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Replication of Porcine Enteric Adenoviruses *in vivo*

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With 6 Figures

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Summary

The replication of an enteropathogenic porcine adenovirus in the intestinal epithelial cells of naturally and experimentally piglets has been studied by transmission electron microscopy (TEM) and by immunoperoxidase (Ip) staining of paraffin sections.

Three types of intranuclear inclusion bodies were observed. Viral particles appeared to be assembled from electron dense crescents which seemed to originate from type II intranuclear inclusion bodies. Virus accumulated in the nucleus of infected cells. It formed paracrystalline arrays in the electron dense centre of the nucleus but was dispersed in the peripheral electron translucent zone. Virus was released from the cells after rupture of the nuclear and plasma membranes.

The results are compared with published data on the replication of adenoviruses in cell cultures.

Introduction

Although adenoviruses were first associated with diarrhea in pigs in 1964 (13) subsequent attempts to reproduce enteritis experimentally have been unsuccessful (9, 14, 15, 23). Hence it has been concluded that adenoviruses are of relatively low pathogenicity for the porcine gastro-intestinal tract (9). Nevertheless adenoviruses are frequently observed in pig fecal material by electron microscopy (4).

It has been shown recently that enteritis can be reproduced consistently in hysterotomy-derived, colostrum-deprived (HDCD) piglets that are infected oronasally with a strain of porcine adenovirus type 3 (7). The lesions are characteristic of an acute viral enteric infection. Similar lesions have been observed in naturally infected two to three week old piglets from field outbreaks of the disease (7). The virus only infects the columnar epithelial cells, unlike enteritic bovine adenovirus which also infects capillary endothelial cells (6). Intranuclear Cowdry type A inclusions have been observed in the enterocytes of the lower small intestine by light microscopy (7).

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This work has now been extended to examine the mode of virus replication in the columnar epithelial cells of experimentally and naturally infected piglets by transmission electron microscopy (TEM) and by immunoperoxidase (Ip) staining of paraffin sections.

Materials and Methods

Virus Source

Porcine adenovirus serotype 3 strain 6618, obtained from Dr. Derbyshire (University of Guelph, Guelph, Canada), was grown in primary porcine kidney cells, maintained in Eagle's minimal essential medium with four per cent fetal calf serum. The second passage was harvested and used as the inoculum.

Experimental Design

HDCD piglets were kept in isolation in Horsfall units. On the second day of life 12 animals were infected oronasally with 4 ml virus suspension (titre $10^{5.3}$ $TCID_{50}$). Two piglets were kept as controls. Animals were killed 3 to 120 days after infection. The sequential pathology of the lesions is described elsewhere (7).

Natural Cases

Two piglets from field outbreaks of diarrhea in two breeding herds were autopsied. Samples from one of these animals were found negative for other infectious agents. The other was only examined morphologically. Adenoviruses were demonstrated in the enterocytes of both animals by TEM and by Ip staining, but the virus was not isolated.

In both the experimental and the naturally infected animals, histological examination of the intestine indicated that the terminal part of the jejunum and the ileum had the most prominent and consistent lesions (7). Therefore, specimens from these regions were taken from TEM and Ip labelling.

Transmission Electron Microscopy (TEM)

Specimens were fixed, dehydrated and embedded in Spurr's resin as previously described (10). Semi-thin and ultra-thin sections were cut and stained as described (10). The ultrathin sections were examined in a Zeiss EM 9 S 2 electron microscope.

Immuno Peroxidase Technique (Ip)

An unlabelled antibody-enzyme technique was used (9). Four µm sections from formalin fixed and paraffin embedded specimens were rehydrated and incubated with the following sera at 22° C:

1. Normal goat serum for 20 minutes.

2. Specific anti-porcine adenovirus strain 6618 serum for 2 hours at a 1/200 dilution or overnight at a 1/800 dilution.

The serum was raised in rabbits by giving 5 fortnightly injections of 10^5 $TCID_{50}$ of the virus. Serum was collected one week after the last injection.

3. Goat anti-rabbit globulin serum (purchased from Pasture, Brussels, Belgium) was used at a 1/20 dilution for 20 minutes.

