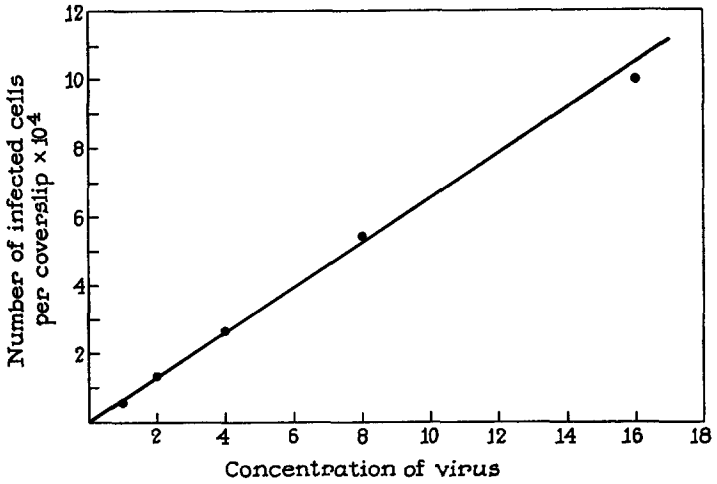
procedure, a small proportion of the virus inoculated remains unmeasured because of failure to adsorb. Similar results have been reported previously with vaccinia virus in chick embryo fibroblast cultures (18). This source of error may be minor compared to the underestimation of the number of infective units in the inoculum which probably results from thermal inactivation of a proportion of the virus particles inoculated. As is shown below, in the absence of protein in the medium, the half-life of NDV at high dilutions is about 2.1 hours at 37°C. Because of the rapidity with which most of the inoculated virus adsorbs to cells, it is unlikely that thermal inactivation leads to greater than 25 to 50 per cent underestimation of the number of infective units in the inoculum.

Since both sources of error are systematic in nature, they affect the absolute but not the relative validity of determinations of infective units.

Thermal Inactivation of NDV.—The rate of thermal inactivation of the infective property of highly diluted NDV was determined at 37°C. in Eagle's medium without protein.

NDV seed suspension was diluted 2.5 × 10^{-4} in protein-free Eagle's medium, and aliquots of the diluted material were distributed into separate screw cap culture tubes. The tubes were gassed with 5 per cent CO_2 in air and incubated at 37°C. At hourly intervals one tube was removed. The concentration of infective virus was determined by the fluorescent cell-counting procedure.

The results of three experiments are summarized in Text-fig. 2. As can be seen the half-life of NDV was 2.1 hours. Some variation in the stability of



TEXT-FIG.3. Linear relationship between concentration of NDV and number of fluorescent cells. The points are based on data summarized in Table I.

NDV was noted in separate experiments; the half-lives based on results of individual experiments were 1.6, 2.25, and 2.6 hours, respectively.

In allantoic fluid the infective half-life of the same strain of NDV was greater than 14 hours (19). It is possible that protein present in allantoic fluid exerted a protective effect. Furthermore, Horsfall's thermal inactivation experiments were performed with a more concentrated suspension of NDV than used in the present studies.

Relationship between Concentration of NDV and Number of Fluorescent Cells.—The relationship between concentration of NDV in the inoculum and the number of infected cells in the first cycle of multiplication was determined by the fluorescent cell-counting procedure.

Twofold dilutions of virus starting with NDV seed diluted 5 × 10^{-4} were prepared and inoculated onto coverslip cultures. One to three coverslips were used per dilution. After a

two hour adsorption period at 37°C., the coverslips were washed in PBS, placed in growth medium and incubation was continued for 8 more hours. The cultures were then collected, fixed, and stained, and the specifically fluorescent cells were counted. Six such experiments were performed on different days.

As can be seen in Text-fig. 3 there was a linear relationship between virus concentration and number of infected cells. The linear relationship held over the entire range examined; *i.e.*, from 0.66 to 11 infected cells per microscopic field, corresponding to a dilution range of 1.2 log units. This relationship indicates that each fluorescent cell was infected by a virus unit not further subdivisible at high dilution (1).

