Ultrastructural Study of Long-Term Canine Distemper Virus Infection in Tissue Culture Cells

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The morphogenesis of canine distemper virus was studied in Vero cell cultures for 43 days post-inoculation. Active replication of the virus was observed by electron microscopy and assay from 12 h after inoculation on, and peak production was observed on days 5, 14, and 22. From day 28 on, constant but smaller amounts of infectious virus were detected. Two ultrastructural types of intracytoplasmic nucleoprotein filaments were observed; although they first appeared at different times, their subsequent chronological patterns of development were similar. The cells apparently became free of virus by a mechanism of vacuolation. Intranuclear filaments were seen about day 11 and appeared to increase in number thereafter, whereas the infectious titer declined. Possible mechanisms of persistence are discussed in the light of these findings.

Paramyxoviruses can cause subacute diseases of the central nervous system, subacute sclerosing panencephalitis (SSPE) in humans (3, 4), and canine distemper in animals (10, 22). Measles virus, which belongs to this group, may produce a slow or persistent infection, as shown by immunopathological findings (6, 13), and has been isolated from patients with SSPE who have had childhood measles (5, 7, 11, 17, 18, 20). The mechanism of slow virus infection is not clear. Adams and Bell (1) suggested that after measles, virus infection RNA undergoes a reverse transcriptase-mediated change to a DNA form, and that this change is brought about by coinfection with a leukovirus.

Paramyxovirus nucleoprotein filaments occur in large intracytoplasmic and intranuclear aggregates. The intracytoplasmic filaments have been described as covered by fuzzy coats which have been resolved in a pentagon shape (15), whereas intranuclear filaments do not have fuzzy coats. In short-term (16) and long-term studies of SSPE and measles virus (19), intranuclear inclusions were observed only in samples from patients with chronic infections. This paper reports the first long-term study of chronic canine distemper virus (CDV) infection of Vero cells to reevaluate the mechanism of virus persistence.

MATERIALS AND METHODS

Virus. The Rockham strain of CDV was obtained as "Vaxitas D" vaccine (Tasman Vaccine Laboratory). This strain, which is grown in dog kidney cells, was passaged in dog kidney cells on four further occasions and in Vero cells on several occasions in our laboratory.

Tissue culture technique. Vero cells (an African green monkey continuous cell line) were obtained from Flow Laboratories, Inc. Bottles (600 ml) were seeded with 7 x 10⁵ cells. Growth and maintenance media were minimal essential medium and Hanks balanced salt solution containing 10 and 2% fetal calf serum, respectively, plus the following antibiotics: penicillin, 100 µg/ml; fungisone, 1 µg/ml; and kanamycin, 50 µg/ml. Confluent monolayers were inoculated with 3 x 10⁸ PFU of CDV. The virus was allowed to adsorb for 1 h at 37°C before maintenance medium was added; the resulting cultures were then incubated at 37°C in 5% CO₂ in air. CDV-infected cultures were sampled daily for up to 43 days after infection. Unoinculated cultures were taken as comparable controls on the same days.

Viral assay. For the assessment of the amount of free infective virus produced each day, tissue culture fluid was harvested daily, and the cells were washed three times with phosphate-buffered saline and replaced with an equal volume of fresh maintenance medium. To determine the amount of all associated infective virus, I scraped the cells in the same amount of maintenance medium after washing them three times with phosphate-buffered saline.

Electron microscopy procedure. Cells were removed from the bottles after trypsinization, washed in Hanks solution, and pelleted at 250 x g for 15 min. A required part of the pellet was fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate solution at pH 7.2 for 1 h, followed by 1% Daltos osmic acid (7) for 2 h. After dehydration, the tissue was embedded in Epon. Thin sections were cut with a diamond knife on an Ultracut (LKB Instruments, Inc.). Sections were stained with uranyl acetate or lead citrate or a combination of both (14) and examined in a Philips 300 electron microscope.

To avoid examining the same cell twice with the electron microscope, I cut 10 blocks of each specimen and based the results on counting 100 cells and the free virus particles between the extracellular spaces.
FIG. 1. Growth of CDV in Vero cell cultures which were inoculated with $2 \times 10^5$ PFU of virus. The supernatant fluids (●) and cell-associated virus (▲) were assayed as described in the text.