4. A commercial peroxidase antiperoxidase complex (9) was diluted 1/300 and incubated for 20 minutes.

Between each incubation the sections were rinsed for 5 minutes on a shaker platform with Tris buffered saline. They were subsequently stained for 7 minutes in a solution of 3,3′-diaminobenzidine (DAB), 25 mg of DAB and 0.04 ml of 30 per cent H_2O_2 in 200 ml of Tris buffered saline. Sections from uninfected control animals were included in the procedure. Adjacent sections were stained to show endogenous peroxidase activity. When necessary, Ip stained sections were counterstained with haematoxylin and eosin.

Results

Transmission Electron Microscopy

Ultrastructural observations on the small intestine of control piglets revealed a normal mucosa with high columnar absorptive epithelial cells and goblet cells.

Similar ultrastructural changes were observed in the intestines of naturally and experimentally infected pigs. A number of enterocytes containing viral particles were observed in the jejunum and ileum (Fig. 1).

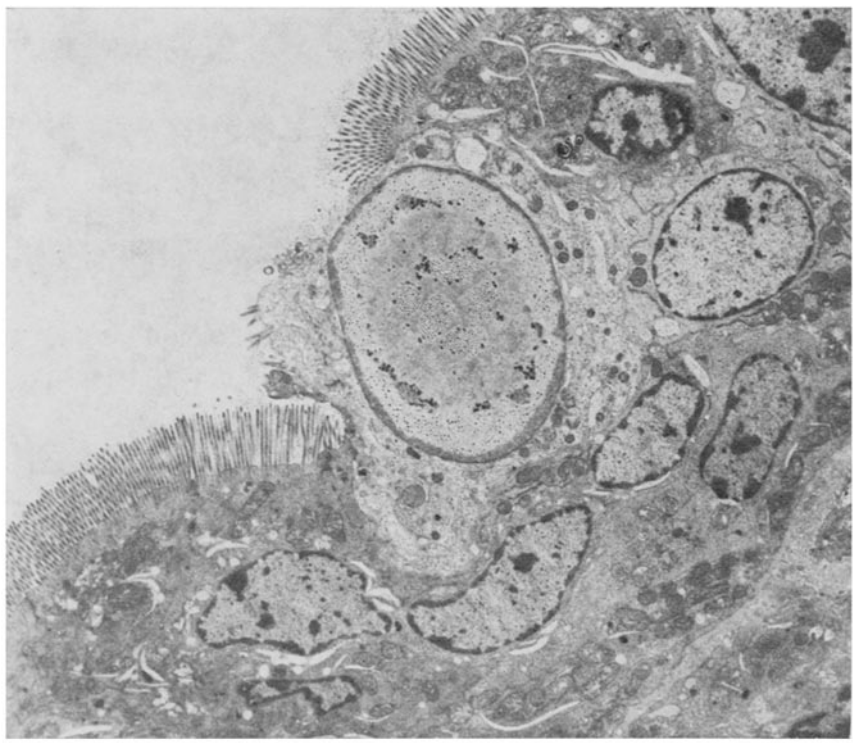

Fig. 1. Infected cells are rounded and have a narrow ring of cytoplasm surrounding a swollen nucleus. Natural infection. ×4875

In addition epithelial cells were seen which did not contain viral particles, but showed ultrastructural alterations which were considered to be virally mediated. These cells were characterized by large irregular intranuclear patches of fairly low electron density. They were composed of fine granules and fibrils which contrasted with the nuclear chromatin by their fine granularity. They sometimes formed a ring with a diameter of up to 3.5 μm (Fig. 2). These structures were designated type II intranuclear inclusions.

Virus particles were confined mostly to the nucleus. When the nucleus contained only a few viral particles, they tended to be distributed towards the nuclear membrane, the central nuclear region being occupied by type II inclusions.

