Cytological Studies of Newcastle Disease Virus (NDV) in HEp-2 Cells.* (28891)

CHARLES F. JOHNSON AND ANTOINETTE D. SCOTT (Introduced by B. Roizman)
Department of Neurological Medicine, Johns Hopkins University School of Medicine, Baltimore, Md.

The growth and alterations caused by Newcastle disease virus in several cell lines (1-10) reveal that the virus multiplies and accumulates in the cytoplasm. During cytological studies of the changes caused by NDV at different temperatures of incubation, we discovered that the virus in HEp-2 cells causes the production of viral antigen in the nucleus. Moreover, 2 types of multinucleated cells or syncytia differing in appearance developed in infected HEp-2 cell cultures. These observations have not previously been reported.

Materials and methods. Solutions. Phosphate buffered saline with 1% bovine albumin (PBS-A), (Armstrong Co., Chicago, Ill.) was used as diluent for virus and antibody. The growth medium consisted of Mixture 199 and 10% calf serum. Two maintenance media (MM) were used: MM-1 consisted of Mixture 199, 4% calf serum, and 1% rabbit-anti-NDV (Ra-a-NDV) except where antibody was eliminated or decreased. MM-2 consisted of Mixture 199 and 1% calf serum.

Virus. The 43rd passage of a virulent chicken-adapted CG strain of Newcastle disease virus (NDV) was used in these experiments.

Virus assay was done by a method similar to that used for assay of Herpes virus(11) but not previously used with myxoviruses. Briefly, monolayer cultures were exposed to virus at 37°C for 1 hour, then, overlaid with MM-1. The plates were fixed with methanol, then stained with Giemsa 4 days after infection. The plaques were approximately ½-¾ mm in diameter (Fig. 1A) in the presence of optimal concentration of antibody (0.8 hemagglutination inhibition units per ml). A linear relationship was observed between the number of plaque forming units (PFU) and the relative virus concentration (Fig. 2). Suboptimal antibody concentration failed to contain the virus in a localized area and characteristic "spreading" was observed (Fig. 1B).

Antibody. Rabbits were inoculated for a period of 6 weeks with 2 injections a week of a mixture of stock NDV (1 part) and Freund's adjuvant (3 parts). The animals were bled from the ear at 4, 5, and 6 weeks. The Ra-a-NDV sera were pooled, filtered, heated at 56°C for 30 minutes and stored at −60°C. The hemaggulination inhibition titer of the pooled serum was 80 units per ml.

Ra-a-NDV serum was conjugated with fluorescein isothiocyanate employing the method of Riggs(12). Conjugated serum was absorbed once with hamster liver powder and twice with HEp-2 cell powder. For fluorescent antibody staining, a 1:4 dilution of the conjugated serum was used.

Cytologic studies. Cytologic studies were

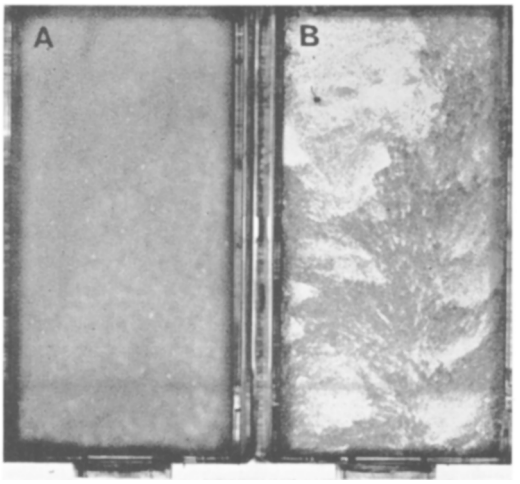

FIG. 1. A. Localized lesions seen on day 5 infected cultures overlaid with maintenance medium containing antibody. B. Spreading effect seen on day 5 in identically-infected cultures without antibody in maintenance medium.

* Supported by grants from U.S.P.H.S. to Dr. C. N. Luttrell and by allocation from Institutional grant of Am. Cancer Soc. to Johns Hopkins Med. Inst.

