Cytopathic Effect of Infectious Laryngotracheitis Virus in Cultures of Chicken Embryo Kidney Cells.* (28002)

A. M. WATRACH AND L. E. HANSON (Introduced by C. A. Brandly)
Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana

Cellular alterations induced by infectious laryngotracheitis (LT) virus in chicken embryo tissue culture systems have been studied by a number of investigators(1,2,3). Among the changes recorded were "granular" degeneration, clumping of cells, necrosis, and the presence of nuclear inclusion bodies. There has, however, been no report on the formation of multinucleate giant cells in tissue cultures infected with the virus. This communication concerns the occurrence of such giant cells and the development and morphologic and biochemical characteristics of nuclear inclusion bodies.

Materials and methods. 1. *Tissue culture.* Primary monolayer cultures of chicken embryo kidney (CEK) tissue were prepared by trypsinization and grown in 15 mm × 175 mm tubes in a medium consisting of 79.5% Hanks' balanced salt solution, 0.5% lactalbumin hydrolysate, 20% sheep serum, and 100 units of penicillin and 100 μg of streptomycin per ml. The same medium, but with 5% sheep serum, was used for maintenance. When confluent sheets of cells had formed, after 3 to 4 days, the cultures were inoculated with virus.

2. *Virus.* The strain of virus (N71851) used in the study was isolated in 1953 in a chicken with a natural case of laryngotracheitis; it has been maintained in a lyophilized state and tested periodically for viability(4). Prior to the experiment, the virus was passed 5 times in CEK cells. Undiluted culture medium from the last passage, harvested on the 3rd day, served as the source of virus for inoculation.

3. *Neutralization tests.* Equal volumes of undiluted virus and serial 2-fold dilutions of serum obtained from an immunized rabbit were mixed and kept at room temperature for an hour; 0.2 ml amounts were then added to tissue culture tubes, and inoculated on the chorioallantoic membrane (CAM) of 9-day-old chicken embryos. Cell cultures and chicken embryos receiving the same quantity of virus only were used as controls. Neutralization end points were read 3 days after the onset of cytopathic effect (CPE) in control cultures and 5 days after the appearance of plaques in control chicken embryos.

4. *Infectivity tests.* Three apparently healthy chickens, 6 weeks old, which had been kept in isolation from the day of hatching, were each inoculated intranasally with 1 ml of undiluted culture medium from the 5th CEK passage of LT virus. Two days after the first signs of respiratory distress, the chickens were killed and portions of their tracheae processed for histopathologic examination. The presence of nuclear inclusion bodies in epithelial cells was regarded as pathognomonic of infectious laryngotracheitis.

5. *Cytopathologic technics.* For detailed study of the pathologic alterations of infected cells, monolayer cultures were obtained on coverslips (11 mm × 22 mm) placed in each of the tubes. Coverslips were removed 12, 18, 24, 36, 48, 60 and 72 hours after inoculation and fixed in methanol for Giemsa staining, in Zenker's fluid for the Feulgen reaction and hematoxylin and eosin method, and in ethanol for the acridine orange technic. Staining was performed according to standard methods.

Results. Normal control cultures examined 5 days after seeding consisted of confluent sheets of epithelial cells arranged in a pattern of distinct groups or whorls (Fig. 1). Individual cells were polygonal or elongate and had a pale staining nucleus containing one or two nucleoli. Small vacuoles were often present in the perinuclear moiety of the cytoplasm.

* This investigation was supported in part by research grant from Nat. Inst. of Allergy & Infect. Dis., U.S.P.H.S.

