EFFECT OF MULTIPLICITY OF INFECTION ON NEWCASTLE DISEASE VIRUS–HeLa CELL INTERACTION*

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Plates 46 to 48

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Investigation of virus-cell interaction has focused for many years on the mechanism of virus reproduction. The cellular response to virus infection, however, has received relatively little attention, and the manner in which cell damage is produced is poorly understood. The present communication relates certain virus-induced alterations in cells to aspects of the virus reproductive sequence, and thus contributes to the understanding of the mechanisms involved in the production of cell damage by viruses.

The Newcastle disease virus (NDV)–HeLa cell system was chosen for study. In preliminary experiments it was observed that with various inocula, each of which was sufficient to infect all cells in the culture, the time at which antigen first appeared and the amount present at subsequent intervals were dependent on the multiplicity of infection. It seemed probable that the time at which alterations in cell structure or functions would appear might also depend on the multiplicity. An investigation was therefore undertaken to determine the effects of virus/cell multiplicity on both virus multiplication and the cellular consequences of virus infection. Of the cellular alterations brought about by virus infection, inhibition of mitosis was of special interest (1).

Some work has been reported describing the effects of multiplicity on various phases of the reproductive cycle of a few viruses, in experiments in which all cells were infected initially (2–6). A few observations are also on record concerning the effects of multiplicity on the development of virus-induced cell damage (2, 7). However, no reports have appeared attempting to relate the effects of multiplicity on virus multiplication to those on cell damage.

In the present report the effects of NDV multiplicity on the production of viral antigen and of infective virus in HeLa cells are described. In addition, the effects of multiplicity on hemadsorption, mitotic activity, and cytopathic changes are reported. The relationships between these events are analyzed, and possible mechanisms of inhibition of mitosis by NDV in HeLa cells are

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discussed. Finally, the effects of multiplicity of NDV on the interference phenomenon with the NWS strain of influenza A virus are reported. Results of interference experiments are used to develop further the analysis of the mechanism of inhibition of mitosis.

Materials and Methods

The materials and experimental procedures outlined in the preceding communication (8) were used in the performance of these studies. In a few experiments, phosphate buffered saline (PBS) (9), rather than protein-free Eagle's medium (10), was used in the preparation of virus dilutions for inoculation of coverslip cultures of HeLa cells. When PBS was used, cultures were incubated in air rather than in 5 per cent CO_2.

In addition to Newcastle disease virus (NDV), the NWS strain of influenza A virus was also employed. The line used had undergone 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 1000 EID_{50} of NWS. After incubation for 38 hours at 35°C., infected eggs were chilled at −26°C. The allantoic fluids were collected, centrifuged, and immediately frozen and stored at −55°C. The same seed of NWS was used in all experiments. It contained 5×10^8 EID_{50} per ml.

Vibrio cholerae Filtrate.—The Inaba strain of *V. cholerae* was used to prepare culture filtrate according to the procedure described previously (11).

Multiplicity.—The term multiplicity refers to the ratio of infective virus particles inoculated to the number of cells in culture. The term multiplicity of adsorption refers to the number of infective particles adsorbed per cell.

EXPERIMENTAL

Interval between Inoculation of NDV and Infection of HeLa Cells.—Preliminary to studies on the effects of multiplicity on several aspects of virus-cell interaction, experiments were carried out to determine the interval between inoculation of NDV and infection of all cells at each of the two multiplicities employed; *i.e.*, 500:1 and 5:1. This was done because it was important to relate the course of events at each multiplicity to the time of onset of infection.

Coverslips with complete monolayers of HeLa cells were placed in a flat bottomed Petri dish and inoculated with 0.1 ml. of either undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in phosphate-buffered saline (PBS), and incubated at 37°C. At intervals following inoculation, a coverslip from each series was washed 6 times in PBS, placed in another Petri dish, and treated for 30 minutes at 37°C. with 0.2 ml. of a 1:2 dilution of anti-NDV immune guinea pig serum in PBS. It had been determined earlier that this dilution of anti-NDV immune guinea pig serum completely neutralized an equal volume of undiluted NDV seed. The coverslip cultures were then washed 6 times in PBS, and returned for further incubation at 37°C. to Leighton tubes containing growth medium to which had been added 10 per cent anti-NDV immune guinea pig serum to prevent dissemination of virus produced in cells which were infected initially to uninfected cells (8). Coverslip cultures were collected 10 hours after inoculation, fixed in acetone, stained with fluorescent antibody (8), and the cells containing viral antigen were counted. Infection of all cells with virus was considered to have occurred when treatment of inoculated cultures with anti-NDV immune serum failed to prevent the subsequent development of virus antigen in any of the cells.

It was found that all cells in cultures inoculated at a multiplicity of 500:1 were infected within 30 seconds after inoculation. All cells in cultures inoculated at a multiplicity of 5:1 were infected within 20 minutes after inoculation, and most cells were infected within 10 minutes.

As is shown below, the effect of a 100-fold difference in multiplicity on the time course of various processes in virus-infected cells is measured in hours. Therefore, the slight difference in the time when all cells become infected at the two multiplicities is disregarded. Clearly, at both multiplicities, all cells became infected within a short time after virus inoculation.

Multiplicity of Adsorption.—The multiplicity of adsorption was determined under the conditions which were later used in experiments on the effects of multiplicity on various aspects of virus-cell interaction. Two multiplicities were employed, 500:1 and 5:1. The amount of virus remaining in the supernatant after a 2-hour adsorption period was compared to that present initially, and the amount adsorbed calculated by difference.

Coverslip cultures of HeLa cells were inoculated with undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in protein-free Eagle's medium. Five coverslip cultures were used at each multiplicity. At the end of a 2-hour adsorption period, 0.1 ml. of protein-free Eagle's medium was added to each coverslip. After thoroughly mixing the medium on coverslips, 0.1 ml. was removed from each coverslip culture. The collected materials were pooled within each of the two groups of cultures, and the viral content of the pools was determined by the procedure of counting fluorescent cells and compared to that of undiluted or 10^{-2} diluted control suspensions of NDV which had been held at 4°C.

The results obtained from 2 such experiments indicated that when the ratio of infective units of virus in the inoculum to cells in the culture was 500:1, 90 infective NDV particles per cell were adsorbed. When the initial multiplicity was 5:1, 4 virus particles per cell were adsorbed. The multiplicities of adsorption obtained are considered to be approximate since the effect of thermal inactivation on the virus inoculated was not taken into account. The multiplicities to which reference is made throughout the present communication are initial multiplicities and not the multiplicities of adsorption.

The Effect of NDV Multiplicity on Formation of Virus Antigen.—The effects of multiplicity were determined on two aspects of the development of viral antigen: the time of first appearance of antigen, and the amount of antigen per cell during the incremental period.

Coverslip cultures of HeLa cells were inoculated with undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in protein-free Eagle's medium and incubated at 37°C. After a 2-hour adsorption period the cultures were washed in PBS and returned to growth medium. At half-hour intervals following inoculation, a coverslip from each multiplicity series was collected, fixed in acetone, and stained with fluorescent antibody.

The results obtained are illustrated in Fig. 1 to 8.

One hour after inoculation, cells which had been exposed to NDV at a multiplicity of 500:1 displayed a moderate number of very small fluorescent foci (Fig. 1). These foci probably represented inoculated virus which had adsorbed to cells, rather than newly formed virus, because such foci could be seen within minutes after virus inoculation. At 1 hour, cells which had been exposed to NDV at a multiplicity of 5:1 showed no virus antigen (Fig. 2).

At 2½ hours, numerous small fluorescent foci were first seen in the cytoplasm of cells exposed to the higher multiplicity (Fig. 3). It should be emphasized that no such foci were seen at 2 hours. These foci were particularly prominent in the perinuclear area, and probably represented newly synthesized NDV antigen. Cells which had been exposed to the lower multiplicity showed no antigen at 2½ hours (Fig. 4).

