Sequential Study on the Development of Infectious Canine Laryngotracheitis Adenovirus

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SUMMARY

Sequential cytological developments in primary dog kidney cells infected with a canine laryngotracheitis virus were studied by examining thin sections with the electron microscope. Early changes in the infected cells were enlargement of the nucleolus and the appearance of heterogeneous dark-staining granules in the nucleus. These granules developed into 'initial bodies' which were shown to contain DNA. The initial bodies increased in size to form 'rings' or more correctly spheres containing less densely staining central cores. The cores disappeared as the inclusions increased in size. Virus particles were occasionally seen at 14 hr and by 16 hr many of the cells contained virus. As the virus particles increased in number they usually aggregated and migrated towards the periphery of the nucleus. Release of the virus into the cytoplasm appeared to be by the formation of protrusions of the nucleus and the pinching off of membrane-bound virus aggregates.

INTRODUCTION

The virus of infectious canine laryngotracheitis has been characterized as an adenovirus serologically related to infectious canine hepatitis (Ditchfield, Macpherson & Zbitnew, 1962). It has also been shown to have a growth cycle similar to other adenoviruses (Yamamoto, 1966) and to be a typical adenovirus with 252 capsomeres with fibres 350 Å long attached to the pentons (Yamamoto & Marusyk, 1968).

Electron- and light-microscope studies on the developmental sequences in human adenovirus replication have been described for type 5 adenovirus by Morgan et al. (1960) and Godman et al. (1960), for human type 1 by Boyer, Leuchtenberger & Ginsberg (1957), and recently for adenovirus type 12 by Martinez-Palomo, Le Buis & Bernhard (1967) and for canine hepatitis virus by Matsui & Bernhard (1967). The present study shows that the virus of canine infectious laryngotracheitis produces cytopathic changes not unlike those produced by human adenovirus types 1, 2, 5 and 6 but that it also exhibits certain differences.

METHODS

Virus. The virus of infectious laryngotracheitis was originally designated as strain TORONTO A26/61 (Ditchfield et al. 1962) and has been subcultivated several times, first in primary dog kidney cells and then in a dog kidney line (Yamamoto, 1966).

Tissue culture. Primary dog kidney cultures were prepared by trypsin dispersion of minced dog kidney. The cells were seeded into 3 oz prescription bottles in Hanks's
lactalbumin hydrolysate medium containing 2.5% calf serum, 50 i.u./ml. penicillin, and 50 μg./ml. streptomycin.

Infection of cells. Monolayers of primary dog kidney cells in 3 oz bottles were drained of medium and 3 ml. of virus suspension added to allow the virus to attach to the cells for 1 hr at 37°. The multiplicity of infection was 10 to 20 as calculated by the limiting dilution method to give a 50% end point. After the adsorption period the fluid was removed and fresh, prewarmed medium was added to the infected cells.

Preparation of specimens for electron microscopy. The infected cells were removed from the bottles with trypsin at 2 or 4 hr intervals and centrifuged at 800 rev./min. for 5 min. The pellets were fixed with buffered osmium tetroxide and sucrose according to the method of Caulfield (1957), dehydrated with a graded series of ethanol, passed to propylene oxide, and embedded in Epon 812 (Fisher Scientific, Fairlawn, New Jersey) in Beam capsules, and hardened at 60° for 24 hr.

Thin sections were cut using a diamond knife (E. I. Dupont de Nemours and Company, Wilmington, Delaware, U.S.A.) on a Porter–Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.). The sections were picked up on carbon-formvar films, stained by treating with CaCl₂ for 3 min., 3% uranyl acetate for 5 min. and lead citrate (Venable & Coggeshall, 1965) for 5 min.

Electron microscopy. The stained sections were examined with a Philips 200 electron microscope at 60 kv with single condenser illumination. Photographs were taken on Kodak 35 mm. fine-grain positive film.