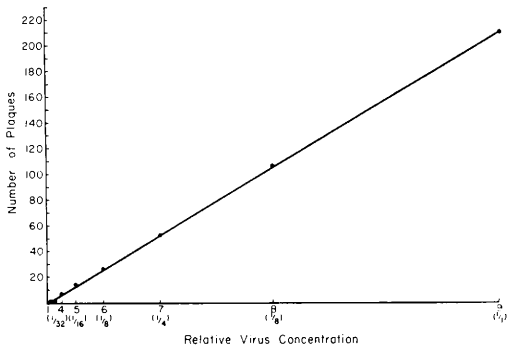

FIG. 2. Proportionality between relative virus concentration and no. of plaques counted.

performed on HEp-2 cell coverslip cultures prepared in Leighton tubes. Each coverslip culture was exposed to 1 ml of PBS-A containing 20,000 PFU and incubated at 37°C. After 1 hour of exposure, the inoculum was aspirated and 1 ml of MM-2 was added to each of the Leighton tubes. The cultures were incubated at 32°, 37°, and 42°C. At 2 hour intervals during the first 24 hours and at 4 hour intervals during the second 24 hours after infection, coverslip cultures were fixed with 95% ethanol at —60°C in individual vials. These were then removed, air dried, and stained with hematoxylin and eosin for inclusion bodies and acridine orange(13) to detect changes of nucleic acid content in infected cells. Fluorescein labeled antibody was incubated with fixed coverslip cultures at 37°C for 90 minutes to detect viral antigen. Most of these coverslip cultures were counterstained with lissamine rhodamine RB200 bovine albumin(14). The coverslips were examined with a Leitz Ortholux microscope fitted with a tungsten and an Osram HBO 200 mercury vapor lamp. Photographs were taken on high speed Ektachrome type A.

Results. With all stains, changes were seen in mononucleate cells and in multinucleate cells or syncytia. It is convenient to consider these changes according to the stain used to visualize them.

1. *Studies on fluorescent antibody stained preparations.* Viral antigen was observed in the nucleus and cytoplasm as specific fluorescence in coverslip cultures incubated at 37° and 42° and fixed 6 hours after infection. In coverslip cultures incubated at 32°, specific fluorescence was observed only in cultures fixed 12 hours or later after infection.

The fluorescence, indicating the presence of viral antigen, was present in the cytoplasm and in the nucleus. In the cytoplasm, it took the form of a halo surrounding the nucleus. Intranuclear fluorescence was present in only half of the fluorescing cells; it took the form of a single central body with several fine radiating strands, most of which could be traced to the nuclear membrane (Fig. 3A, D, E). The perinuclear halos which at first appeared diffusely fluorescent became granular 8 to 10 hours after infection. Subsequently, between 24 and 48 hours after infection the granules increased in size and the intranuclear fluorescence disappeared. As the granules became larger, the margins of the granules fluoresced with greater intensity than their centers, giving the impression the centers were devoid of viral antigen, or were impenetrable to the labeled antibody (Fig. 3B, C).

It is interesting to note that single cells and syncytia in cultures infected with NDV take up large quantities of rhodamine conjugated albumin and fluoresce intensely with a red color (Fig. 3F).

2. *Studies on hematoxylin and eosin stained preparations.* When incubated at 37° and 42°, infected coverslip cultures could not be differentiated from uninfected cultures earlier than 12 hours after infection. At 32°, infected and uninfected cultures could not be differentiated until 20 hours after infection.

In the infected cells, the nuclei were prominent and appeared to consist of basophilic strands which were thicker than those seen in uninfected nuclei. Subsequently, the reticulum of each cell stained more basophilic and became more granular. In addition, basophilic granules appeared first at the nuclear membrane and later in the cytoplasm. These granules increased in number and size until about 48 hours after infection. At the same time or shortly thereafter, the infected cells showed disruption of nuclear membranes and bubbling of their cytoplasms (Fig. 4A).