RESULTS

Viral assay. The replication of small amounts of CDV in Vero cells, (Fig. 1 and 2) was detected as early as 12 h postinfection. Significant amounts (Fig. 1 and 2) of virus were recovered from the tissue culture media up to 28 days postinfection, and then low titers were observed on the remaining 15 days of the study (Fig. 1 and 2). Virus was consistently associated with the tissue culture cells in titers ranging from $10^{-3}$ to $10^{-6}$ (Fig. 1).

FIG. 2. Continuous production of CDV nucleoprotein filaments and virus particles, as determined by electron microscopy over 43 days. The percentage of cells containing different types of nucleoprotein filaments and virus particles on each day postinfection is shown. Symbols: ○, free and budding particles; ▲, cell-associated virus type I; ●, cell-associated virus type II; □, nuclear filaments.

FIG. 3. Electron micrograph of Vero cells at day 4 postinfection. Note that every cell contains at least one CDV nucleoprotein inclusion body (arrows). $\times 3,000$; bar = 3,000 nm.
Cytoplasmic changes. On examination of sections after 12 h of incubation of the virus, about 1 of 10 inoculated cells showed small intracytoplasmic CDV nucleoprotein filaments. Some of these infected cells also showed budding particles on the surface. After 25 h of incubation, the number of infected cells increased, and by day 4, almost every cell cross section contained at least one intracytoplasmic inclusion of nucleoprotein filaments (Fig. 3 and 4). Longitudinal and transverse sections of the CDV nucleoprotein filaments showed an electro-dense core coated with fuzzy material, as described previously for measles virus (15). Cross sectioned, the central core of the nucleoprotein measured about 16 nm.

The number of budding particles seen also increased with the incubation period and was maximal by day 5 (Fig. 2). The number of particles seen correlated with the viral assay (Fig. 1 and 2).

From days 5 and 6 on, some of the infected cells developed intracytoplasmic vacuoles which increased in size and were present in the majority of cells by day 8 postinfection (Fig. 5). As the size and number of vacuoles in each infected cells increased, the virus filamentous inclusions were retained the cytoplasmic strands (Fig. 5), which eventually broke off from the main cell body: the cells thus became free of the inclusions (Fig. 6). At this stage, the cells became much smaller, about two-thirds the normal size, and the number of cells containing the virus nucleoprotein inclusions decreased. The cells then increased in size, and, after division, confluent monolayers were produced. The second cycle started when these inclusion bodies reappeared in most of the cells, and peaks occurred on about days 14 and 22 (Fig. 2). From day 22 on, the number of these inclusion bodies decreased.

On the day 7 postinfection, besides the typical CDV nucleoprotein inclusion body described above (Fig. 3 through 5), another type of filamentous intracytoplasmic inclusion body appeared in some of the cells (Fig. 7A and B). A few of the cells contained both types of inclusions. The number of cells containing the latter type of inclusion reached a maximum by about
day 10 (Fig. 2). At low magnification, this inclusion body appeared granular (Fig. 7A) and stained less intensely. At higher magnification, the nucleoprotein filaments were more loosely packed and had ill-defined outlines, compared with the first type of inclusion (Fig. 8A and B). The filaments measured about 14 nm in diameter (Fig. 7B). This type of inclusion, as visualized by electron microscopy, showed peaks on days 10 and 18 postinfection (Fig. 2).

Nuclear changes. Nuclear changes became apparent with the appearance of intranuclear filaments on about day 11. These changes began with the transformation of nucleoli into granular reticulated masses or several small clumps of granular material. The longer the incubation period, the more filaments were contained in the infected nuclei. (Fig. 2). Each filament appeared to be made by a helical tube about 16 nm in diameter and a clear central core (Fig. 9). These intranuclear tubes were distinguishable from their intracytoplasmic counterparts by their lack of an outer pentagonal coat (Fig. 9).

FIG. 5. Electron micrograph of a Vero cell at day 8 postinfection showing extensive vacuolation of cytoplasm. Note that the viral material is being held by cytoplasmic strands (arrow). ×6,600; bar = 1,500 nm.

DISCUSSION

The results of the present experiments show that CDV replicated in Vero cells throughout the 43 days of the study. Synthesis of infectious virus was continuous; peak production on certain days indicated that production was cyclical. The cells appeared to become free of virus after vacuolation. The virus was continuously detected in the supernatant fluid, although on certain days, titers were low. No ultrastructural differences were seen in the old Vero cell cultures, the virus nucleoprotein present in them, or the structure of the budding virus particles.