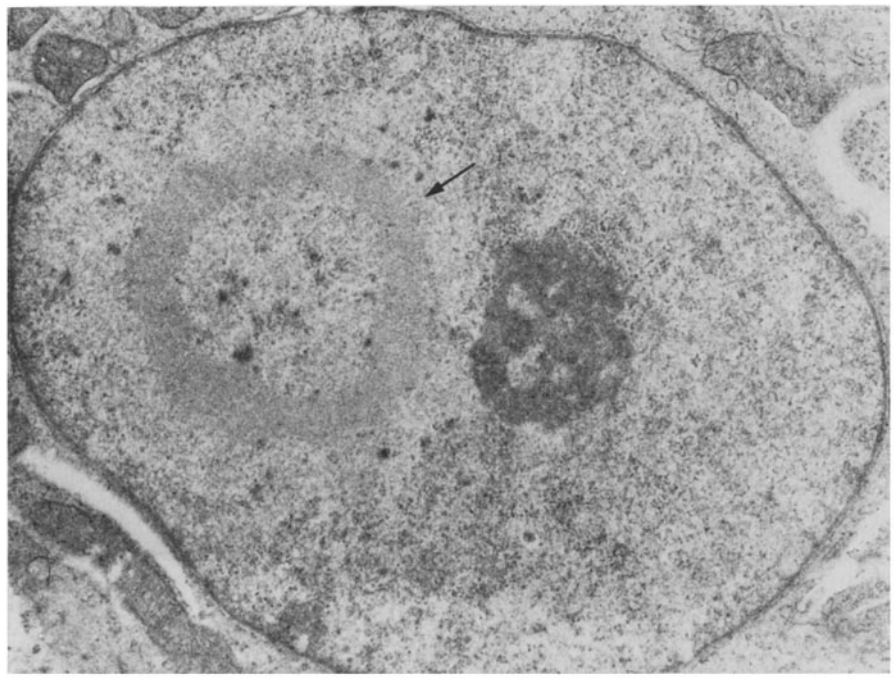

Fig. 2. Ring shaped type II intranuclear inclusion (arrow), viral particles are absent. Intestinal epithelial cell 4 days after inoculation. × 14,400

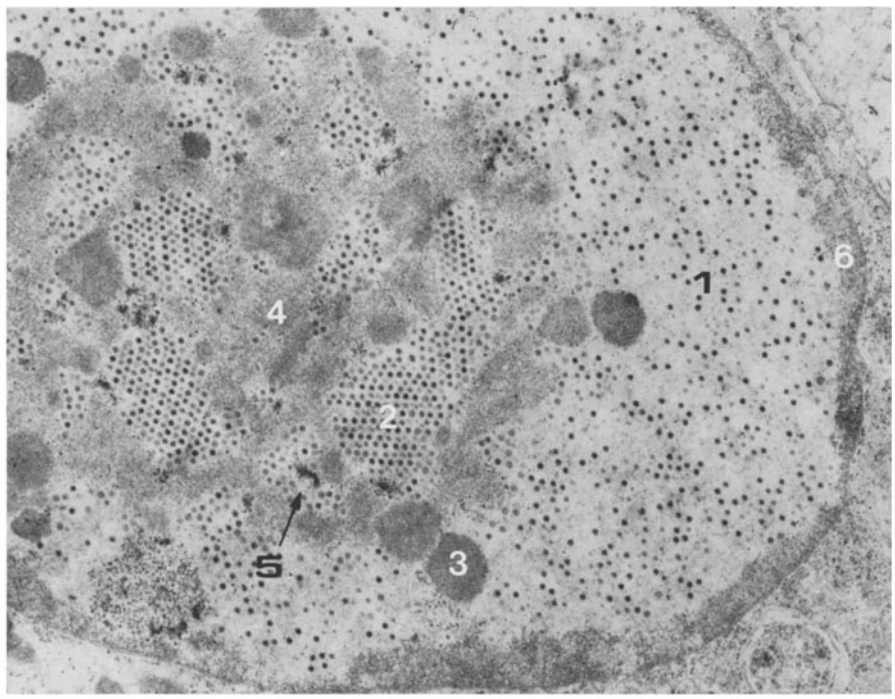

Fig. 3. Heavily infected intestinal cell nucleus. *1* Electron translucent peripheral zone. *2* Paracrystalline arrays of viral particles in the central zone. *3* Electron dense type I intranuclear inclusions. *4* Type II intranuclear inclusions. *5* Type IV intranuclear inclusions. *6* Marginated chromatin. Natural infection. × 14,400