Syncytia with nuclear and cytoplasmic changes similar to those seen in infected

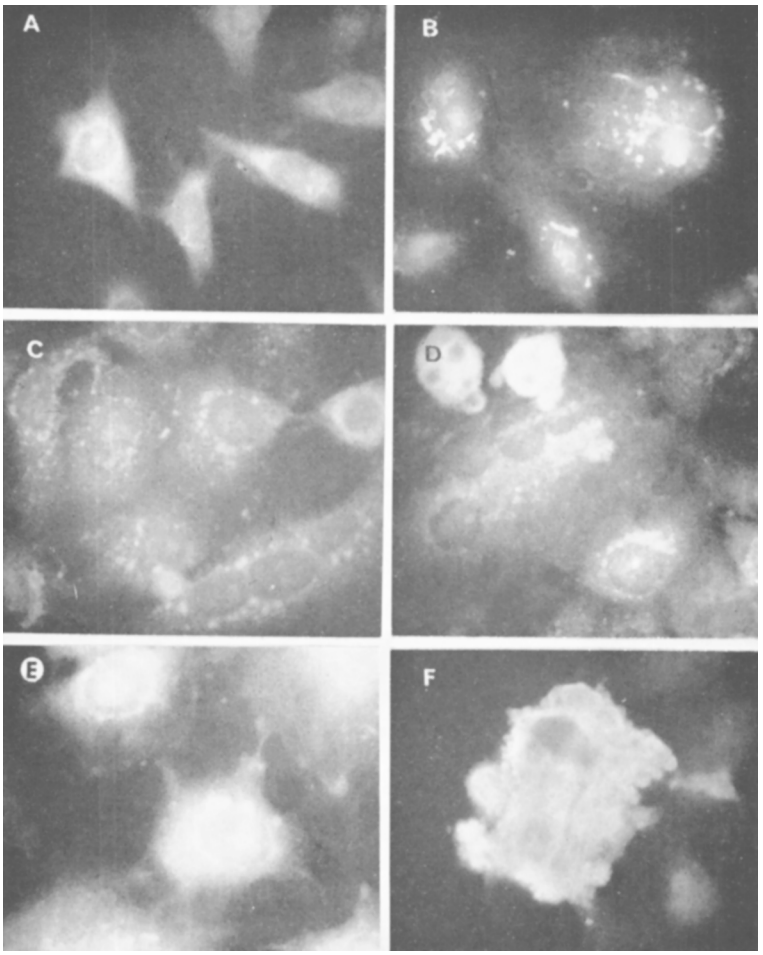

FIG. 3. Infected cells incubated at 37°C and stained with fluorescein conjugated antibody and rhodamine conjugated albumin. A. Mononucleated cells, one of which has a prominent perinuclear halo and intranuclear fluorescent body. 225 ×. 16 hr after infection. B. Mononucleated cells with large cytoplasmic granules. 225 ×. 28 hr after infection. C. Mononucleated cells and syncytium with cytoplasmic granules of varying sizes. 225 ×. 32 hr after infection. D. Mononucleated cells, one of which has a prominent intranuclear staining body. Strands connecting central fluorescent mass with nuclear membrane were prominent, but reproduced poorly in the photograph. 225 ×. 28 hr after infection. E. Mononucleated cells, one of which has both intranuclear and cytoplasmic fluorescence. 225 ×. 24 hr after infection. F. Syncytium showing loss of structural detail and intensely staining bubbling cytoplasm. 225 ×. 48 hr after infection.

mononucleate cells were first seen at 14 hours in 37° and 42° cultures, but not until 22 hours in the 32° cultures.

The syncytia in HEp-2 cultures may be classified in two groups. Those composing one group contained usually no more than 8 nuclei arranged in a circle or crescent (Fig. 4B). The nuclei of these syncytia appeared smaller in size than those of uninfected mononucleate cells. These syncytia were present in both infected and uninfected cultures. In the infected cultures, the nuclei of these syncytia showed changes similar to those of mononucleate cells. Basophilic granules were seen around the nuclei and then in the cytoplasm of these syncytia.

