Occurrence of Intranuclear Inclusions in Tissue Cultures Infected with Virus of Infectious Bovine Rhinotracheitis. (23531)

WILLIAM J. CHEATHAM AND ROBERT A. CRANDELL (Introduced by Fred D. Maurer)
Armed Forces Institute of Pathology, Washington, D.C.

The disease now known as infectious bovine rhinotracheitis (IBR) appears to have first been recognized in 1950 in Colorado(1). Since that time it has been reported from a number of states in the western and midwestern part of the United States. Recent papers (2,3) have described the isolation and propagation of the etiological agent in tissue cultures of bovine cells. However, inclusion bodies have not been described in tissue culture or in naturally or experimentally infected animals(4).

It is the purpose of this report to describe the intranuclear inclusions which are a constant feature of the cytopathogenicity of this virus in cultures of bovine kidney and human amnion.

Materials and methods. Tissue cultures of bovine kidney were prepared by a modification of the trypsin digestion technic of Youngner(5). Cells were grown in tubes 16 x 150 mm incubated initially as stationary slant cultures at 35°C; after inoculation they were maintained as either stationary or roller cultures. The nutrient medium consisted of 95% lactalbumin hydrolysate-tris solution (0.5% lactalbumin hydrolysate in modified Hanks buffered salt solution containing 0.01 M tris [hydroxymethyl aminomethane] adjusted to pH of 7.4) and 5% lamb serum. Cultures of human amnion were prepared by the method of Zitcer, Fogh and Dunnebacke (6). Media consisted of 95% of either lactalbumin hydrolysate-tris solution or bovine amniotic fluid and 5% horse serum. Phenol red (0.002%), penicillin (100 U/ml), streptomycin (100 μ/ml) and mycostatin (100 U/ml) were incorporated in all media. Three strains of virus were studied. The first was provided through the courtesy of Dr. W. R. Pritchard, Purdue University, as fluid from the 15th tissue culture passage and is referred to as the Pritchard strain. The American and Cooper strains were obtained from Dr. T. L. Chow, Colorado A & M College, as nasal washings from clinical cases of IBR. Cytopathogenic agents were isolated and passed serially from both nasal washings in cultures of bovine kidney and the American strain in cultures of human amnion. Titration of virus was carried out using cells homologous for the tissue culture system being studied; calculation of ID_{50} was based on an inoculum per culture of 0.1 ml. After fixation in Bouin's fluid hematoxylin-and-eosin stained preparations were made employing either coverslips or the collodion membrane technic(7).

Results. With an inoculum of 100 ID_{50} of Pritchard strain of IBR lesions were first seen in cultures of bovine kidney at 18-24 hours but were generally somewhat more delayed in cultures of human amnion. In both tissues the lesions consisted of small foci of degeneration (Fig. 1). These foci slowly increased in size and new or secondary foci began to appear in an additional 24 hours in kidney and in 36-72 hours in human amnion. The degeneration generally became more diffuse in bovine kidney, involving the entire culture in about 72 hours, while in human amnion the focal nature of the change persisted longer, frequently requiring 10-14 days for total involvement of the cultures. The affected cells were rounded or spindle-shaped with elongated, occasionally beaded, processes. The cytoplasm was opaque and showed fine perinuclear granularity in contrast to the relative nuclear pallor. Nucleoli were small or inapparent. As the degeneration proceeded and foci became confluent the growth was converted into a network of these spindle-shaped cells which remained intact and adherent to the glass for many hours.

As indicated by the pH of the media there was increased acid production in the infected cultures.

A constant feature of the cytopathogenicity of all 3 strains studied was the occurrence in stained preparations of typical intranuclear inclusions in a large proportion of the degen-

erating cells (Fig. 2,3,4). These bodies began to appear at about the time degenerative changes were first detected in the unstained preparation. The earliest change in the nucleus usually consisted of a single small irregular aggregate of amphophilic to eosino-