TABLE I

Reproducibility of Determinations of Cell-Infecting Units (CIU) of NDV Virus

Experiment	No. of fluorescent cells per coverslip culture × 10^3					No. of CIU per ml. of virus seed × 10^9
	Dilution of virus in the inoculum*					
	3×10^{-5}	6×10^{-5}	1.2×10^{-4}	2.5×10^{-4}	5×10^{-4}	
A	4.5	10	21	48	103	1.96
B	6.9	15	23	52	107	2.04
C		14	28	59	92	2.08
D	7.2	15	32			2.60
E	4.8	12	26	57		2.24
F		15	28	55		2.20
Mean....	5.9	13.5	26.3	54.2	100	2.19

* Cultures were inoculated with 0.1 ml.

The data from the six experiments, on which the curve shown in Text-fig. 3 is based, are summarized in Table I. As can be seen the determinations of NDV concentration by the fluorescent cell counting procedure in experiments done on different days were highly reproducible at all concentration levels employed.

Precision of Fluorescent Cell Counts.—To obtain an estimate of the precision of fluorescent cell counts in a single experiment, a series of replicate determinations was performed on 1 day.

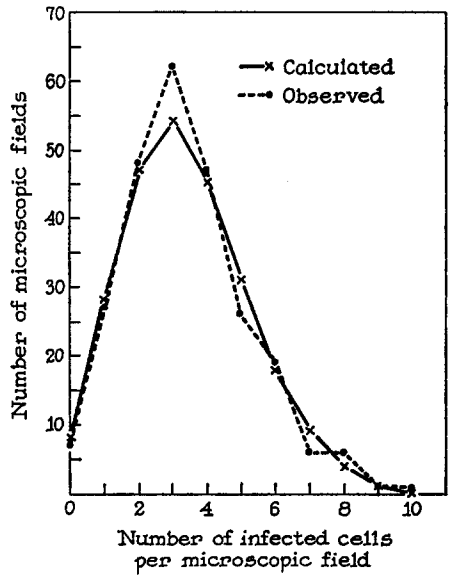
Thirty-five coverslip cultures containing complete monolayers of HeLa cells were infected with a 1.25×10^{-4} dilution of NDV seed. The inoculum contained sufficient virus to infect approximately 7 per cent of cells in the monolayer. After a 2 hour adsorption period at 37°C. the cultures were washed in PBS, placed in growth medium, and incubated for an additional period of 8 hours. They were then collected and stained. In each determination a separate coverslip culture was used, and the fluorescent cells in 50 microscopic fields were counted.

The mean number of fluorescent cells in 50 fields per coverslip was 170 with a range from 131 to 199. The standard deviation was 14.2. Expressed as

a percentage of the mean, the standard deviation was 8.2. This compares favorably with the precision of plaque assay procedures (1, 15, 18). Ninety-five per cent of the determinations were within 1.96 standard deviations on either side of the mean, indicating a normal distribution.

Distribution of Infected Cells in a Monolayer Culture.—The mode of distribution of single infected HeLa cells in a monolayer was determined.

A coverslip culture composed of a complete monolayer of HeLa cells was inoculated with a 1.25×10^{-4} dilution of NDV seed. The amount of virus inoculated was sufficient to infect



TEXT-FIG. 4. Distribution of NDV-infected HeLa cells in a coverslip culture. The amount of virus inoculated was sufficient to infect 7 per cent of cells.

approximately 7 per cent of cells in the monolayer. Under these conditions it would be expected that of the fluorescent cells 95 per cent were singly infected. After incubation at 37°C. for 2 hours the cultures were washed in PBS, placed in growth medium, and incubated for an additional period of 8 hours. The cultures were then collected and stained, and the number of microscopic fields containing specifically fluorescent cells in various numbers were recorded. Two hundred and fifty fields were counted and the results compared with the expected numbers as calculated for a theoretical Poisson distribution of infective virus particles according to the expression $\frac{e^{-m} m^x}{x!}$.

As can be seen in Text-fig. 4 the observed frequencies corresponded closely to the calculated frequencies. In addition, the X^2 test of goodness of fit of the experimental data to the theoretical Poisson distribution was calculated, and it gave a probability of 0.73. The distribution of singly infected cells in the

coverslip cultures, therefore, corresponds to the calculated Poisson distribution for independently distributed particles.

Using the plaque technique, similar results have been obtained with herpes simplex virus and HeLa cells (14), and vaccinia virus and chick fibroblasts (18).