At 4 hours, numerous fluorescent foci of moderate size were present in cells exposed to the higher multiplicity (Fig. 5) indicating that the amount of viral antigen had increased considerably during the preceding 1½ hours. At 4 hours, a moderate number of very small fluorescent foci were seen in the cytoplasm of cells which had been exposed to the lower multiplicity (Fig. 6). These foci probably represented the first appearance of newly made virus antigen. It should be emphasized that at the lower multiplicity no fluorescent foci were seen at 3 or 3½ hours.

Photomicrographs taken at 6 hours reveal that more antigen had been formed in each series during the preceding 2-hour interval (Figs. 7 and 8). Cells which had been exposed to the higher multiplicity contained large clumps of antigen (Fig. 7); the amount, at 6 hours, of antigen in cells which had been exposed to the lower multiplicity (Fig. 8) was similar to that observed at 4 hours in cells which had been exposed to the higher multiplicity (Fig. 5).

At 8 hours, cells in the higher multiplicity series showed a further, but not striking increase in the amount of specific fluorescence, whereas those in the lower multiplicity series contained considerably more antigen at 8 hours than at 6 hours. The difference between the higher and lower multiplicity series was less marked at 8 hours than it had been earlier; and still less at 10 hours. In the lower multiplicity series the antigen appeared to have increased somewhat between the 8th and 10th hour.

In summary, antigen appeared in cells which had been exposed to NDV at a multiplicity of 500:1 about 1.5 hours earlier than it did in cells exposed to NDV at a multiplicity of 5:1. During the period of rapid increase, there was an approximately 2 hour lag in antigen production between the higher and lower multiplicity series, but the rate of accumulation of antigen was apparently similar in the two series.

It should be emphasized that the fluorescent foci representing NDV antigen were of variable size in cells exposed to either of the two multiplicities used and examined at different times after virus inoculation. Of special interest is the finding that variability in the size of fluorescent foci was noted at the time when the antigen was first visualized.

It was stated above that at the time of first appearance of newly made virus antigen a considerable number of fluorescent foci were present at either multiplicity. Examination of singly infected HeLa cells has also shown multiple fluorescent foci at the time of first appearance of antigen.

At no time was newly formed NDV antigen observed in the cell nucleus in either multiplicity series. This confirms the observations reported earlier (1). Similar findings have been made with NDV in Ehrlich ascites tumor cells (4), in the chorioallantoic membrane of the chick embryo and the ciliated nasal epithelium from chicks (12), and in chick embryo lung cells (13).

Effect on NDV Multiplicity on Production of Infective Virus.—In studies on the multiplication of NDV virus, HeLa cells were exposed to virus multiplicities of 500:1 and 5:1, and at frequent intervals cell-associated virus (CAV), and released virus (RV) were measured by the fluorescent cell-counting procedure.

Coverslip cultures of HeLa cells were inoculated with undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in protein-free Eagle's medium, and incubated at 37°C. After a 2 hour adsorption period, the cultures were washed in PBS and treated for 30 minutes at 37°C. with 0.2 ml. of a 1:2 dilution of anti-NDV immune guinea pig serum in PBS. The immune serum was then washed off by immersion of cultures for 30 minutes at 37°C. in a series of tubes containing PBS. Each coverslip was placed in a fresh Leighton tube with 1 ml. of growth medium containing 5 per cent human serum, and incubation at 37°C. was continued. At appropriate intervals following inoculation, 3 cultures from each multiplicity series were transferred to 6°C.

When all cultures were collected, the medium was removed from each group of 3 tubes and pooled, and each culture washed with 1 ml. of medium which was added to the pool. The 6 ml. samples of medium were then centrifuged at 600 *g* for 15 minutes at 4°C., and the supernates were collected and designated RV. The pellets were resuspended in 3 ml. volumes of medium and added to Leighton tubes containing the cells on coverslips.

Each set of Leighton tubes was frozen and thawed four times to disrupt most cells. The frozen and thawed material was centrifuged at 600 *g* for 15 minutes at 4°C., and the supernates were collected and designated CAV:frozen-thawed.

The pellets were resuspended in 1 ml. of 0.04 per cent trypsin in an appropriate saline solution (8), and incubated for 30 minutes at 33°C. Growth medium, 3 ml. per tube, was then added to neutralize the trypsin. Following centrifugation at 600 *g* for 15 minutes at 4°C., the supernates were collected and designate CAV:trypsinized.

The pellets were resuspended in 2 ml. of medium and subjected to sonic vibration for 1 minute.[1] The sonicated samples were designated CAV:sonicated.

The amount of virus present in each virus sample was then determined by the fluorescent cell-counting procedure. In the assay procedure, following adsorption of virus, 5 per cent anti-NDV immune guinea pig serum was present in the medium (8).

The distribution of CAV among the three fractions, frozen-thawed, trypsinized, and sonicated, is shown in Table I. The per cent values recorded in Table I are based on sums of determinations of cell-infecting units of virus carried out on samples collected at 16 time intervals in the growth curve experiment. As can be seen, freezing and thawing alone released only about ⅖ of the total CAV. Trypsin treatment of the cell material from the freeze-thawing procedure released an additional ⅖ of CAV. Finally, sonication of frozen and thawed and trypsin-treated cell material released about ⅕.

The total CAV at each time interval in the growth curve experiment was computed by adding the cell infective units of virus found in each fraction.

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[1] A Raytheon 200 watt 10 kc. magnetostrictive oscillator was used at 9000 cycles per second.

The results of the growth curve experiment with NDV are shown in Text-fig. 1. The virus yield is expressed in terms of infective NDV produced per HeLa cell, and the cell associated and released virus are plotted separately.

As can be seen, at the 500:1 multiplicity, a small amount of CAV was detected at 1½ hours. This may have represented new virus or residual inoculated virus. Between 1½ and 4 hours CAV increased to reach a maximal yield of approximately 6 CIU per cell. The rapid rise was followed by a gradual decline in CAV. The decline could not be accounted for on the basis of release of virus from cells and, therefore, it was probably due to inactivation of infective CAV. It is of interest that the apparent rate of inactivation was considerably slower than that of thermal inactivation of NDV suspended in protein-free Eagle's medium. There are several possible reasons for this: (*a*) production of new virus may have continued after the peak in virus concentration was reached; (*b*) new virus originating in HeLa cells may have a longer thermal half-life; (*c*) inactivation of CAV may be slower than inactivation of free virus in

TABLE I

Multiplicity	Distribution of cell-associated virus (CAV) in consecutively treated fractions expressed as per cent of total CAV		
	Frozen-thawed	Trypsinized	Sonicated
500:1	47	30	23
5:1	35	41	24
Mean.....	41	35.5	23.5

suspension; or (*d*) presence of serum in the medium may have had a protective effect on CAV.