The second group was composed of syncytia of a different type or a more advanced stage of the syncytia already described. These contained as many as 50 nuclei or nuclear fragments (Fig. 4C). They stained intensely basophilic and little nuclear detail could be

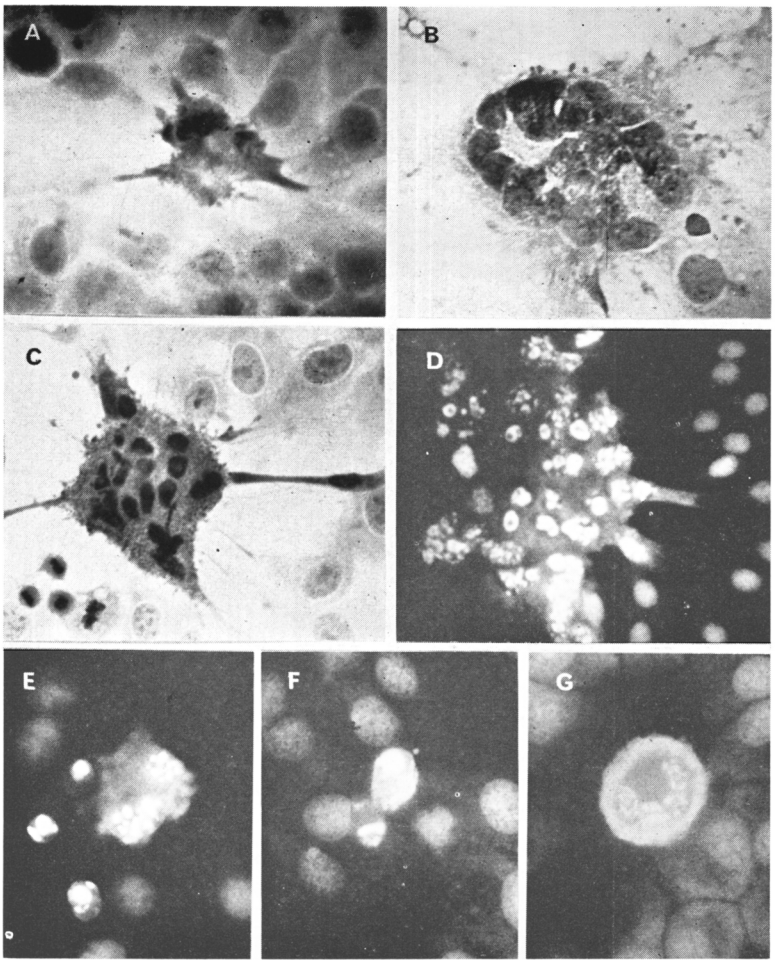

FIG. 4. Infected cells incubated at 37°C and stained with either hematoxylin and eosin or acridine orange. A. Mononucleated cell showing basophilic cytoplasmic granules and nuclear and cytoplasmic disruption. 225 ×. 22 hr after infection. H & E. B. Large syncytium with circular nuclear configuration showing basophilic cytoplasmic granules. 225 ×. 48 hr after infection. H & E. C. Syncytium with numerous basophilic cytoplasmic granules, nuclear fragmentation, and cytoplasmic bubbling with prominent intercytoplasmic bridging. 225 ×. 22 hr after infection. H & E. D. Large syncytium with alterations in neighboring cells, prominent intercytoplasmic bridge, and acridine orange positive material in the cytoplasm. 80 ×. 36 hr after infection. Acridine orange. E. Two mononucleated cells showing nuclear changes and acridine orange positive cytoplasmic granules. 225 ×. 36 hr after infection. Acridine orange. F. Mononucleated cells showing circular intranuclear pattern of chromatin margination and an intranuclear structure adjacent to the nuclear membrane. 225 ×. 36 hr after infection. Acridine orange. G. Mononucleated cell with nuclear margination and bubbling cytoplasm. 225 ×. 36 hr after infection. Acridine orange.

discerned. On the basis of observations made of coverslip cultures stained at different times after infection, it would appear that the cytoplasms of these syncytia tended to migrate toward their centers leaving behind strands of cytoplasm which gave the appearance of being intercellular bridges. Mononucleate cells adjacent to syncytia showed evidence of viral infection more frequently than cells more distal. These cells also showed similar cytoplasmic processes which seemed to extend to adjacent cells. Frequently small basophilic granules were seen in these processes.