Determination of Concentration of Infective NDV by Three Assay Procedures.—Using the infected allantoic fluid seed of NDV as test virus, the sensitivity of the fluorescent cell-counting procedure was compared to that of the plaque titration procedure in chick embryo cells in Petri dish cultures, and also to the sensitivity of the 50 per cent end-point titration procedure in embryonated chicken eggs.

TABLE II

Determination of Concentration of Infective NDV by Three Assay Procedures

Host system and procedure	Virus content per ml. of seed preparation
HeLa cell monolayer; enumeration of infected cells stained with fluorescent antibody	2.2×10^9 CIU
Chick embryo fibroblast monolayer; enumeration of plaques	1.8×10^9 PFU
Allantoic cell layer of embryonated eggs; 50 per cent infectivity end-point titration; result converted to egg infective units	1.8×10^9 EIU

The techniques used have been described above. For purposes of direct comparison of the titer obtained in embryonated eggs with the number of infective units determined by the other procedures, one EID_{50} was considered equivalent to 0.69 egg infective doses (EID_{100}).

As can be seen in Table II the three techniques used to measure the concentration of infective NDV in the allantoic fluid seed showed no significant differences in sensitivity. Thus, the fluorescent cell-counting procedure is as sensitive as the techniques used previously. As was pointed out above, the precision of the fluorescent cell-counting procedure compares favorably to that of the plaque assay. The precision of both procedures is much greater than that of the 50 per cent end-point titration procedure.

Microepidemiology of NDV in HeLa Cells.—During the development of the fluorescent cell-counting procedure for determination of cell-infecting units of NDV, it became necessary to distinguish between cells infected with inoculated virus and those infected with new virus produced in the culture. Also, the question arose whether specific immune serum would prevent secondary infection. Therefore, a study of the microepidemiology of NDV in HeLa cells was undertaken.

Coverslip cultures were inoculated with a 3×10^{-6} dilution of NDV seed. The amount of virus inoculated was sufficient to infect approximately 0.17 per cent of cells in the monolayer.

After a 2 hour adsorption period the coverslips were divided into three groups: A. Washed 6 times in PBS and returned to tubes containing growth medium; B. Washed 6 times in PBS and then treated with undiluted anti-NDV immune guinea pig serum for 15 minutes, washed ten times in PBS, and returned to growth medium; C. Washed six times in PBS and returned to growth medium containing 20 per cent anti-NDV immune serum. The tubes with coverslips were then placed in a stationary position in a 37°C. incubator, and coverslips from each group removed at 10, 15, and 24 hours after virus inoculation, fixed in acetone, and stained with fluorescent antibody.

The results are shown in Figs. 3 to 8.

In group A at 10 hours single cells containing moderate amounts of antigen were present surrounded by a large number of uninfected cells (Fig. 3). At 15 hours cells containing small quantities of viral antigen were seen next to and surrounding single cells containing large amounts of antigen (Fig. 4). At 24 hours large clusters of infected cells were present (Fig. 5). Most of the infected cells contained large amounts of NDV antigen. These findings strongly suggest that the fluorescent cells seen at 10 hours were infected with virus in the inoculum, and that the satellite cells seen at 15 hours were infected with new virus produced by cells infected initially.

Group B gave the same result as group A, and therefore photomicrographs of group B are not shown. Group B eliminates the possibility that cells in group A which first displayed antigen at 15 hours or later, were infected by inoculated virus which may have adsorbed but not penetrated during the 2 hour adsorption period.

Group C establishes the mechanism of viral dissemination. At 10 hours following inoculation the picture (Fig. 6) was identical with that shown by both A and B. At 15 and subsequent hours, however, there was no increase in the numbers of infected cells in group C (Figs. 7 and 8), indicating that antibody had neutralized all virus released from primarily infected cells. The failure of viral spread from infected cells to contiguous cells in the presence of antibody clearly establishes an extracellular stage in the microepidemiology of NDV in HeLa cells.

DISCUSSION

The quantitative procedure described above for determination of infective units of Newcastle disease virus is unique in that the very cells infected by the virus in the inoculum are visualized and counted. The concentration of virus in the inoculum can thus be calculated and expressed in terms of cell-infecting units (CIU). It should be emphasized that the term CIU refers strictly to the ability of virus particles to interact with cells and to initiate formation of virus antigen which can then be readily visualized by staining with fluorescent antibody. Production of infective virus particles in cells is not a necessary requirement for the CIU, nor is the presence of such progeny essential for successful assay by the fluorescent cell-counting procedure.