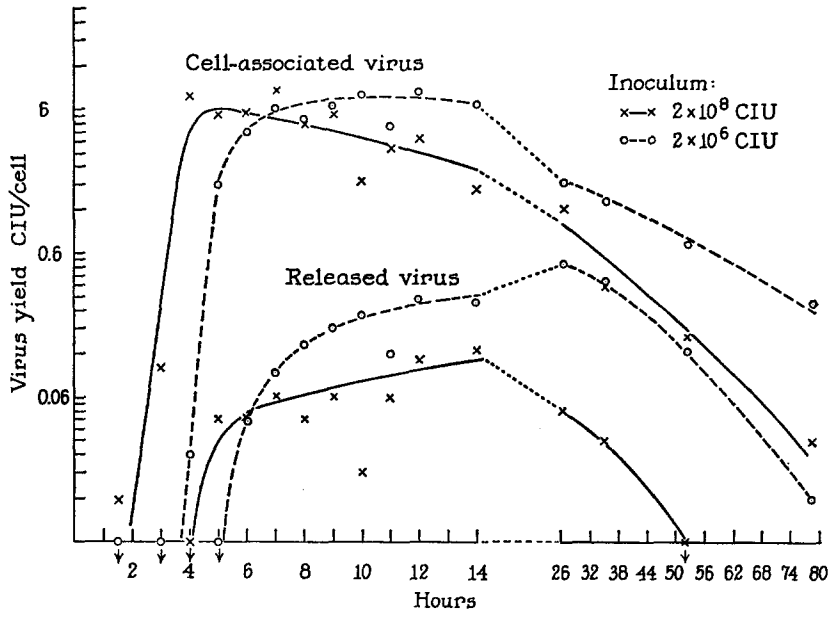
At the 5:1 multiplicity no detectable amounts of CAV were found 1½ or 3 hours after inoculation. This provides strong evidence of an "eclipse" of the infecting virus. A small amount of CAV was present at 4 hours. The virus then increased rapidly reaching a yield of approximately 6 particles per cell at 7 hours. Cultures collected during the next 6 hours all contained approximately 6 CIU of NDV per cell. It seems likely that continued production and inactivation balanced each other and were responsible for the plateau in virus concentration. Beginning at 14 hours a gradual decline in CAV virus was observed.

In summary, there was an approximately 2 hour lag between CAV growth curves obtained with the multiplicities of 500:1 and 5:1. However, the maximal yield of virus was closely similar at the two multiplicities.

Virus became demonstrable in the medium approximately 2 hours later than in cells, and less than 5 per cent of the total virus produced at each multiplicity was released into the medium. RV became demonstrable in the higher multiplicity series 1 hour earlier than in the lower multiplicity series, but more

virus was released in the latter than in the former. Data are presented below concerning the time course of development of marked cell damage in NDV-infected cultures. These data, together with those shown in Text-fig. 1, indicate that development of marked cell damage had no effect on the slow release of virus from cells.

To determine whether the low yields of NDV in HeLa cells were associated with the production of considerable amounts of non-infective hemagglutinating particles, hemagglutination titrations were carried out with CAV and RV



TEXT-FIG. 1. Effect of NDV multiplicity on production of infective virus in HeLa cell monolayer cultures.

samples collected at 10, 14, and 35 hours. No demonstrable amounts of hemagglutinating virus were found. Thus, the yields of total virus as well as of infective virus were low with the strain of NDV and the type of cell used.

The low average yield of NDV per cell could be due either to a low virus-producing capacity of all cells in the culture, or to failure of a considerable proportion of cells to produce any infective virus. To decide between these possibilities, experiments were carried out to determine the percentage of NDV-infected cells which were releasing infective virus. This was done by studying the development of NDV antigen-containing satellite cells in the neighborhood of cells which were infected by inoculated virus.

On the basis of previous results (8) it was expected that cells infected with

the inoculated virus, *i.e.* primarily infected cells, could be readily visualized 10 hours after infection, and that at 16 hours many would be surrounded by secondarily infected cells. Furthermore, it was expected that the number of primarily infected cells would not change between 10 and 16 hours, because by the 10th hour all cells infected with the inoculated virus have developed sufficient antigen to be readily detectable by the fluorescent antibody staining procedure (8).

Coverslip cultures of HeLa cells were inoculated with a 1×10^{-5} dilution of NDV-infected allantoic fluid in protein-free Eagle's medium. The amount of virus inoculated was sufficient to infect approximately 1 per cent of cells on coverslip cultures. After a 2 hour adsorption period the coverslips were washed with PBS and returned to growth medium. Beginning at 10 hours after virus inoculation, coverslips were collected at hourly intervals and stained with fluorescent antibody. The cultures collected at 10 and 16 hours are discussed in detail because they provided a basis for conclusions concerning the question under study.

Examination of the coverslip which had been incubated for 16 hours showed that of the 150 primarily infected cells which were counted, 132 had secondarily infected satellite cells. The remaining 18 cells contained large amounts of virus antigen, but there were no secondarily infected cells in their immediate vicinity. The number of primarily infected cells at 16 hours was equal to that found in cultures at 10 hours.

This experiment demonstrates that at least 88 per cent of NDV-infected HeLa cells have the capacity to produce and release infective virus progeny. The other 12 per cent may have in time also released infective virus. Whether at higher virus multiplicities a similar proportion of infected cells produce and release infective virus remains to be determined.

Nature of Cell-Associated Virus.—In experiments with NDV in monolayer cultures of chick embryo lung epithelium, most of the newly formed infective CAV could be neutralized at various times throughout the growth cycle by treatment of the infected cells with anti-NDV immune serum before disrupting them (14). Thus, most of newly formed CAV appeared to be at the surface of infected cells rather than within cells.

Experiments were carried to determine the localization of newly formed CAV in the NDV-HeLa cell system. Infected HeLa cells were treated with anti-NDV immune serum or *V. cholerae* filtrate to see whether such treatments would lower the amount of CAV.

In experiments with immune serum, the procedure followed was that used in growth curve experiments. Coverslip cultures of HeLa cells were infected with NDV at a multiplicity of 5:1. After a 2 hour adsorption period, the cultures were treated for 30 minutes with anti-NDV immune guinea pig serum to neutralize residual inoculated virus. They were then washed for 30 minutes with PBS and incubated in growth medium. Nine hours after virus inoculation 8 coverslips were washed 8 times in tubes containing PBS. They were then placed in Petri dishes and one-half of the cultures treated with a 1:2 dilution of anti-NDV immune guinea pig serum

in PBS, 0.2 ml. per coverslip. The other half received PBS. Thirty minutes later the immune serum was washed off by immersion of cultures for 30 minutes in a series of tubes containing PBS. They were then placed in new Leighton tubes containing 1 ml. of growth medium. The cells were disrupted by freezing and thawing followed by trypsinization and sonication, as described above, and the amount of CAV determined by the fluorescent cell-counting procedure.

In experiments with *V. cholerae* filtrate the preliminary steps were carried out as described above. Nine hours following virus inoculation, the medium was aspirated from 8 tubes, and the cultures were washed twice with PBS. One-half of the cultures received a 1:5 dilution of *V. cholerae* filtrate in protein-free Eagle's medium, 1.0 ml. per tube, and the other half received a 1:5 dilution of broth. The cultures were then incubated at 37°C. for 30 minutes, the media removed, and the cultures washed with 4 changes of PBS. One ml. of growth medium was placed in each tube and the cells were disrupted in a manner described above. The amount of CAV was determined by the fluorescent cell-counting procedure. The yield of virus in controls was 11 CIU per cell.

These experiments showed that treatment of infected cells at 9 hours with anti-NDV antibody neutralized 99.3 per cent of CAV. In contrast, treatment with *V. cholerae* filtrate had no effect. It appears that newly produced CAV occupies a position at the surface of the cell membrane in which it is accessible to antibody. This finding confirms the earlier results (14) to which reference was made above. The precise nature of the bonds between virus particles and the cell surface is unclear. Failure of treatment with *V. cholerae* filtrate to release CAV does not exclude the possibility that newly formed CAV is combined with sialic acid–containing virus receptors on the cell surface, but it does suggest that the relationship between virus particles with newly acquired infectivity (14) and the cell surface may be more complex.

Effect of NDV Multiplicity on Hemadsorption.—It has been demonstrated that cells in cultures infected with a number of different viruses including NDV, are capable of adsorbing erythrocytes (15). To determine whether in HeLa cell cultures infected with NDV only the NDV antigen-containing cells would adsorb erythrocytes, experiments were performed in which erythrocytes were added to NDV-infected coverslip cultures of HeLa cells, and the cultures studied by fluorescent antibody technique.