3. *Studies on acridine orange stained preparations.* Cultures incubated at 37° and 42° showed changes characteristic of viral infec-

tion 12 hours after infection when stained with acridine orange. Equivalent changes were not seen in 32° cultures until 20 hours after infection. In mononucleate cells and syncytia, these changes consisted of nuclear margination of chromatin and the development of yellow-orange staining perinuclear cytoplasmic granules which were presumed to be RNA (Fig. 4D, E, F, G). These granules corresponded in number, size, and location with those seen in H & E and fluorescent antibody stained preparations. Granules increased in size and number until approximately 48 hours after infection, at which time nuclear fragmentation and bubbling cytoplasm became prominent features.

Discussion. The main features of our results are the formation of viral antigen in nuclei of infected cells and the finding of two different kinds of syncytia in infected cultures.

An intranuclear phase of fluorescence has not previously been described for NDV. A related myxovirus, the influenza virus, has a prominent phase of intranuclear antigen production(9). Our studies show that NDV antigen may also form in the nucleus. The nature of the antigen, particularly whether it is a structural subunit of the virus, is uncertain.

Inclusions and cytoplasmic fluorescence in other cells infected with NDV have been reported(1,4,5,6,8,9,15). The correlation of basophilic, RNA positive, and fluorescent cytoplasmic granules as described here suggest that these structures are NDV antigen.

We have observed 2 kinds of syncytia but are not certain whether they are distinct types or represent different stages of development of a single type. Different syncytial types in measles infected cell cultures have been described(16,17). It has been postulated that one type of syncytium arises by nuclear division while the other type results from recruitment of cells.

Summary. HEp-2 coverslip cultures were infected with the CG strain of Newcastle disease virus and incubated at 32°, 37°, and 42°C. Infected cultures contained infected mononucleate and multinucleate cells when stained at frequent intervals with hematoxylin and eosin, and acridine orange stains. It was found that fluorescein conjugated antibody stain demonstrated the presence of viral antigen in the nucleus and cytoplasm as early as 6 hours after infection in cultures incubated at 37° or 42°. At 32°, antigen was demonstrable 12 hours after infection. The nuclear fluorescence was transient, disappearing between 24 and 48 hours after infection. In H & E and acridine orange stained coverslip cultures, infected cells could not be differentiated until 12 hours after infection. These stains revealed the presence of granules possibly containing RNA corresponding in position and size to those staining with fluorescent antibody in the perinuclear zone. Syncytia could be classified into 2 groups. They could differ in origin or be different stages of infection of one type. The cultures incubated at 37° and 42° could not be differentiated from each other. The appearance of viral antigen as detected with fluorescein labeled antibody and the cellular alterations appeared to be delayed by approximately 6 hours in infected cultures incubated at 32°.

We are indebted to Dr. Charles N. Luttrell, Dr. Bernard Roizman, and Philip R. Roane for their counsel during experimentation and in preparation of this manuscript.

1. Oh, J. O., *Brit. J. Exp. Path.*, 1961, v42, 424.
2. Bankowski, R. A., Hyde, J., *Am. J. Vet. Res.*, 1957, v18, 743.
3. Das, M. S., Goldberg, H. S., *J. Bact.*, 1961, v82, 151.
4. Wheelock, E. F., Tamm, I., *J. Exp. Med.*, 1961, v113, 317.
5. Adams, W. R., Prince, A. M., *ibid.*, 1957, v106, 617.
6. Prince, A. M., Ginsberg, H. S., *J. Immunol.*, 1957, v79, 107.
7. Love, R., *Ann. N. Y. Acad. Sci.*, 1959, v81, 101.
8. Tanaka, N., Yamaguchi, H., Kobayashi, M., *Jap. J. Microbiol.*, 1959, v3, 345.
9. Traver, M. I., Northrop, R. L., Walker, D. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 268.
10. Zusehek, F., Hanson, R. P., Brandly, C. A., *Am. J. Vet. Res.*, 1959, v20, 111.
11. Hoggan, M. D., Roizman, B., *Virology*, 1959, v8, 508.
12. Marshall, J. D., Eveland, W. C., Smith, C. W.,

PROC. SOC. EXP. BIOL. AND MED., 1958, 98, 898.