RESULTS

The infected cells underwent a sequence of changes within the nuclei but no marked alterations were noted in the cytoplasm either before or during the period of virus synthesis. Because of the lack of synchrony in infection, several stages of virus-induced changes could be seen at any one time. The sequential changes which the cells undergo could be deduced from the number of cells showing these characteristics at succeeding times.

At 4 hr following infection the virus penticeps were observed to be in membrane-bound vesicles in the cytoplasm often positioned near the nuclear membrane (Pl. 1 b). By the 8th hr both the nuclei and the nucleoli had increased in size with the nucleoli exhibiting a variegated heterogeneous appearance (Pl. 1 c). The earliest developmental stage noted was the appearance of granules in the nucleus at 8 hr after infection (Pl. 1 c) with subsequent enlargement into initial bodies (Pl. 2 a). These granular bodies first appeared at 12 hr and the number of cells containing these increased rapidly by the 16th hr. The initial bodies increased in size and rapidly changed to densely staining ‘rings’ or more correctly spheres with lightly staining centres (Pl. 2 b). These ‘rings’ increased in size and became thicker (Pl. 3 a) until eventually they merged to form one or two large inclusions (Pl. 3 b).

A few virus particles were first observed in a small number of cells at 14 hr during the time when there was an increase in the size of the ring and by the 16th hr cells containing some virus particles were readily detected (Pl. 3 b). As the number of virus particles increased they tended to aggregate into crystalline structures which usually moved towards the periphery of the nucleus (Pl. 3 c). Associated with areas of virus synthesis were areas consisting of diffuse, dark granular particles (Pl. 4 a) which appeared to be virus precursor. In addition to the virus inclusions, two different non-
Electron micrographs of sections of canine kidney cells in culture infected with canine laryngotracheitis adenovirus. The following abbreviations are used: nu, nucleolus; ib, initial body; si, spherical inclusion; mi, mature inclusion; di, dark inclusion; li, light inclusion; gi, granular inclusion; vi, virus inclusion.

(a) Uninfected normal control dog kidney cell.
(b) Adenovirus particles in cytoplasm 4 hr after infection.
(c) Cell 8 hr after infection showing enlarged nucleoli and granular material in nucleoplasm.

T. YAMAMOTO  (Facing p. 398)
(a) Cell 12 hr after infection. Granules have increased in size and appear as dark initial bodies.
(b) Cell 16 hr after infection. Typical dark granular spherical inclusion with lighter staining cells which have developed from the initial bodies.

T. YAMAMOTO
(a) Cell 16 hr after infection. The ‘rings’ have increased in size and are expanding in towards the centre.

(b) Cell nucleolus 16 hr after infection. The dark ring inclusions have filled the light staining centres to produce mature inclusions. Few virus particles may be seen.

(c) Cell 20 hr after infection containing partially aggregated virus particles and a very dark round inclusion. The mature inclusion occupies most of the nucleus.

T. YAMAMOTO
(a) Portion of cell nucleus 24 hr after infection with granular inclusion and dark round inclusions within a lighter staining inclusion. A granular inclusion which may be composed of incomplete virus particles is present.

(b) Virus aggregates in space between the nuclear membranes 60 hr after infection; the virus inclusions are surrounded by a single membrane.

(c) Virus aggregate in cytoplasm 60 hr after infection surrounded by a double membrane.

(d) Free virus particles in the cytoplasm 60 hr after infection.

T. YAMAMOTO
virus inclusions were found closely associated with the virus aggregates. One type was a light staining homogeneous inclusion of inconstant size, shape and number (Pl. 4a), the other was a completely distinct circular or oval intranuclear inclusion consisting of darkly staining material (Pl. 3c).

Release of the virus from the nucleus into the cytoplasm, at least in the later stages, was by the formation of protrusions of the nuclear membrane which when pinched off released either a single-membrane-bound aggregate into the vacuole formed by the separation of the nuclear membrane (Pl. 4b) or a double-membrane-bound aggregate of virus particles into the cytoplasm (Pl. 4c). In the cytoplasm it appeared that the membranes surrounding the virus were destroyed, freeing the virus and allowing its release from the cytoplasm as individual particles.