It has been reported that measles virus shows varying degrees of persistence and latency after primary infection. Raine et al. (19), who have studied long-term measles infection in cultures of hamster dorsal root ganglion, did not observe cycles in the production of infectious measles virus or cytoplasmic nucleoprotein, but they did show that synthesis of infectious measles virus continues until day 25 postinfection, as com-
pared with day 43, when, in the present study, observations were discontinued.

The results of the present long-term tissue culture study demonstrate how cells manage to survive CDV infection. This peculiar method of shedding virus by vacuolation has not been observed for any other virus. It is difficult to say that these cells become completely free of virus, but it is certain that they divide because the cell sheet becomes confluent again.

Raine et al. (19) explained that the low titers in the tissue culture fluid after day 26 postinfection are due to the trapping of the virus particles within the interstices of the tissue. This phenomenon was not observed for CDV. However, very few cells contained CDV nucleoprotein, and in only a few of the cells were budding particles present.

Although a number of hypotheses have been proposed to explain the mechanism of persistence of measles virus in tissue culture and animal and human brain tissue (1, 3, 5), these hypotheses are not supported by experimental data. The present ultrastructural studies, combined with viral assay, demonstrate that virus multiplication slows down during a long incubation period, and a state of balance is achieved between virus multiplication and survival of the cell. The results of the viral assay of fresh Vero cells did not indicate that the virus was growing more slowly; therefore, it seemed that the virus had not changed its character to a slow type by this time. It seems likely that the cells acquired a mechanism of self-protection. There are various possibilities, although for tissue culture, one can discount involvement of antibody or cell-mediated immunity. It is possible that in tissue culture, the infected cells repeatedly free themselves of virus, divide, and are reinfected so that a cell population is selected which is more resistant to reinfection by the virus.

The second possibility involves the production of interferon (10). It has been shown that this class of substances is produced by cells in tissue culture or animals infected by almost any animal virus containing either DNA or RNA. Wong et al. (23) showed that primary African green monkey cell cultures infected at a low or high multiplicity of infection produce small or large amounts of interferon, respectively, and that after infection at a high multiplicity, interferon production continues as long as the cells

FIG. 6. Electron micrograph of a Vero cell at day 9 postinfection showing a few dense bodies (arrows). Note that there is no filamentous material in the cell. ×11,800; bar = 1.000 nm.
FIG. 7. Electron micrograph of a Vero cell at day 7 postinfection showing type II CDV nucleoprotein filaments in the cytoplasm. (A) ×11,000; bar = 1,000 nm. (B) ×21,000; bar = 500 nm.
FIG. 8. Electron micrographs of Vero cells showing types I (A) and II (B) at the same magnification (×52,000). Compare the sharp outline of type I both in longitudinal and transverse sections with the outline of type II (arrows). Bar = 500 nm. (C), Higher magnification (×80,000) of (A); (D) higher magnification (×80,000) of (B). Bar = 250 nm.
survive. The authors suggested that persistent infection of African green monkey cell cultures by rubella virus is associated with persistent activity of the interferon system.

Of the two types of filamentous inclusions seen on various days, type I, which appeared first, ultrastructurally resembled in shape and size the inclusions reported in previous studies (9, 12, 22).

The second filamentous inclusion, type II, which appeared on about day 7, has not been described before. The filaments, although slightly smaller in diameter than those of type I, also appeared to be loosely packed. On the basis of ultrastructure, they can be identified as nucleoprotein filaments. It is quite possible that the CDV used in the present study was a mixture of two strains with different incubation periods. Recently, Allen et al. (2), using an experimental hamster model, isolated large and small plaque-forming strains from Onderstepoort CDV, but as yet, these researchers have not demonstrated the ultrastructural features of the strains.

The intranuclear aggregates of filamentous material which appeared late on about day 11 persisted throughout the entire subsequent period of infection. The late appearance of intranuclear filaments also occurs in measles virus, as described by Raine et al. (19). It is important to point out that when the number of intracytoplasmic inclusion bodies and the viral titer were decreasing, the number of nuclei containing intranuclear filaments were increasing. I have previously shown (15) that the intranuclear filaments lack the fuzzy "m" protein coat; therefore, it appears that these filaments represent incomplete nucleoprotein.

LITERATURE CITED