Coverslip cultures of HeLa cells were inoculated with a 1×10^{-3} dilution of NDV seed. The amount of NDV inoculated was sufficient to infect approximately 50 per cent of the cells. After incubation at 37°C. for 9 hours, coverslips were washed 10 times in PBS and placed, for 10 minutes, in a tube containing 3 ml. of a 1 per cent dilution of chick erythrocytes in cold PBS. The cultures were then gently washed 10 times in PBS, fixed, and stained with fluorescent antibody.

As can be seen in Fig. 9 only cells containing NDV antigen adsorbed erythrocytes.

Next it was of interest to determine whether multiplicity would affect the time course of development of the hemadsorption phenomenon.

Coverslip cultures of HeLa cells were inoculated in the usual way with a 500:1 or 5:1 multiplicity of NDV. After a 2 hour adsorption period, the coverslip cultures were washed in PBS and returned to growth medium for further incubation at 37°C. At appropriate intervals a coverslip from each series was collected, and the hemadsorption procedure performed as described above. The cultures were then examined under the microscope.

It proved difficult to determine accurately the earliest time when erythrocytes became adsorbed to cells in infected cultures, or to follow the increase in the hemadsorbing ability of cells as infection progressed. However, it was possible to make a reliable estimate of the earliest time when all HeLa cells in the culture adsorbed erythrocytes. At a multiplicity of 500:1 hemadsorption was complete at 5 hours, whereas at a multiplicity of 5:1 it was complete at 7 hours.

Effect of NDV Multiplicity on Incidence of Mitosis and Cytopathic Alterations in HeLa Cells.—Mitotic activity in HeLa cell cultures infected with NDV at the multiplicities of 500:1 and 5:1 was determined and compared with development of virus-induced cytopathic alterations in the same cultures.

Coverslip cultures of HeLa cells were placed in flat bottomed Petri dishes and inoculated with undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in protein-free Eagle's medium, and incubated at 37°C. After a 2 hour adsorption period, the coverslips were washed in PBS and returned to growth medium for further incubation. Uninfected control cultures were treated in a similar manner.

The coverslip cultures were examined microscopically at hourly intervals, and the number of cells in mitosis, and the number of markedly damaged cells per coverslip culture were determined for each multiplicity series. Cells in metaphase, anaphase, and telophase were readily identified. Cells undergoing marked cytopathic alterations were rounded or fragmented and could also be readily identified. Mitotic or damaged cells were counted within a square formed by a microgrid inserted in the ocular of the microscope. The same 3 coverslip cultures for each multiplicity and the control cultures were examined hourly, and 2 squares per culture were selected at random and scored. The mean numbers of mitotic or damaged cells per square were calculated and multiplied by the number of squares per coverslip. The results could then be expressed as numbers of mitotic or markedly damaged cells per coverslip culture. The cultures originally contained approximately 4×10^5 cells. The degree of mitotic activity in infected cultures was related to that in uninfected control cultures.

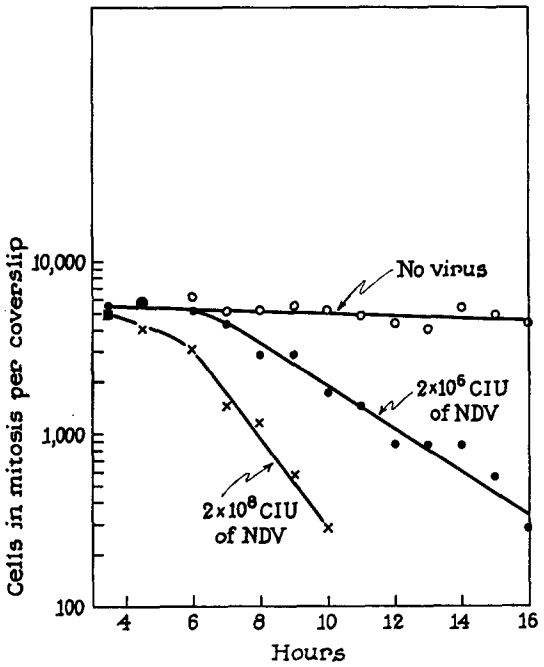
As can be seen in Text-fig. 2, cultures exposed to the higher multiplicity showed a small decrease in the incidence of mitosis at 5 to 6 hours and the last mitotic cell was seen at 10 hours after infection. Between the 6th and 10th hour the mitotic rate decreased exponentially.

Cells exposed to the lower multiplicity showed slightly reduced mitotic activity at 8 hours and the last mitosis was seen 16 hours after infection. Between the 8th and 16th hour the rate decreased exponentially. The slope of the decrease was less steep than that observed at the higher multiplicity.

At the higher multiplicity, the first cells showing marked damage were seen at 6 hours after inoculation. As is shown in Text-fig. 3, the number of markedly damaged cells increased exponentially between 7 and 13 hours. At 13 hours, 50 per cent of cells

were affected. During subsequent hours, many cells separated from glass, and therefore it was not possible to determine accurately either the number or per cent of cells affected.

At the lower multiplicity, no markedly damaged cells were seen at 6 hours, but beginning at 7 hours, when a significant number of damaged cells were observed, the number of damaged cells increased exponentially until at 16 hours 50 per cent of cells present were affected. Quantitative observations were not possible at subsequent intervals.

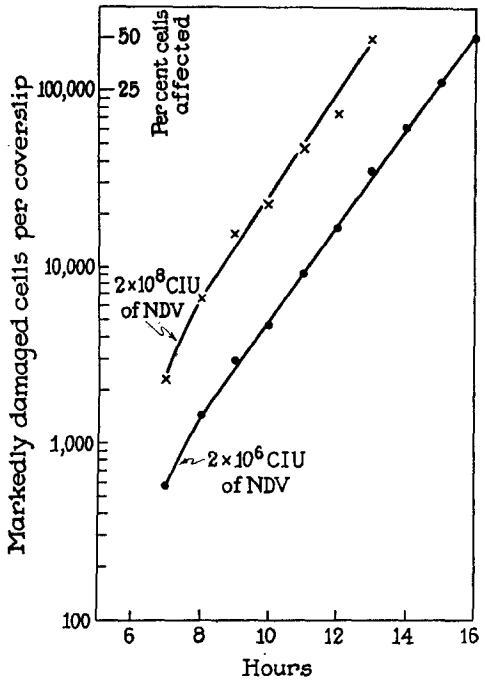


TEXT-FIG. 2. Effect of NDV multiplicity on the incidence of mitosis in HeLa cell monolayer cultures.

In summary, markedly damaged cells were seen 1 hour earlier in cultures exposed to the higher multiplicity of NDV. The exponential increase in the number of affected cells followed a parallel course at the two multiplicities, but there was a lag of approximately 1½ hours between the higher and lower multiplicity curves.

It may be pointed out that 10 hours after inoculation only 6 per cent of cells had been markedly damaged in cultures exposed to the higher multiplicity, but mitotic activity had dropped 94 per cent. This demonstrates that virus-infected cells are inhibited from undergoing mitosis before gross cellular damage occurs.