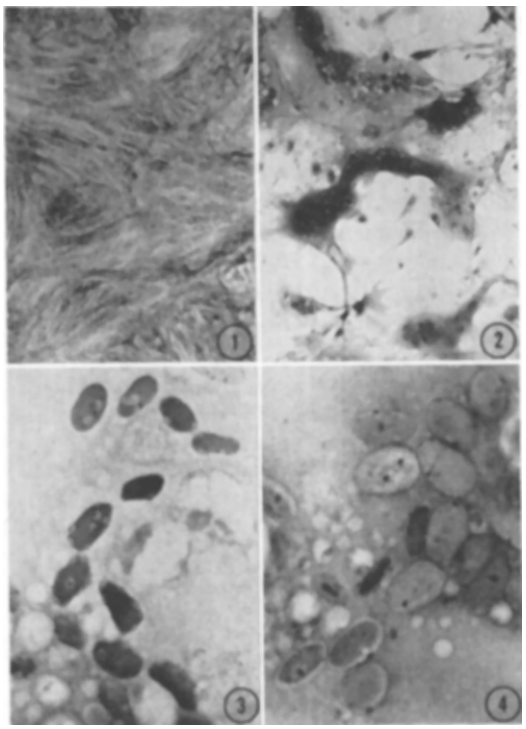

FIG. 1. Normal chicken embryo tissue culture cells. Giemsa stain. Magnification 50 ×.

FIG. 2. Multinucleate giant cells in chicken embryo kidney tissue culture. Giemsa stain. Magnification 50 ×.

FIG. 3. Twenty-four hr after infection. Inclusion bodies fill the entire nuclei. There is no separation between nuclear membrane and the mass of inclusion. Giemsa stain. Magnification 320 ×.

FIG. 4. Forty-eight hr after infection. Some of the inclusions (lower left) are separated from nuclear membrane by a clear zone. Degenerating nuclei containing inclusion bodies are shown in center. Giemsa stain. Magnification 320 ×.

The cytopathic effect of LT virus was characterized by a) degeneration and necrosis, b) formation of multinucleate giant cells, and c) nuclear inclusion bodies. Degenerative changes were detectable as early as 12 hours after infection. Some of the cells, singly or in small groups, showed increased granularity and vacuolation, and rounding off of their cytoplasm. These changes were followed by pyknosis, condensation of the cytoplasm and, eventually, by detachment from the coverslip. While these alterations were clearly evident and continued to take place throughout the entire period of observations, they were not a predominating feature of CPE.

Eighteen hours after infection, the first multinucleate giant cells began to form apparently by fusion of individual cells. The process increased progressively in intensity and by the 36th to 48th hour many giant cells, some with 80 to 100 nuclei, had formed (Fig. 2). The cells varied greatly in size and in number of nuclei, and most of them had numerous, large cytoplasmic vacuoles, usually containing a small amount of finely granular, eosinophilic material. Vacuolation was followed by more pronounced degenerative changes and by detachment of cells. The latter process was manifested in its early stages by sharp demarcation of the periphery of the cytoplasm, with formation of distinct bays and promontories. At the 72nd hour after inoculation, many cells had fallen off the coverslips.

The appearance of nuclear inclusion bodies was an early phenomenon. At the 12th hour after infection, small, irregularly shaped aggregates of basophilic material had formed in some of the nuclei and had begun to coalesce into larger, Feulgen-positive bodies. The deoxyribonucleic acid (DNA) content of these bodies was also verified by the acridine orange technic. Within the next 24 hours, the affected nuclei were filled to capacity with basophilic, DNA-containing material. At this stage, there was no detectable separation between the nuclear membrane and the inclusion body (Fig. 3). Nucleoli were either pushed toward the periphery or remained in their original location, but were separated from the mass of the inclusion by a clear zone. The nuclear membrane was often more prominent, due to margination of chromatin material. At the 36th hour after infection, some of the inclusions appeared to be more condensed and somewhat reduced in size with resulting separation from the nuclear membrane. The process continued with increasing intensity, and at the 48th and 72nd hours many inclusion bodies were separated from the nuclear membrane by a distinct, clear zone. Concurrent with these changes, there was gradual loss of basophilic staining affinity of the inclusion bodies and reduction in their DNA content. At the 72nd hour after infection, many inclusions tended to be

eosinophilic and apparently devoid of DNA.