When the nucleus contained many viral particles, as shown in Fig. 3, they tended to be located in closely packed groups in the centre of the nucleus where they formed paracrystalline arrays. These arrays were sometimes very large and composed of virions with a centre-to-centre spacing of 80 nm. Type II inclusions were found between the arrays. Small spherical inclusions, approximately 0.5 to 1 μm in diameter, were also present. They consisted of a fine granular electron dense matrix (Fig. 3) and were mainly located at the boundary of the central nuclear region. They were designated type I inclusions. In addition, small, granular, highly electron dense inclusions were seen in close apposition to the paracrystalline arrays. These were termed type IV inclusions (Fig. 3).

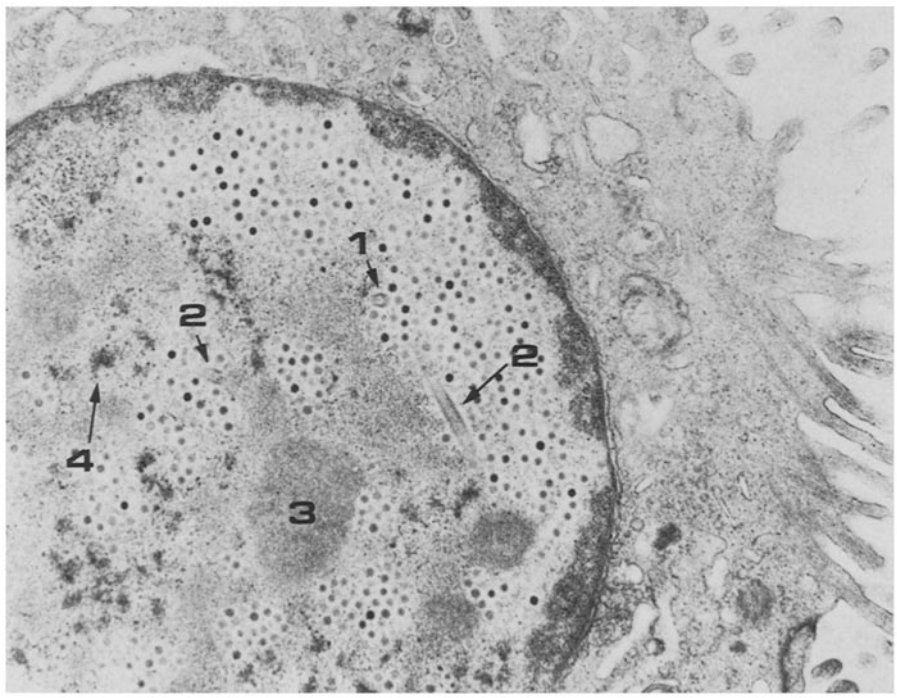

Fig. 4. Transverse *(1)* and longitudinally *(2)* sectioned tubular structures in an infected intestinal cell nucleus. Note type II *(3)* and IV *(4)* intranuclear inclusions, 4 days after experimental infection. × 20,425

Inclusions were not usually observed at the periphery of highly infected nuclei. This zone was electron translucent containing only dispersed fine granular material and many viral particles. The latter were randomly distributed in this area. Rough granulo-fibrillar material, resembling chromatin, was observed adhering to the nuclear envelope. In these infected cells the nucleolus was also usually located at the periphery of the nucleus. Both in the peripheral area and in the central part of these heavily infected nuclei, tubular structures with a diameter of approximately 90 nm were occasionally seen (Fig. 4). They had a thin moderately electron dense coat surrounding an inner highly electron dense tubule which was filled with fine granular material. The lenght of these tubules varied greatly. They were always observed in close association with type II inclusions and with viral particles.

The viral particles were of two types. The first had an electron dense core measuring approximately 50 nm surrounded by an outer capsid. The second had a moderately electron dense core bounded by an inner electron dense shell which was separated from the capsid by a narrow clear space (Fig. 5). The virions had an overall diameter of 65 nm. Empty particles with only a capsid shell were occasionally seen. Virions were found in the vicinity of type II inclusions close to crescent-like structures which seemed to derive from the granulo-fibrillar material of the inclusion (Fig. 5).