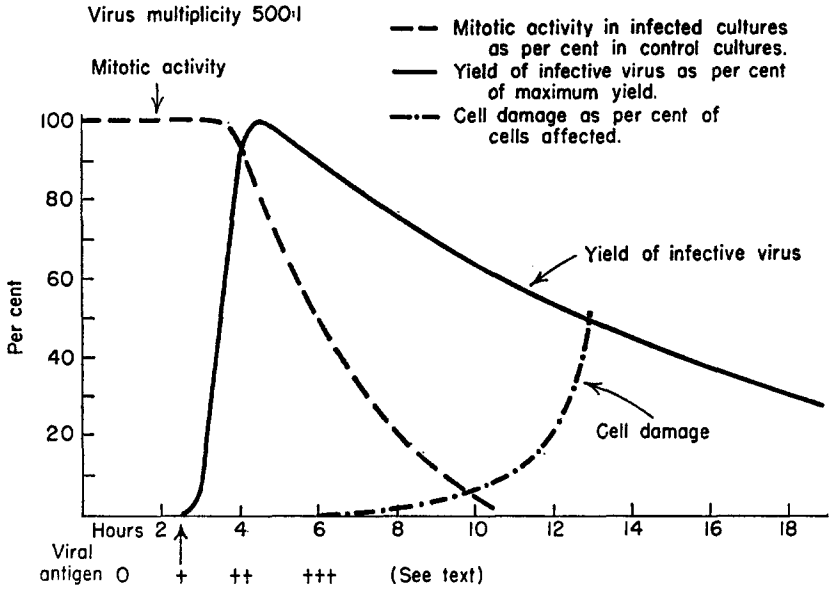
Summary of Events in NDV-Infected HeLa Cells.—The temporal relationships between the various events in NDV-infected HeLa cells are presented in Text-figs. 4 and 5, which summarize the results of studies of production of viral antigen and infective virus, and of mitotic activity and development of marked cell damage. Except for the data on antigen production, all results are shown on percentage scales to facilitate comparisons.



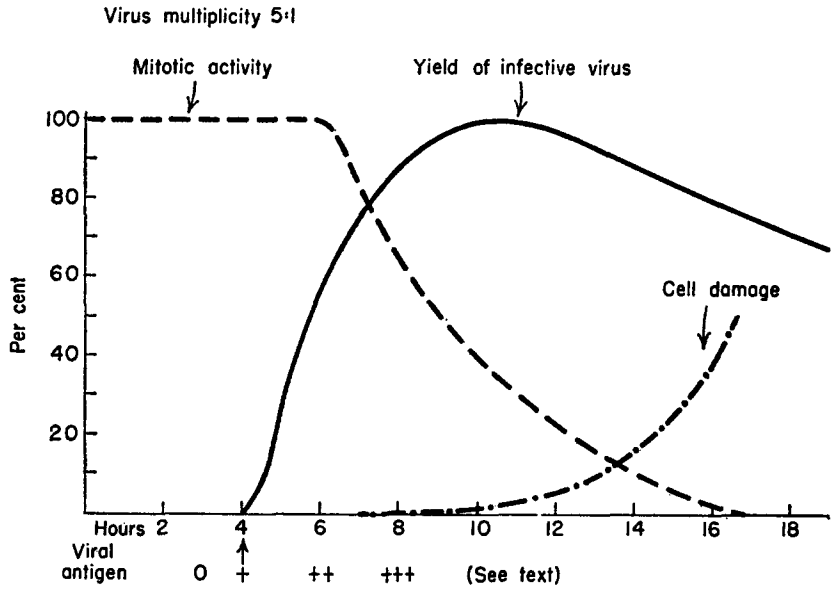
TEXT-FIG. 3. Effect of NDV multiplicity on the number of markedly damaged HeLa cells in monolayer cultures.

As can be seen in Text-fig. 4 which shows the results obtained in cultures exposed to NDV multiplicity of 500:1, newly produced viral antigen and infective virus became detectable at approximately the same time and both increased during the following 2 hours. However, after this, a marked difference is noted in these two aspects of viral reproductive activity: whereas viral antigen continued to increase during the subsequent 4 hour period, infective virus not only failed to increase but showed a gradual decline.

It is possible, but unlikely that the decline in infective virus was entirely due to rapid inactivation of newly made virus. It seems necessary to postulate that after the initial period of rapid virus production, the mechanism for the pro-



TEXT-FIG. 4



TEXT-FIG. 5

TEXT-FIGS. 4 and 5. Summary of several aspects of NDV-HeLa cell interaction studied at virus multiplicities of 500:1 or 5:1.

duction of infective virus became defective, and that little new infective virus was produced after 4 to 5 hours.

The time relationship between production of viral antigen and mitotic activity is of considerable interest. A drop in mitotic activity was evident shortly after the first appearance of viral antigen. Over the next several hours viral antigen increased and mitotic activity decreased. There appeared to be an inverse relationship between antigen production and mitotic activity. It is also true that mitotic activity began to decrease shortly after the major portion of the maximum yield of virus had been produced.

Development of marked cytopathic damage was clearly a late phenomenon. Markedly damaged cells became evident in significant numbers only after much viral antigen had been produced, and several hours after the peak in infective virus had been reached. Mitotic activity had dropped to very low levels before significant numbers of markedly damaged cells were observed.

As can be seen in Text-fig. 5, the temporal relationships between the various events in cultures infected with a 5:1 multiplicity of NDV were essentially similar, although all events were shifted to later times.

Double Infection of HeLa Cells with NDV and Influenza Virus.—Preliminary to experiments on the effects of multiplicity on interference between Newcastle disease and influenza viruses, it was important to determine whether cells could be doubly infected with these viruses. Means for investigation of this possibility were available because the intracellular distribution of Newcastle disease and influenza virus antigens, as revealed by the fluorescent antibody technique, is strikingly different (1).

Coverslip cultures of HeLa cells were inoculated with either a 2.5×10^{-4} dilution of NDV seed or a 10^{-1} dilution of NWS seed in protein-free Eagle's medium, 0.1 ml. per culture. After a 2 hour adsorption period at 37°C., the coverslips were washed in PBS and returned to growth medium for further incubation. Fourteen hours after inoculation, coverslip cultures were collected, fixed, and stained with the appropriate virus-specific immune rabbit serum followed by fluorescein isothiocyanate-labeled sheep anti-rabbit globulin.

In addition, some cultures were inoculated simultaneously with a 10^{-1} dilution of NDV and a 5×10^{-1} dilution of NWS in protein-free Eagle's medium, 0.05 ml. of each virus per coverslip culture. After a 1-hour adsorption period at 37°C., the coverslips were washed in PBS and returned to growth medium for further incubation. Twelve hours after inoculation, coverslip cultures were collected, fixed in acetone, and stained with both anti-NDV and anti-NWS immune rabbit sera followed by sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate.

As can be seen in Fig. 10, in cells infected with NDV alone, large clumps of antigen were present in the cytoplasm; the nucleus did not contain detectable amounts of NDV antigen. In contrast, Fig. 11 demonstrates the predominantly nuclear localization of influenza virus antigen in cells infected with NWS alone. Some influenza virus antigen was also present in the cytoplasm, where its fine distribution could be readily visualized.

Fig. 12 shows double infection of HeLa cells with Newcastle disease and influenza viruses. NWS antigen can be visualized in the nucleus and NDV antigen in the cytoplasm of the same cell.

The Effect of Multiplicity of NDV on the Establishment of Interference against NWS.—In interference experiments, the interval between interfering virus (NDV) and challenge virus (NWS) was varied, and the inability of challenge virus antigen to develop in cells infected with the interfering virus was considered to indicate interference. Cultures were exposed to NDV at two multiplicities, 500:1 and 5:1.

Coverslip cultures of HeLa cells were placed in flat-bottomed Petri dishes and inoculated with undiluted NDV-infected allantoic fluid or a 10^{-2} dilution of NDV seed in PBS. After incubation for 15 minutes at 37°C. the coverslips were washed in PBS and returned to growth medium. In the time allowed for adsorption all cells were infected at the higher multiplicity, and more than 95 per cent of cells at the lower multiplicity. On return to growth medium and at hourly intervals thereafter, coverslips were placed in a flat-bottomed dish and inoculated with 0.1 ml. of a 5×10^{-1} dilution of NWS in PBS. This inoculum contains sufficient virus to infect approximately 50 per cent of all cells. After an incubation period of 30 minutes at 37°C., the coverslips were washed in PBS and returned to growth medium. 5½ hours after inoculation of NWS virus all cultures were collected and fixed in acetone. Fluorescent antibody staining was done with a 1:2 mixture of anti-NDV and anti-NWS immune rabbit sera followed by sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate.