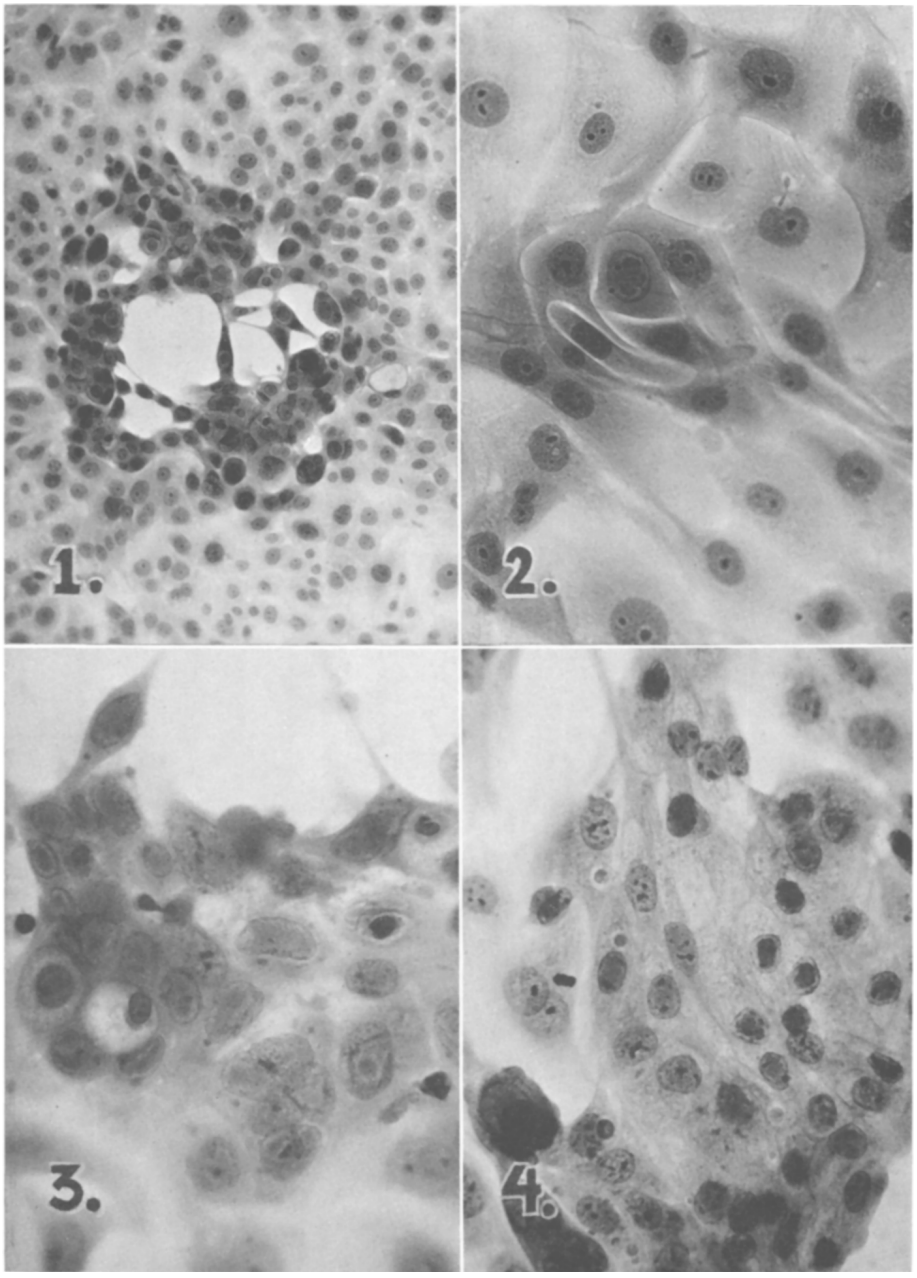

FIG. 1. Early lesion seen in tissue culture of human amnion infected with Pritchard strain of IBR. H & E × 130. AFIP neg. 57 2079.

FIG. 2. Intranuclear inclusion in tissue culture of human amnion infected with Pritchard strain of IBR. H & E × 265. AFIP neg. 57 2072.

FIG. 3. Intranuclear inclusions in culture of human amnion infected with Pritchard strain of IBR. H & E × 450. AFIP neg. 57 2080.

FIG. 4. Intranuclear inclusions in culture of bovine kidney infected with Pritchard strain of IBR. H & E × 450. AFIP neg. 57 4748.

philic, finely granular material among the nuclear chromatin. It appeared that the size of the particles responsible for the granularity of the inclusions was at about the limit of resolution of the light microscope, so that in some preparations the inclusions were homogeneous. The aggregate slowly increased in size until it occupied a major portion of the nucleus. By the time inclusions were well developed, nucleoli were generally small or had disappeared. These mature inclusions retained the amphophilic to eosinophilic staining of the earlier ones and were either round, oval or irregular in contour, generally corresponding to the shape of the nucleus. Although there was not the intense reaction of the nuclear chromatin characteristic of viruses of the herpes group, there was margination of the chromatin toward the nuclear membrane. Even in the late stages of degeneration the nuclear membrane usually remained intact and was separated from the inclusion by a thin clear zone or halo. Intense cytoplasmic eosinophilia accompanied the nuclear changes, but no specific cytoplasmic inclusions were seen. Changes similar to those observed with the Pritchard strain were also seen with the American and Cooper strains.

Cytopathogenicity, including the development of intranuclear inclusions, could be prevented by convalescent serum from clinical cases of rhinotracheitis. No evidence of neutralizing substances was found in the single pool of human gamma globulin tested.

Discussion. Previous reports of rhinotracheitis in tissue culture and of necropsy of naturally and experimentally infected animals have not described the presence of specific inclusions in this disease. In spite of these negative reports the demonstration of distinct intranuclear inclusions in tissue culture of three different strains of the virus of IBR, their consistent association with the cytopathogenicity and the prevention of their development by immune serum leave little doubt that they are a specific manifestation of cellular infection by this virus.

Although the metabolic activities responsible for the decreased pH of cultures inoculated with IBR have not been investigated, it is of interest that similar observations have been made in cultures of HeLa cells infected with adenovirus(8).

We have been able to demonstrate similar intranuclear inclusions in necropsy material fixed in Zenker's and Bouin's fluids. This material was obtained from calves killed during the early stages of disease following intranasal inoculation with the 3 strains of IBR. It appears likely that demonstration of such inclusions in nasal smears or biopsy specimens may facilitate diagnosis of this disease.

Summary. The virus of infectious bovine rhinotracheitis was isolated and passed serially in cultures of human amnion with the production of lesions similar to those occurring in cultures of bovine kidney. Specific intranuclear inclusions were noted in tissue culture and in cases of the disease experimentally produced with the 3 strains of virus studied.

1. Miller, N. J., *J. Am. Vet. Med. Assn.*, 1955, v126, 463.

2. Madin, S. H., York, C. J., and McKercher, D. G., *Science*, 1956, v124, 721.

3. York, C. J., Schwarz, A. J. F., and Estela, L. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v94, 740.

4. McKercher, D. G., Moulton, S. E., Madin, S. H., and Kendrick, J. W., *Am. J. Vet. Res.*, 1957, v18, 246.

5. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

6. Zitcer, E. M., Fogh, J., and Dunnebacke, T. H., *Science*, 1955, v122, 30.

7. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

8. Fisher, T. N., and Ginsberg, H., *ibid.*, 1957, v95, 47.

Received August 14, 1957. P.S.E.B.M., 1957, v96.