The ability to recognize and count individual cells infected by the virus in the inoculum provides certain advantages in the determination of virus concentration which are lacking in the widely used plaque technique. In the plaque technique cells initially infected produce new virus which infects contiguous cells in the monolayer. To prevent generalized spread of virus throughout the culture, an overlay of agar is usually employed. The plaques consist of discrete loci of degenerated cells. The plaque technique has provided an effective means for the determination of virus concentration in numerous virus-cell systems where considerable numbers of infective progeny per cell are produced, with ready transmission from cell to cell, followed by degenerative changes in infected cells. However, there are many viruses which lack one or more of the necessary attributes, and therefore fail to produce plaques on monolayers of host cells. It is understood, of course, that in the plaque technique the cell is an important variable, and that there may be great differences among different cell types in their response to a given virus.

The fluorescent cell-counting procedure appears to be suitable for the enumeration not only of viruses which are capable of forming plaques, but also for those which in a particular cell system fail to give rise to high yields of infective virus, are poorly transmitted from cell to cell, or do not cause marked changes in host cells. The fluorescent cell counting procedure would not, however, be applicable in those virus-cell systems in which fluorescent staining characteristics of newly made virus antigen do not permit ready identification of infected cells.

It is apparent that the fluorescent cell-counting procedure allows considerable freedom in the choice of the cell type to be used in virus measurements. With many virus-host combinations infection may not proceed beyond the synthesis of virus precursor materials (20), or may lead to production of particles lacking infectivity (21). A continuous passage cell line with highly desirable growth and maintenance characteristics may be effectively used to measure infective virus by the fluorescent cell-counting procedure in spite of the fact that only an abortive infection may take place.

It should be emphasized that results of determinations of infective NDV by the present procedure were in excellent agreement with infectivity titrations in chicken embryos, and with plaque counts on monolayer cultures of chick embryo fibroblasts. Furthermore, a linear relationship between concentration of NDV in the inoculum and the number of fluorescent cells was demonstrated. The precision of the fluorescent cell-counting procedure was equal to or higher than that of plaque procedures, and the reproducibility on different days with different batches of HeLa cell cultures was excellent. It should be emphasized that an incubation period of 10 hours was sufficient, whereas plaque assays require anywhere from a few to several days.

Thus, the fluorescent cell-counting procedure is a sensitive, precise, and rapid

technique for virus enumeration. Furthermore, it can be used in situations in which other existing techniques for virus enumeration cannot be applied.

In studies described in the communication which follows, the new experimental and assay procedures were applied to an investigation of the effects of multiplicity of infection on NDV-HeLa cell interaction with special emphasis on mitosis and its inhibition in infected cells.

In the course of the present study it became desirable to prevent, if possible, the dissemination of NDV produced in cells infected with virus in the inoculum. The finding that specific NDV immune serum completely prevented the dissemination of virus in the culture is of interest because it indicates that NDV does not spread directly from cell to cell through intercellular processes, but through the extracellular medium. In this regard it is similar to poliovirus (22) which also has an extracellular stage in its micro-epidemic cycle. In contrast, herpes simplex (14, 23), herpes B (22), varicella (24), herpes zoster (24), and measles (25) viruses spread from cell to cell without release into the extracellular milieu.

SUMMARY

A procedure has been developed for the determination of the concentration of infective Newcastle disease virus (NDV) based on the enumeration of singly infected and distributed HeLa cells which are visualized by staining with fluorescent antibody. Infective virus assayed by the fluorescent cell-counting procedure is expressed in terms of cell-infecting units (CIU).

Adsorption of NDV to HeLa cell monolayers reached a plateau 1 to 1.5 hours after inoculation of coverslip cultures, and 12 per cent of the infective particles inoculated failed to adsorb. The half-life of NDV in protein-free Eagle's medium at 37°C. was 2.1 hours. There was a linear relationship between virus concentration and the number of infected cells. The coefficient of variation of the mean of replicate determinations of infective NDV was 8.2 per cent. The distribution of single infected HeLa cells in the monolayer corresponded to the Poisson distribution. With NDV the cell-infecting unit (CIU) determined in HeLa cells is equivalent to the plaque-forming unit in chick embryo cells and the egg infective dose.