TABLE II

Effect of Multiplicity of NDV on Establishment of Interference against NWS Virus

Interval between NDV and NWS	Presence of detectable amounts of NWS antigen	
	NDV inoculum and multiplicity	
	2×10^8 CIU 500:1	2×10^6 CIU 5:1
hrs.		
0	+	+
1	0	+
2	0	+
3		+
4		+
5		+

As can be seen in Table II, when NWS was inoculated immediately after the termination of the NDV adsorption period, the cells were able to support the synthesis of both NDV and NWS antigen regardless of the multiplicity of NDV.

However, when NWS was inoculated 1 hour after the termination of the NDV adsorption period, the ability of cells to support the synthesis of NWS antigen was dependent on the multiplicity of NDV. In cultures infected at the

higher multiplicity of NDV, NWS antigen failed to develop to detectable levels, whereas at the lower multiplicity of NDV, NWS antigen did reach detectable levels.

Furthermore, at the lower multiplicity of NDV, NWS antigen developed even when the interval between NDV and NWS was prolonged to 5 hours. However, it was noted that both the number of cells containing NWS antigen, and the intensity of the fluorescence in these cells decreased in direct relationship to the length of the interval between NDV and NWS virus inoculation. Thus, the degree of interference was quantitatively related to the amount of NDV antigen produced prior to infection of cells by the challenge virus (NWS). This observation supplements the finding described above that interference was dependent also on the multiplicity of interfering virus.

Mitosis in Cells Which Have Produced Infective NDV.—In a previous communication (1), it was demonstrated that HeLa cells containing newly made NDV antigen can undergo mitosis and divide. The results which have been described in the present communication are in agreement with the earlier finding. Furthermore, they provide suggestive evidence that cells which have produced infective virus are able to undergo mitosis and divide.

To obtain proof of this in individual cells, a coverslip culture collected at 15 hours in the experiment concerned with the proportion of infected cells capable of releasing infective progeny was examined for mitotic cells which had been infected by the virus in the inoculum. The 15 hour time interval was chosen because secondarily infected satellite cells become detectable at around this time. A cell in metaphase containing large amounts of antigen was found surrounded by 8 cells containing small quantities of antigen (Fig. 13).

Because several hours are required for detectable amounts of antigen to be produced in infected cells, it follows that the satellite cells were infected several hours before the slide was collected. Furthermore, since a cell remains in metaphase for 1½ hours at most, the parent cell which was in metaphase at the time the culture was collected clearly must have produced and released infective virus before entering metaphase.

DISCUSSION

The present studies demonstrate that the time course of virus reproduction and its cellular consequences depend on the multiplicity of infecting virus. The outstanding difference in the time course, following exposure of HeLa cells to Newcastle disease virus (NDV) at multiplicities of 500:1 or 5:1, was the accelerated occurrence of various processes at the higher multiplicity. These included the following; production of new viral antigen and of new infective virus, hemadsorption phenomenon, inhibition of mitosis, and cytopathic alterations in cells. A 1 to 2 hour acceleration was observed in the development of each process. The final outcome at the two multiplicities was in

all respects similar, however, in that the maximal amounts of viral antigen and infective virus produced were identical, and in each instance complete hemadsorption, inhibition of mitosis, and destruction of cells occurred eventually.

The characteristics of the time curves of increase in viral antigen and in infective virus in cells exposed to different multiplicities strongly suggest that once detectable amounts of antigen or virus have been produced, the rates at which further quantities are made are similar regardless of multiplicity.

The large number and size of specifically fluorescent sites in NDV-infected HeLa cells suggest that infection ultimately leads to the production of large quantities of viral antigen. If this be true, it follows that in HeLa cells there is a marked discrepancy between the amounts of antigen and virus that are produced, since only 6 to 11 CIU of infective virus per cell and no detectable hemagglutinating virus was produced. Thus it appears that more virus material is synthesized than is incorporated into virus particles. In an earlier study (16) performed with a different strain of NDV and a different HeLa cell line, the yield was 0.8 infective particles per cell. Over 95 per cent of the virus material released was non-infective and only detectable as hemagglutinin. Similar low yields of NDV, ranging from 2 to 37 infective particles per cell, have been reported in other *in vitro* cell systems (14, 17–19). On the other hand, it has been estimated that in the allantoic sac of the chick embryo the yield per cell is approximately 1000 infective particles (20, 21). It therefore appears that, in all cultured cells studied thus far, the mechanism of reproduction of NDV is defective.

The mechanism of inhibition of mitosis in NDV-infected HeLa cells presents an intriguing problem. The demonstration that in the NDV-HeLa cell system mitosis and division can occur several hours after infection (1), combined with the finding that division is eventually inhibited (22), indicated that there is a critical point during or after production of NDV antigen at which sufficient cell damage occurs to prevent mitosis (1). The results obtained in the present investigation serve to exclude some factors from among the possible causes of inhibition of mitosis and to focus attention on certain others which may be responsible for the inhibition.

First, it should be emphasized that inhibition was not due to marked cytopathic changes, since these occurred much later. Second, the results obtained show that production of infective virus does not *per se* affect the ability of cells to undergo mitosis and to divide. The development of marked cell damage is also, apparently, not directly related to production of infective NDV. Influenza viruses which fail to produce any infective progeny in HeLa cells nevertheless cause degeneration and death of cells (23).

It should be emphasized that production of viral antigen continued for several hours after maximal yields of infective virus had been reached. The fact that inhibition of mitosis began and became marked during the period

of progressive increase in the amount of intracellular virus antigen raises the possibility that inhibition of mitotic activity may have been caused by the continued production of viral antigen, which may also have been responsible for the eventual development of marked degenerative changes in the cells.

As to the intimate mechanism and time course of inhibition of mitosis in NDV-infected cells, the following can be stated at this time: Virus multiplication must reach a critical phase to produce damaging effects on cellular processes preceding, and necessary for, successful mitosis. Clearly, each cell has certain pre-mitotic reactions which are sensitive to this damaging effect of virus multiplication. Inhibition occurs if virus multiplication has reached the critical phase before a cell has completed the sensitive steps. Conversely, a cell which has completed virus-sensitive pre-mitotic steps before the critical phase in virus multiplication has been reached, will proceed to undergo mitosis and divide in spite of the presence of replicating virus.

It has been shown that in HeLa cells the pre-mitotic resting phase, *i.e.* the interval between the end of DNA synthesis and the onset of mitosis may vary in length between 3 and 10 hours, and the other intermitotic phases may vary also, but considerably less (24). Therefore, the time before mitosis at which a specific sensitive step may occur in cells dividing asynchronously, may vary from cell to cell. Furthermore, the critical point in virus multiplication is probably not reached at the same time in all cells. This is indicated by asynchrony in the production of virus antigen. These appear to be the main reasons for the gradual rate of decline in mitotic activity in NDV-infected HeLa cell cultures.

Ability of a cell to undergo mitosis represents a sensitive index of the functional integrity of the cell, and therefore viral inhibition of mitosis provides an approach to the complex problem of the nature and mechanism of virus-induced alterations in cells. There is evidence that the effects of different viruses on mitosis in cultured cells are variable. In the NDV-HeLa cell system mitosis and division can occur several hours after infection even at high multiplicities. It should be mentioned that in singly infected cells many hours are necessary before virus multiplication can cause inhibition of mitosis. In contrast, herpes simplex virus at a multiplicity of 1.1 causes inhibition of HeLa cells within 1 hour after infection (25). Rous sarcoma virus has no inhibitory effect on mitotic activity of host cells (26), whereas pseudorabies causes amitotic nuclear division of RK cells (27). With polyoma virus in cultures of embryonic mouse and hamster cells, a complex picture was observed (28). The polyoma virus gave rise to two types of virus-cell interaction; a cytocidal interaction, leading to extensive virus synthesis and cell degeneration, and a moderate interaction leading to the transformation of the cells into neoplastic cells. The cytocidal interaction was most frequent in the mouse cultures, whereas the moderate interaction predominated in the hamster cultures.