**DISCUSSION**

The study on the growth of infectious canine laryngotracheitis virus in canine kidney cells (Yamamoto, 1966) and the electron-microscope study presented in this paper show it to be a typical adenovirus.

The entry and movement of the infectious canine laryngotracheitis adenovirus appears to be by a phagocytic type of process since the virus was found within membrane-bound vesicles. This finding agrees with that of Dales (1962) and of Fong, Bensch & Hsiung (1968), who found that adenovirus is transported in packets within membrane structures to the nuclear membrane. The mode of entry into the nucleus, either of the whole virus or after stripping of the capsid from the intact virus, could not be visualized. Occasionally, some virus particles could still be found within the cytoplasm 8 hr after infection, indicating that entry into the nucleus takes a long time and may explain the delay before the first signs of virus replication. The fact that the virus is eclipsed over a period of 8 hr in a population was shown earlier in one-step growth experiments (Yamamoto, 1966) and accounts for the lack of synchrony in adenovirus multiplication.

Comparative light-microscope studies of human adenoviruses by Boyer et al. (1957) and Boyer, Denny & Ginsberg (1959) showed that the human adenoviruses could be divided into 2 types—those which produced multiple eosinophilic Feulgen-negative inclusions within the nuclei about 14 hr after infection as characterized by types 1, 2, 5 and 6, and those which produced irregular granular masses as characterized by types 3, 4 and 7. This study shows the virus of infectious canine laryngotracheitis in canine kidney cells to be more like that of types 1, 2, 5 and 6 rather than that of types 3, 4 and 7.

The notable feature observed in the canine adenovirus development was the appearance of dense granular spheres, which is characteristic of this virus. Similar inclusions were observed previously and the Feulgen-positive nature of the 'rings' described in the light-microscope study of Boyer et al. (1957) in the early stages of infection. Although Morgan et al. (1966) also described ring-like structures with the electron microscope in the cells infected with human adenovirus type 5, their study did not reveal the temporal relationship to virus synthesis. The 'ring' structures were also observed in the nuclei of dog kidney cells infected with infectious canine hepatitis virus by Garg, Moulton & Sekhri (1967). Similar structures were observed by Sharpless et al. (1961) in chicken cells infected with GAL virus. These studies show that the
formation of spheres in the adenovirus infected cells prior to the appearance of the virus particles indicates that it may be a common feature of adenovirus cytopathology.

Light-microscopic studies of primary dog kidney cells infected with infectious canine laryngotracheitis virus show that the outer portion of the sphere is basophilic while the core is eosinophilic (personal observation). In the study of human adenovirus type 1 by Boyer et al. (1957) multiple inclusions with basophilic virus were described similar to those described here. However, in the subsequent description of the adenovirus group by Ginsberg & Dingle (1965) the basophilic nature of the inclusion is not mentioned. Since the early inclusions or initial bodies contain DNA as determined by the Feulgen reaction (personal observation) before the formation of virus particles it appears that the virus DNA as it is synthesized forms a sphere which is then filled in to form a mature virus-producing inclusion.

In contrast to the development of most adenoviruses, adenovirus type 12 developed much more slowly in KB cells and dense inclusions were not observed until 18 hr and virus particles after 24 hr (Martinez-Palomo et al. 1967). Although a direct comparison of cytopathology of canine adenovirus cannot be made with that produced by human adenovirus type 12, the initial bodies which form 'rings' are probably equivalent to their type 1 inclusion since they are both known to contain DNA. Nuclear bundles observed by Kalnins, Stich & Yohn (1966), and Martinez-Palomo et al. (1967) in the adenovirus type 12 infected KB cells were not seen in the canine adenovirus infected cells and may only be a structural characteristic of the slower replicating, highly oncogenic adenoviruses.

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REFERENCES


Development of infectious canine laryngotracheitis adenovirus


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