In experiments on the mechanism of dissemination of NDV in monolayer cultures of HeLa cells, NDV was found to spread from cell to cell through the extracellular milieu.

BIBLIOGRAPHY

1. Dulbecco, R., and Vogt, M., Plaque formation and isolation of pure lines with poliomyelitis viruses, *J. Exp. Med.*, 1954, **99**, 167.
2. Schwerdt, C. E., Quantitative relationships between virus particles and their functional activity, *in* The Viruses; Biochemical, Biological and Biophysical

Properties, (F. M. Burnet and W. M. Stanley, editors), New York, Academic Press, Inc., 1959, **1,** 329–358.

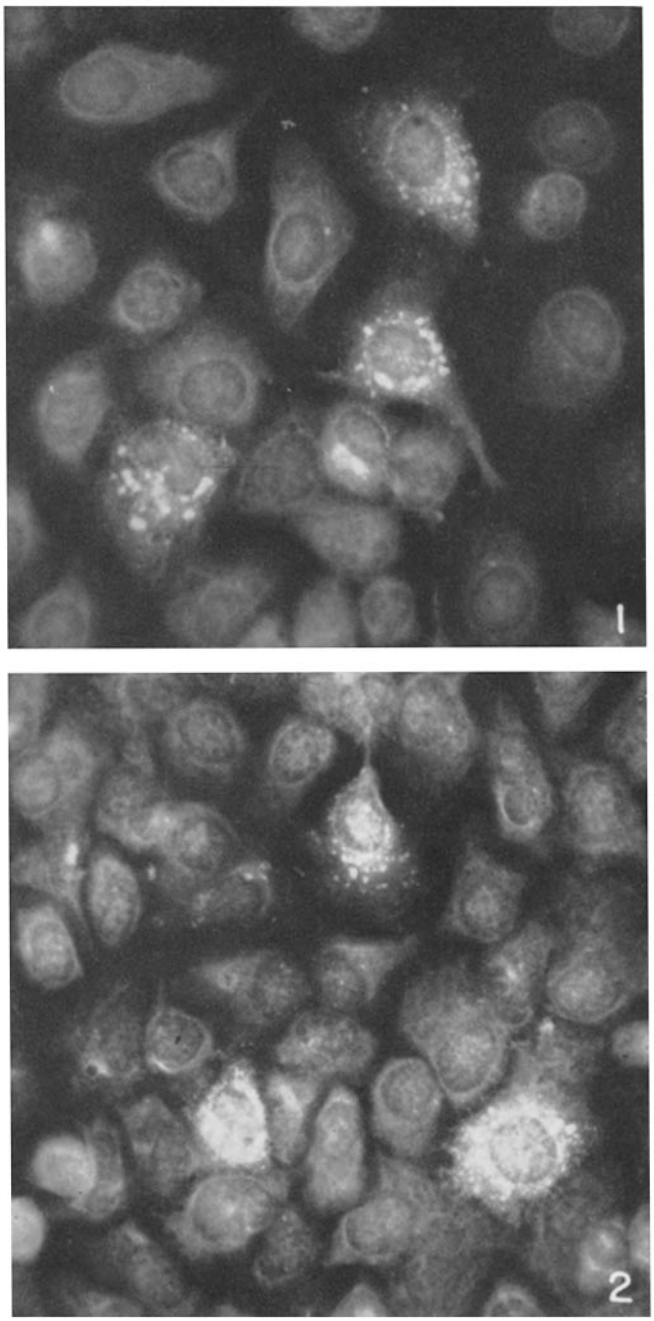
3. Rapp, F., Seligman, S. J., Jaross, L. B., and Gordon, I., Quantitative determination of infectious units of measles virus by counts of immunofluorescent foci, *Proc. Soc. Exp. Biol. and Med.*, 1959, **101,** 289.
4. Hotchin, J. E., Deibel, R., and Benson, L. M., Location of noncytopathic myxovirus plaques by hemadsorption, *Virology*, 1960, **10,** 275.
5. Deibel, R., and Hotchin, J. E., Quantitative applications of fluorescent antibody technique to influenza-virus-infected cell cultures, *Virology*, 1959, **8,** 367.
6. Marcus, P. I., and Puck, T. T., Host-cell interaction of animal viruses. I. Titration of cell-killing by viruses, *Virology*, 1958, **6,** 405.
7. Wheelock, E. F., and Tamm, I., Effect of multiplicity of infection on Newcastle disease virus-HeLa cell interaction, *J. Exp. Med.*, 1961, **113,** 317.
8. Eagle, H., The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture, *J. Exp. Med.*, 1955, **102,** 37.
9. Puck, T. T., Cieciura, S. J., and Fisher, H. W., Clonal growth *in vitro* of human cells with fibroblastic morphology. Comparison of growth and genetic characteristics of single epithelioid and fibroblast-like cells from a variety of human organs, *J. Exp. Med.*, 1957, **106,** 145.
10. Weller, T. H., and Coons, A. H., Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86,** 789.
11. Franklin, R. M., Rubin, H., and Davis, C. A., The production, purification, and properties of Newcastle disease virus labeled with radiophosphorus, *Virology*, 1957, **3,** 96.
12. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints, *Am. J. Hyg.*, 1938, **27,** 493.
13. Kaplan, A. S., A study of the herpes simplex virus-rabbit kidney cell system by the plaque technique, *Virology*, 1957, **4,** 435.
14. Farnham, A. E., The formation of microscopic plaques by herpes simplex virus in HeLa cells, *Virology*, 1958, **6,** 317.
15. Osterhout, S., and Tamm, I., Measurement of herpes simplex virus by the plaque technique in human amnion cells, *J. Immunol.*, 1959, **83,** 442.
16. Henry, C., and Franklin, R. M., Growth of mouse encephalomyelitis virus in Earle's L cells, *Virology*, 1959, **9,** 84.
17. Rubin, H., Franklin, R. M., and Baluda, M., Infection and growth of Newcastle disease virus (NDV) in cultures of chick embryo lung epithelium, *Virology*, 1957, **3,** 587.
18. Postlethwaite, R., A plaque technique for the titration of vaccinia virus in chick embryo cells and some features of vaccinial infection in this system, *Virology*, 1960, **10,** 466.
19. Horsfall, F. L., Jr., Studies on animal virus reproduction, *Science*, 1954, **120,** 781.
20. Franklin, R. M., and Breitenfeld, P. M., The abortive infection of Earle's L-cells by fowl plague virus, *Virology*, 1959, **8,** 293.
21. Henle, G., Girardi, A., and Henle, W., A non-transmissible cytopathogenic effect of influenza virus in tissue culture accompanied by formation of non-infectious hemagglutinins, *J. Exp. Med.*, 1955, **101,** 25.