13. Armstrong, J. A., Niven, J. S. F., *Nature,* 1958, v180, 1335.

14. Smith, C. W., Marshall, J. D., Eveland, W. C., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 179.

15. Brandt, C., *Virology,* 1958, v5, 177.

16. Roizman, B., Schluederberg, A. E., *Nat. Cancer Inst.,* 1962, v28, 35.

17. Thomison, J. B., *Lab. Invest.,* 1962, v11, 211.

Received September 3, 1963. P.S.E.B.M., 1964, v115.

Preparation of Highly Potent Human Pituitary Gonadotrophins.* (28892)

LEO E. REICHERT, JR. AND ALBERT F. PARLOW (Introduced by V. Popovic)

Departments of Biochemistry and Physiology, Division of Basic Health Sciences, Emory University, Atlanta, Ga.

There have been numerous reports on the preparation of human pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH, ICSH) having relatively high degrees of purity(1,2,3,4). Recently, Reichert and Parlow(5) described a simple, efficient method for a high degree of separation and purification of human pituitary FSH and LH, involving differential adsorption on DEAE-Cellulose followed by stepwise elution with NaCl. The resulting preparations of lyophilized human FSH (29 times as potent as NIH-FSH-S1) and LH (0.9 times as potent as NIH-LH-S1) were more active than any previously isolated(5). Since then, it has been possible to obtain human FSH and LH fractions which are even more active than those of our previous report.

Methods and results. LH activity was determined by the method of Parlow (ovarian ascorbic acid depletion in pseudopregnant rats)(6). FSH activity was determined by the HCG-augmentation method of Steelman and Pohley(7). Descriptions of the methods, design, and statistical analysis of these bioassays have been reported(8,9). *Gel filtration.* Five mg of a partially purified human LH fraction, F-1-2, (0.9 times as potent as NIH-LH-S1), prepared by chromatography on DEAE-Cellulose(5), was applied to a 0.9 × 120 cm column of the hydrated dextran gel Sephadex G-100, previously equilibrated with 0.005 M phosphate buffer, pH 6.0. This buffer was also the solvent for the protein fraction. The column was developed at a flow rate of 6 ml/hour, with collection of 1.5 ml samples per tube. This and all other experiments were conducted at 3-5°C. The ultraviolet elution profile, measured at 275 mμ, is shown in Fig. 1. Protein content of fractions in solution was determined by the ultraviolet absorption method of Waddell (10) and Murphy and Kies(11). A detailed outline of this procedure as we use it has appeared elsewhere(12). The results of bioassays of various fractions are shown in Fig. 1 and Table I. The most potent fraction, #23, was 2.9 times more active than NIH-LH-SI, representing a 3-fold increase in activity over its precursor.

Three mg of a partially purified human FSH fraction F-1-4 (initial potency: 29 times NIH-FSH-S1), prepared by chromatography on DEAE-Cellulose(5), was treated under conditions identical to those described above for gel filtration of the human LH fraction. The ultraviolet elution pattern at

TABLE I. Gel Filtration of Human Pituitary LH on Sephadex G-100.

Fraction	No. of assays	Mean potency (units/mg)*	95% limits
Precursor F-1-2	5	.9	.8–1.1
Tube 17	1	<.2	
18	1	<.2	
19	1	.5	.3– .8
20	2	.9	.6–1.5
23	3	2.9	2.1–4.0
26	2	1.4	.8–2.2
29	1	.3	.2– .5
38	1	<.1	

* One unit = activity in one mg NIH-LH-S1.

* This study was supported by U.S.P.H.S. grants. This is publication number 610, of the Division of Basic Health Sciences, Emory University, Atlanta 22, Ga.