It is probable that inhibition of mitosis in virus-infected cells may be brought about by a variety of mechanisms. There is no direct evidence concerning the mechanism whereby NDV infection causes inhibition of mitosis in HeLa cells. However, the results of interference experiments with NWS virus suggest that infection with NDV results in deficiencies in cells which may be metabolic in nature. Such deficiencies may also preclude successful mitosis in infected cells.

It seems clear that studies of the effects of virus infection on mitosis may yield important information about both processes. Using mitotic activity as an index of functional integrity of cells, studies can be undertaken to determine which events in the virus reproductive cycle are capable of causing alterations in this important biological activity of cells. Conversely, using viral infection as a probe, experiments can be performed to identify and characterize the stages in the mitotic cycle which can be influenced by viral activity.

SUMMARY

The effects of a hundred-fold difference in virus/cell multiplicity on the interaction of Newcastle disease virus (NDV) with HeLa cells were studied, and various phases of the virus reproductive cycle were related to cellular consequences of infection. At both multiplicities used all cells were infected. The following events occurred 1 to 2 hours earlier in cells which were inoculated with the higher multiplicity: (*a*) first appearance of newly made virus antigen, and the amount present at any time during the period of rapid increase; (*b*) onset and time course of production of infective virus; (*c*) development by infected cells of hemadsorbing ability; (*d*) onset and time course of inhibition of mitosis; and (*e*) onset and time course of marked cell damage.

Double infection of HeLa cells with NDV and NWS was demonstrated by the fluorescent antibody technique, and was used to show that the establishment of interference against NWS was also dependent upon the multiplicity of NDV.

In cells inoculated at each multiplicity, newly made virus antigen appeared at the same time as the first infective virus particles. Infective virus rapidly reached a peak, and then declined. Viral antigen continued to increase for several hours after the decline in infective virus had begun. Thus, only a small fraction of the virus antigen produced was incorporated into new infective particles. The maximal yield of such particles was only 6 to 11 per HeLa cell. Over 95 per cent of new virus was cell-associated, but could be neutralized by treatment with antiserum before disruption of cells.

Mitosis occurred in cells which had produced and released infective NDV. Progressive inhibition of mitotic activity in infected cells was correlated with continued production of viral antigen. Marked cytopathic changes developed after mitotic activity had decreased to low levels.

The mechanism by which NDV inhibits mitosis in HeLa cells is discussed.

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EXPLANATION OF PLATES

PLATE 46

FIGS. 1 to 8. Photomicrographs of HeLa cell monolayer cultures inoculated with either a 500:1 or 5:1 multiplicity of Newcastle disease virus (NDV), and incubated for varying periods. Cultures stained with anti-NDV immune rabbit serum followed by fluorescein isothiocyanate–labeled sheep anti-rabbit globulin. The term "fluorescent" when used below refers to specific fluorescence. × 440.

FIG. 1. At 1 hour, cells which had been exposed to NDV at high multiplicity display numerous diffusely distributed fluorescent foci. These foci probably represent inoculated virus which has adsorbed to cells.

FIG. 2. At 1 hour, cells which had been exposed to NDV at low multiplicity display no fluorescent foci.

FIG. 3. At 2½ hours, high multiplicity cells contain large numbers of small fluorescent foci of variable size in the cytoplasm, principally in the perinuclear area. These foci probably represent newly formed NDV antigen.

FIG. 4. At 2½ hours, low multiplicity cells display no fluorescent foci.

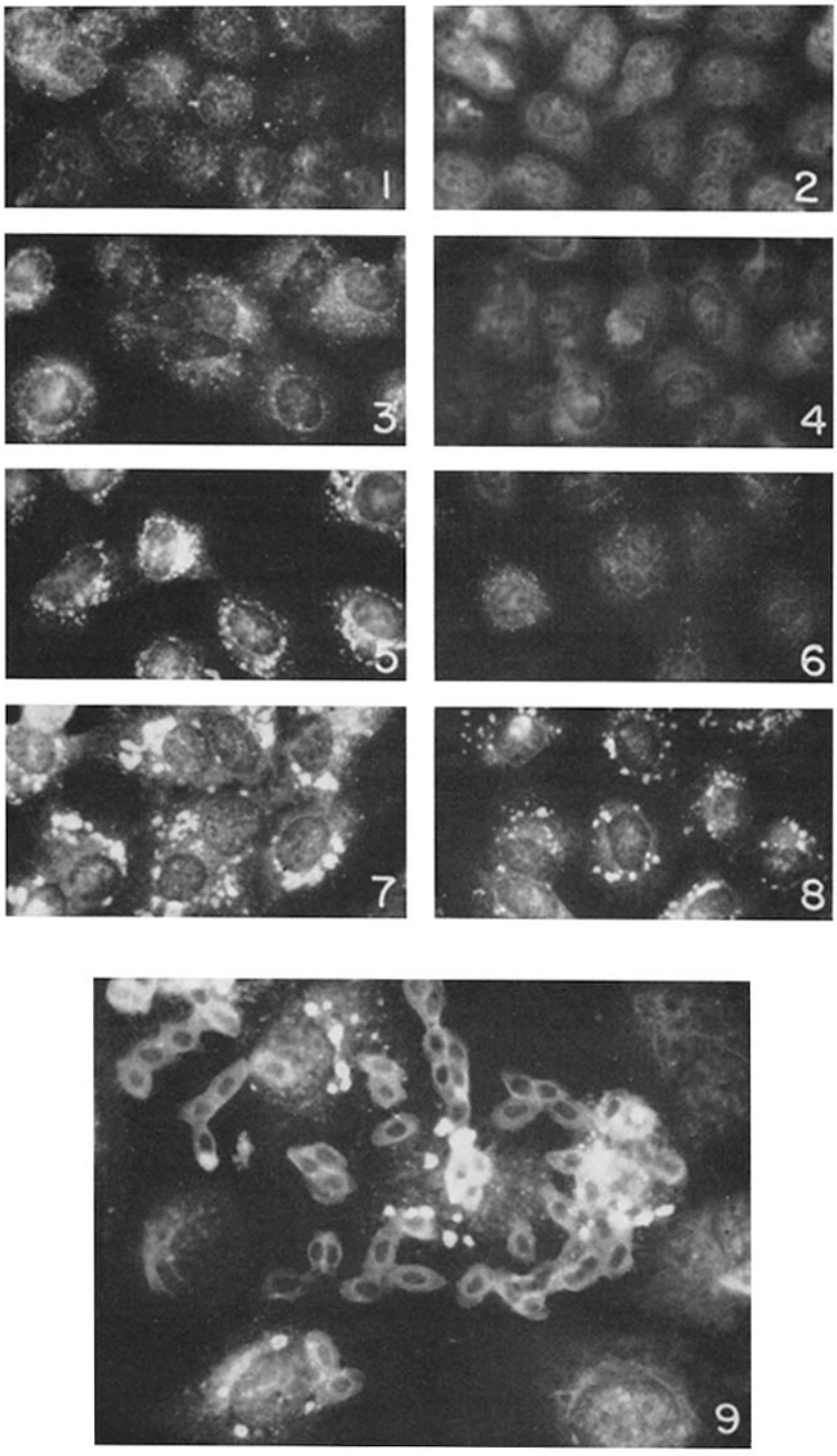
FIG. 5. At 4 hours, in high multiplicity cells, fluorescent foci are larger than those present at 2½ hours.