22. Black, F. L., and Melnick, J. L., Micro-epidemiology of poliomyelitis and herpes-B infections. Spread of the viruses within tissue cultures, *J. Immunol.*, 1955, **74,** 236.
23. Wheeler, C. E., Further studies on the effect of neutralizing antibody upon the course of herpes simplex infections in tissue culture, *J. Immunol.*, 1960, **84,** 394.
24. Weller, T. H., Serial propagation *in vitro* of agents producing inclusion bodies derived from varicella and herpes zoster, *Proc. Soc. Exp. Biol. and Med.*, 1953, **83,** 340.
25. Rapp, F., Gordon, I., and Baker, R. F., Observations of measles virus infection of cultured human cells. I. A study of development and spread of virus antigen by means of immunofluorescence, *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 43.

EXPLANATION OF PLATES

PLATE 44

FIGS. 1 and 2. Photomicrographs of HeLa cell monolayer cultures infected with Newcastle disease virus (NDV), incubated 10 hours, and stained with anti-NDV immune rabbit serum followed by fluorescein isothiocyanate-labeled sheep anti-rabbit globulin. The photomicrographs are representative of fields scanned during counting of specifically fluorescent cells for the determination of cell-infecting units of NDV. Fig. 1 shows four single NDV-infected cells containing viral antigen in the cytoplasm, and Fig. 2 shows three such cells. × 660.



(Wheelock and Tamm: Cell-infecting particles of Newcastle disease virus)

PLATE 45

Photomicrographs of HeLa cell monolayer cultures infected with NDV and incubated in the absence or presence of anti-NDV immune guinea pig serum. Cultures stained with anti-NDV immune rabbit serum followed by fluorescein isothiocyanate-labeled sheep anti-rabbit globulin. × 280.

FIGS. 3 to 5. Cultures incubated in the absence of anti-NDV immune serum (group A in text).