In some heavily infected cells, a few viral particles were also observed in the cytoplasm. They were located between the lamellae of the endoplasmic reticulum and in small vacuoles in the apical cell cytoplasm.

Virus release seemed to occur after rupture of the nucleus and the apical cell membrane.

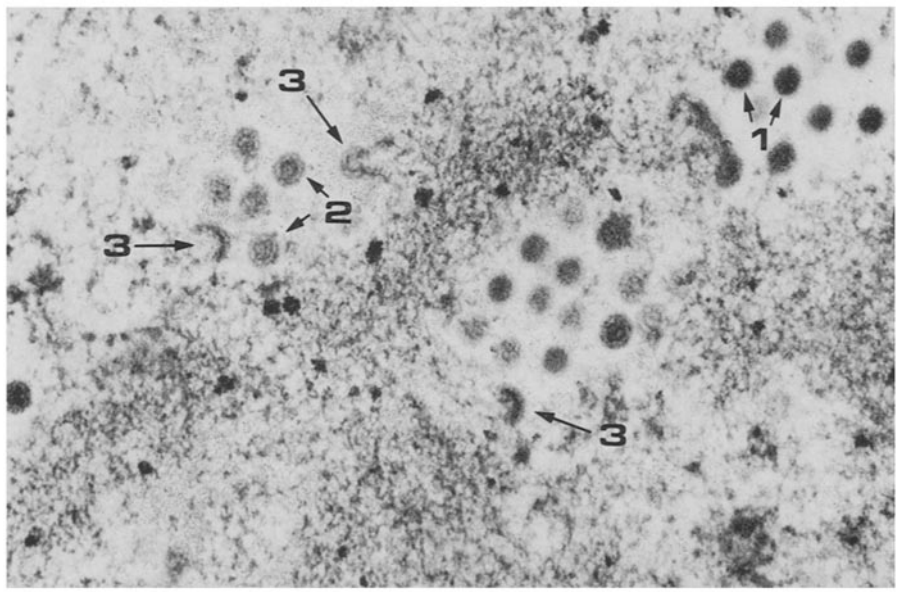

Fig. 5. Two types of viral particles in the nucleus of an infected cell (*1* and *2*). Crescent-shaped structures originating from type II inclusion body material *(3)*, 4 days after experimental inoculation. ×78,400

Immuno Peroxidase

Sections of intestine from control piglets were negative on Ip staining.

This technique revealed a difference between the experimentally infected animals and the field cases; in the latter, infected intestinal epithelial cells were more weakly stained. Therefore, the description given here will refer mainly to the experimental animals.

Ip positive cells were only found in the epithelium of the villi. Positive cells were occasionally found in the crypts but the majority were found higher up the villi. In a number of cells staining was restricted to the supranuclear cytoplasmic zone. However, most positive cells were stained throughout their cytoplasm. This

cytoplasmic staining had a fibrillar arrangement (Fig. 6). The intracytoplasmic localisation of the stain was confirmed by counterstaining Ip labelled sections with H. & E. and by comparing the relationship of infected to non-infected cells at the ultrastructural level (Fig. 1). In some cells with a positive Ip cytoplasmic stain, the nucleus was faintly stained (Fig. 6). A patchy nuclear staining pattern was observed in other cells but, on the whole, nuclei showed intense uniform staining.

In the Ip stained sections it was never possible to recognize the peripheral clear nuclear halo seen in H. & E. stained sections of infected tissue cultures (7). The different stages of Ip staining described above could often be seen in neighbouring cells on the same villus.