Serum obtained from a rabbit immunized against LT virus neutralized the effect of that virus in tissue culture cells and in CAM of chicken embryos up to a dilution of 1:64. The identity of the virus was further confirmed by infectivity tests. Inclusion bodies characteristic of infectious laryngotracheitis were observed in the tracheae of 6-week-old chickens infected with the virus.

Discussion. The formation of multinucleate giant cells in tissue culture systems used in the study of viruses is not uncommon. It has been observed following infections with viruses of measles(5,6), herpes simplex(7,8), herpes B(9), mumps(10), canine distemper (11), infectious bovine rhinotracheitis(12), Newcastle disease(13), parainfluenza 2(13), pseudorabies(14), and mouse hepatitis(15). However, the cytopathic effect of some of these viruses has not been consistently characterized by the appearance of giant cells.

Among the factors which are generally considered to be capable of determining the character and intensity of cellular alterations in virus-host cell systems are variations in cytopathogenicity of different strains of the agent, degree of cellular susceptibility, and environmental conditions. The influence of metabolic factors has been demonstrated by Reissig *et al.*(16), and Melnick *et al.*(17). Recently, Moura(5) has shown that formation of giant cells in infection with measles virus is enhanced by the use of medium containing sheep serum instead of serum from other species.

The prominent and distinguishing feature of the cytopathic effect of LT virus described here was the formation of multinucleate giant cells. Since the formation of cells of this type has not been previously reported in cell cultures infected with LT virus, it would seem reasonable to assume that a factor or factors mentioned above may have been involved in bringing about the dissimilarity of the cytopathic effects.

Summary. The cytopathic effect of infectious laryngotracheitis virus in chicken embryo kidney tissue culture cells was characterized by a) degeneration, necrosis and detachment of cells, b) formation of giant cells, some of which contained 80 to 100 nuclei, and c) appearance of nuclear inclusion bodies. In the early stages of formation, the inclusions contained deoxyribonucleic acid as demonstrated by the Feulgen reaction and the acridine orange technic.

The assistance of Mrs. Marilyn Wingard in preparation of tissue cultures is gratefully acknowledged.

1. Atherton, J. G., Anderson, W., *Austral. J. Exp. Biol. Med. Sci.*, 1957, v35, 335.

2. Chomiak, T. W., Luginbuhl, R. E., Helmboldt, C. F., *Avian Dis.*, 1960, v4, 235.

3. Pulsford, M. F., *Austral. J. Exp. Biol. Med. Sci.*, 1960, v38, 153.

4. Watrach, A. M., Hanson, L. E., Vatter, A. E., Watrach, M. A., Rhoades, H. E., *Am. J. Vet. Res.*, 1959, v20, 537.

5. Moura, R. A., *Arch. f. Virusforsch.*, 1961, v11, 487.

6. Rapp, F., Gordon, I., Baker, R. F., *J. Biophys. Biochem. Cytol.*, 1960, v7, 43.

7. Crandell, R. A., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 508.

8. Hoggan, M. D., Roizman, B., *Am. J. Hyg.*, 1959, v70, 208.

9. Falke, D., *Virology*, 1961, v14, 492.

10. Gresser, I., Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 804.

11. Rockborn, G., *Arch. f. Virusforsch.*, 1958, v8, 485.

12. Armstrong, J. A., Pereira, H. G., Andrewes, C. H., *Virology*, 1961, v14, 276.

13. Brandt, C. D., *ibid.*, 1961, v14, 1.

14. Reissig, M., Kaplan, A. S., *ibid.*, 1962, v16, 1.

15. Mosley, J. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v108, 524.

16. Reissig, M., Black, F. L., Melnick, J. L., *Virology*, 1956, v2, 836.

17. Melnick, J. L., Hsiung, G. D., Rappaport, C., Howes, D., Reissig, M., *Texas Rep. Biol. & Med.*, 1957, v15, 496.

Received October 12, 1962. P.S.E.B.M., 1963, v112.