FIG. 3. At 10 hours, a single infected HeLa cell containing moderate amounts of antigen is surrounded by a large number of uninfected cells.

FIG. 4. At 15 hours, satellite cells containing small amounts of viral antigen are seen next to and surrounding a single cell containing large amounts of antigen.

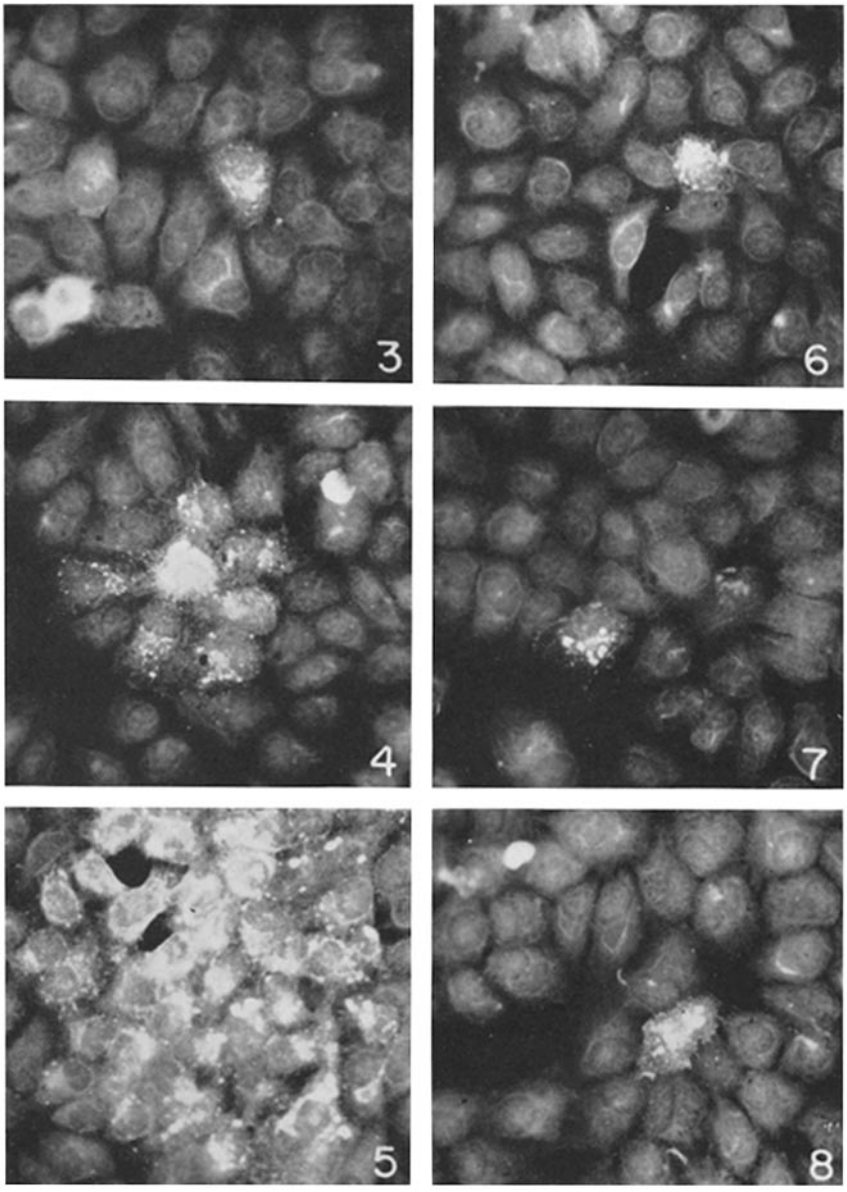
FIG. 5. At 24 hours, a large cluster of infected cells is present; most of the infected cells contain large amounts of NDV antigen.

FIGS. 6 to 8. Cultures incubated in the presence of anti-NDV immune serum (group C in text).

FIG. 6. At 10 hours, a single infected HeLa cell containing moderate amounts of antigen is surrounded by a large number of uninfected cells.

FIG. 7. At 15 hours, a single infected cell containing a moderately large amount of antigen is present. No satellite cells are seen.

FIG. 8. At 24 hours, a single infected cell containing large amounts of antigen is present. No satellite cells are seen.



(Wheelock and Tamm: Cell-infecting particles of Newcastle disease virus)