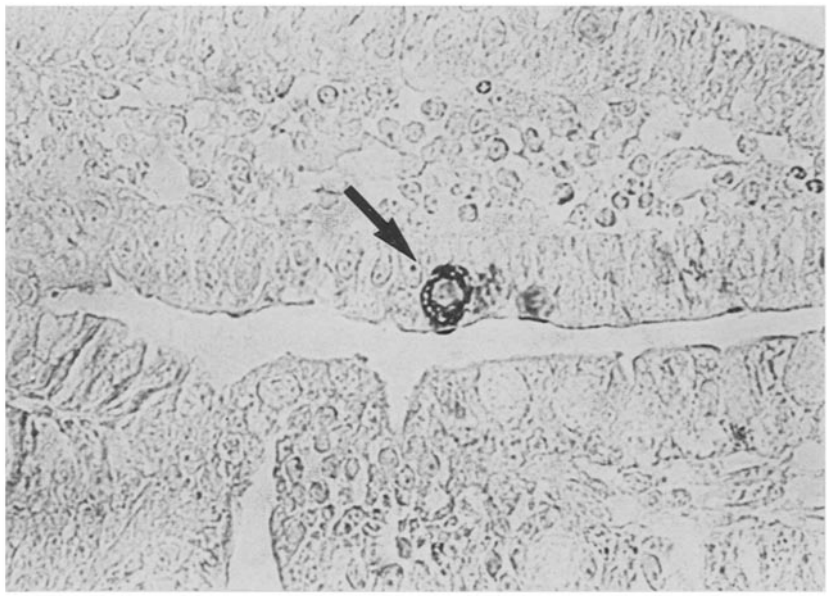

Fig. 6. Ip positive cell on an intestinal villus. Note the fibrillar arrangement of the cytoplasmic stain. ×400

Discussion

Adenovirus replication has been studied mainly in tissue culture (1, 2, 3). Porcine adenovirus replication has been investigated in tissue culture by histological (8), immunofluorescence (16, 20, 21) and TEM (8, 17) techniques. This is the first *in vivo* study of the replication of a porcine adenovirus. The first ultrastructural change observed in the enterocytes of infected pigs was the appearance of type II intranuclear inclusion bodies. The nomenclature used for the different ultrastructural intranuclear inclusion bodies in the literature is confusing. The one used here is comparable to that used for human adenovirus intranuclear inclusion bodies in tissue culture (18, 26). The ring formation of the type II inclusion has also been observed in tissue cultures infected with equine adenovirus (22). Type III inclusion bodies (18) were not observed in the present study. Type I inclusions were frequently seen, but were never found in the absense of viral particles and type IV inclusions were observed in close association with paracrystalline arrays

of viruses. The parallel fibre inclusions described in bovine adenovirus (3, 24) and in human adenovirus 12 infected cell cultures (18) were not observed.

Rectilinear profiles have been described in tissue culture cells infected with porcine adenoviruses (8, 17) but were not seen in our *in vivo* infected material, however, the tubular structures described above may bear some resemblance to these rectilinear profiles. The budding of viral particles from rectilinear profiles has been described (17) but our tubular structures do not appear to be involved in a similar process. The tubular structures in the present material were quite similar to those seen in cell cultures infected with fowl adenoviruses (1).

Histological investigations of cell cultures infected with canine adenovirus have shown a halo around the periphery of infected nuclei and this has been attributed to chromatin margination (19). Similar nuclear chromatin margination and the formation of a peripheral electron lucent zone were observed in the present material. Viral particles, both in the central and peripheral regions of infected nuclei, were of two types. Two types of viral particles have also been observed in bovine adenovirus infected cell cultures (2) and in experimental enteric adenovirus infection of mice (25), whereas in pig kidney cell cultures infected with porcine adenovirus strain 25R three types of viral particles have been described (17).

The appearance of crescent-shaped structures at the margin of type II inclusions have to our knowledge not been described before. They may represent the assembly of the viral particles. The initial appearance of viruses in the vicinity of type II inclusions has been described for pig adenovirus strain 25R (17) and for bovine adenovirus (5) replicating in cell cultures.

The intranuclear distribution of viral antigen as demonstrated by the Ip technique is comparable to immunofluorescence studies on porcine (16, 20, 21) and other adenoviruses (1, 2) in cell cultures and on mouse adenovirus *in vivo* (20). The patchy distribution probably corresponds to type I and II inclusions. Studies on cultured cells infected with human adenovirus have shown that these inclusions contain viral protein (15). The strong Ip positive reaction in the cytoplasm may be due to the production of excess viral protein, as has been suggested for adenoviruses in cell cultures (12).

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