FIG. 6. At 4 hours, low multiplicity cells contain moderate numbers of fluorescent foci of variable size in the cytoplasm; these foci represent newly formed NDV antigen.

FIG. 7. At 6 hours, in high multiplicity cells, fluorescent foci are larger than those present at 4 hours.

FIG. 8. At 6 hours, in low multiplicity cells, fluorescent foci also appear larger than those present at 4 hours.

FIG. 9. Photomicrograph of HeLa cell monolayer culture infected with NDV. Culture was treated with chicken erythrocytes, fixed in acetone, and stained with fluorescent antibody. Only those HeLa cells which contain virus-specific fluorescent foci in the cytoplasm have adsorbed erythrocytes. × 660.



(Wheelock and Tamm: Newcastle disease virus-HeLa cell interaction)

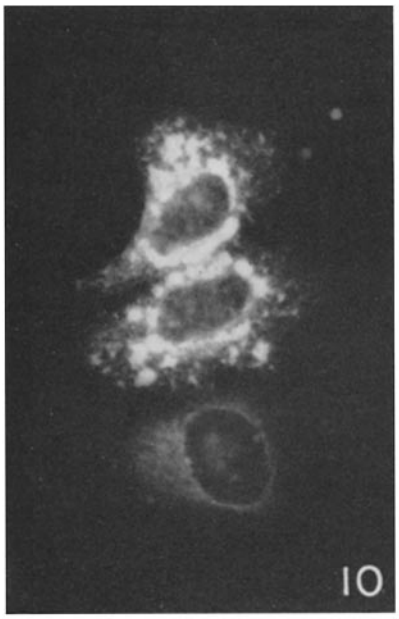
PLATE 47

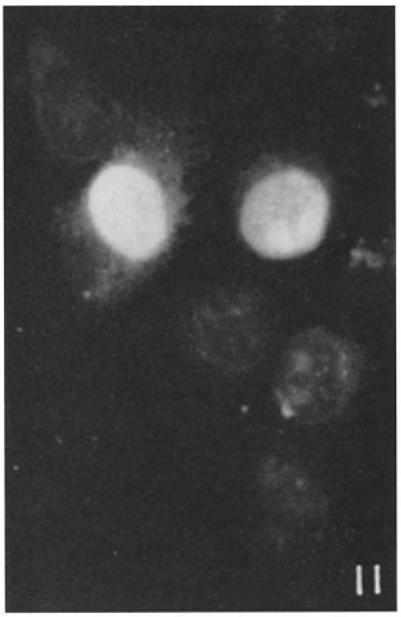
FIGS. 10 to 12. Photomicrographs of HeLa cell monolayer cultures infected with either Newcastle disease virus (NDV), or influenza virus (NWS), or both. Staining was done with anti-viral immune sera followed by sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate. × 660.

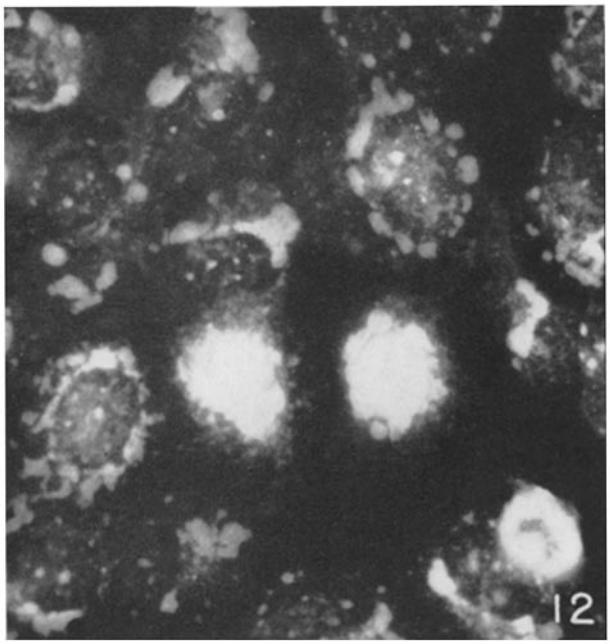
FIG. 10. HeLa cells infected with NDV. In the staining anti-NDV serum was used. Large clumps of NDV antigen are present in the cytoplasm, but none in the nucleus.

FIG. 11. HeLa cells infected with NWS. In the staining anti-NWS serum was used. NWS virus antigen is localized primarily in the nucleus; some is also present in the cytoplasm.

FIG. 12. HeLa cells infected with both NDV and NWS. In the staining, anti-NDV and anti-NWS sera were used. Large clumps of NDV antigen are present in the cytoplasm of cells which also show intense nuclear fluorescence due to NWS antigen.



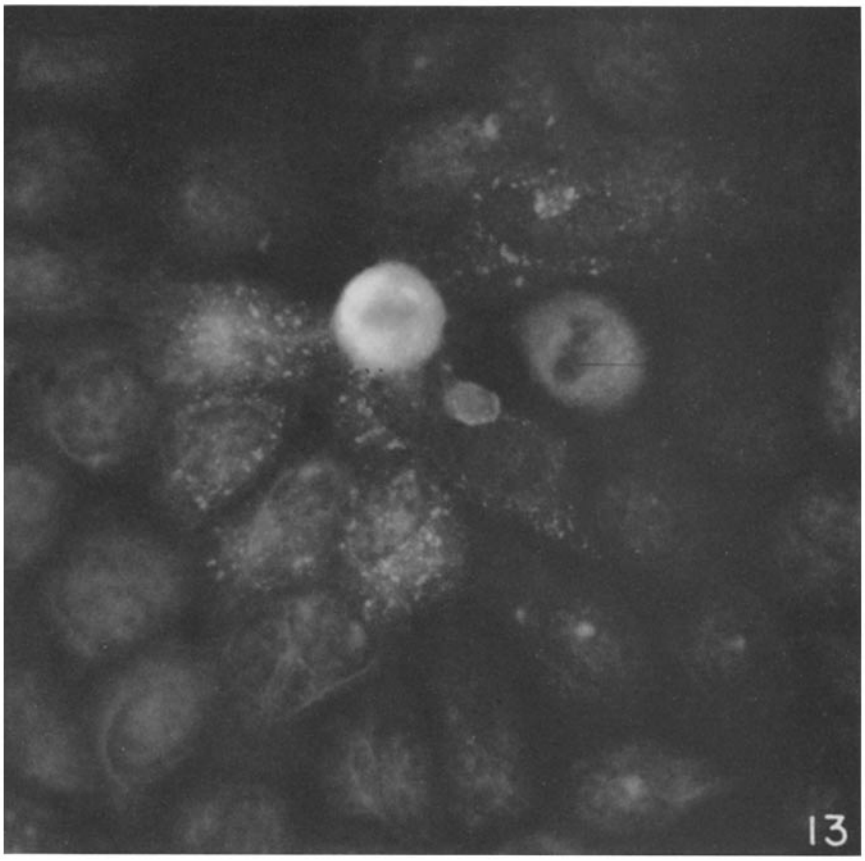




(Wheelock and Tamm: Newcastle disease virus-HeLa cell interaction)

PLATE 48

FIG. 13. Photomicrograph of HeLa cell monolayer culture infected with NDV and stained at 15 hours with anti-NDV immune rabbit serum followed by fluorescein isothiocyanate–labeled sheep anti-rabbit globulin. A cell in metaphase contains large amounts of virus antigen and is surrounded by 6 to 8 satellite cells containing smaller amounts of antigen. One of these satellite cells is also in metaphase. × 850.



(Wheelock and Tamm: Newcastle disease virus-HeLa cell interaction)