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Growth of canine distemper virus in cultured astrocytes: relationship to *in vivo* persistence and disease

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Canine distemper virus (CDV) causes an encephalomyelitis in dogs which varies with the viral strain. The CDV Cornell A75-17 strain produces a delayed, subacute to chronic, demyelinating CNS disease. In contrast, the Snyder Hill (CDV-SH) strain-associated neurological disease is more acute in onset, is usually non-demyelinating and primarily produces lesions in the gray matter. In these studies we describe the effects of these two virulent and one avirulent CDV strain, Rockborn (CDV-RO), on astrocytes in dissociated canine brain cell cultures. In multiple replicate experiments, astrocytes were infected most rapidly by CDV-RO [100% of astrocytes were infected by 14 days post-inoculation (p.i.)]. This strain caused severe cytopathic effect (CPE) and cytolysis. CDV-SH similarly produced a rapid infection of the astrocytes. In contrast, CDV A75-17 infected less than 25% of the astrocyte population during the first 28 days p.i. ($\pm$7 days); after 28 days p.i., a rapid rise in astrocyte infection occurred. Both virulent viruses caused astrocytic syncytial formation but did not cause cytolysis of the astrocyte population as was observed with the attenuated virus. Titers of infectious virus, released into the supernatant fluid, reflected the degree of astrocyte infection. Virus released by the cultures late in CDV A75-17 infection showed enhanced ability to infect newly derived astrocytes; in contrast, brain cell passaged CDV-SH did not show increased growth in these cells. These results show that (1) there is a difference in growth rate, CPE and capacity for adaptation of three different CDV strains in astrocytes *in vitro*, and (2) some aspects of the disease (such as persistence in white matter) produced by the virulent strains *in vivo* may be related to the course of astrocyte infection observed *in vitro*.

Key words: canine distemper virus; astrocytes; persistent infection; encephalomyelitis.

Introduction

Canine distemper virus (CDV) is a morbillivirus closely related to measles virus and is an important pathogen in the dog. Following infection, systemic illness is often followed by neurological disease which may be acute, subacute or chronic. Experimental studies have characterized the pattern of central nervous system (CNS) disease induced by various strains of CDV.[1–3] In comparative studies[4] we demonstrated that both the course of the disease and the target areas within the CNS are virus-strain dependent. The Snyder Hill strain (SH) produces an acute encephalitis with predominantly gray matter injury, and only in rare instances results in demyelinating white matter lesions.[5] This contrasts with the CDV A75-17 strain which usually results in a more delayed,

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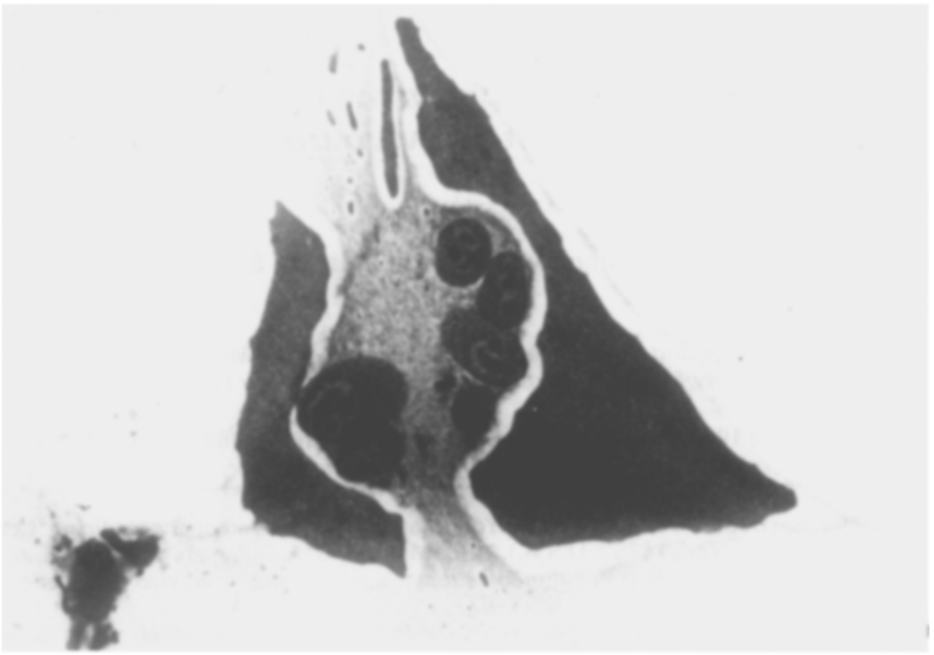


Fig. 1. Multinucleated syncytia and CDV inclusions stained with Shorr stain in cultured canine brain cells. Cultures were derived from newborn dogs and inoculated with CDV at 7–10 days *in vitro*. Infected cultures were fixed in 50% ethanol/50% ether, stained for 1 min in Shorr stain (Harleco Corp.) and counterstained with hematoxylin. CDV-RO 21 days p.i. 250×.

subacute to chronic encephalitis in which myelinated areas of the CNS are more strikingly affected than gray matter.

These naturally occurring viral strains, which result in different forms of encephalomyelitis, are of interest as models for examining mechanisms which may function in similar chronic human diseases of the CNS such as multiple sclerosis (MS), postvaccinal measles encephalitis, and subacute sclerosing panencephalitis (SSPE). To this end we have compared the effects of CDV strains on astrocytes in dissociated canine brain cell cultures. Infection of other glial cells and neurons has also been examined and will comprise a separate report.

Results

Expression of viral antigen and cytopathic effects (CPE) in astrocytes

Astrocytes were the most numerous cell type (50–80% of the cells observed) in the dissociated canine brain cell cultures derived as described in the Materials and methods section. Astrocytes were identified by labelling with antisera to glial fibrillary acidic protein (GFAP) in an indirect immunofluorescent stain and they varied from broad epithelioid to star-shaped cells. Other cells present in these cultures were oligodendrocytes, neurons, fibroblasts and macrophages which were identified with antisera to cell specific antigens. Infection of these cells by all CDV strains except CDV A75-17 was detected and will be reported separately (manuscript in preparation).

Viral inclusions and CPE were readily observed in brain cells stained with Shorr stain (Fig. 1). Astrocytes were among the first cells infected by the three viral strains used (Fig. 2) and by 7 days post-inoculation (p.i.) exhibited both intranuclear and cytoplasmic viral protein labelling of inclusions (Figs 3–5). As cytoplasmic inclusion

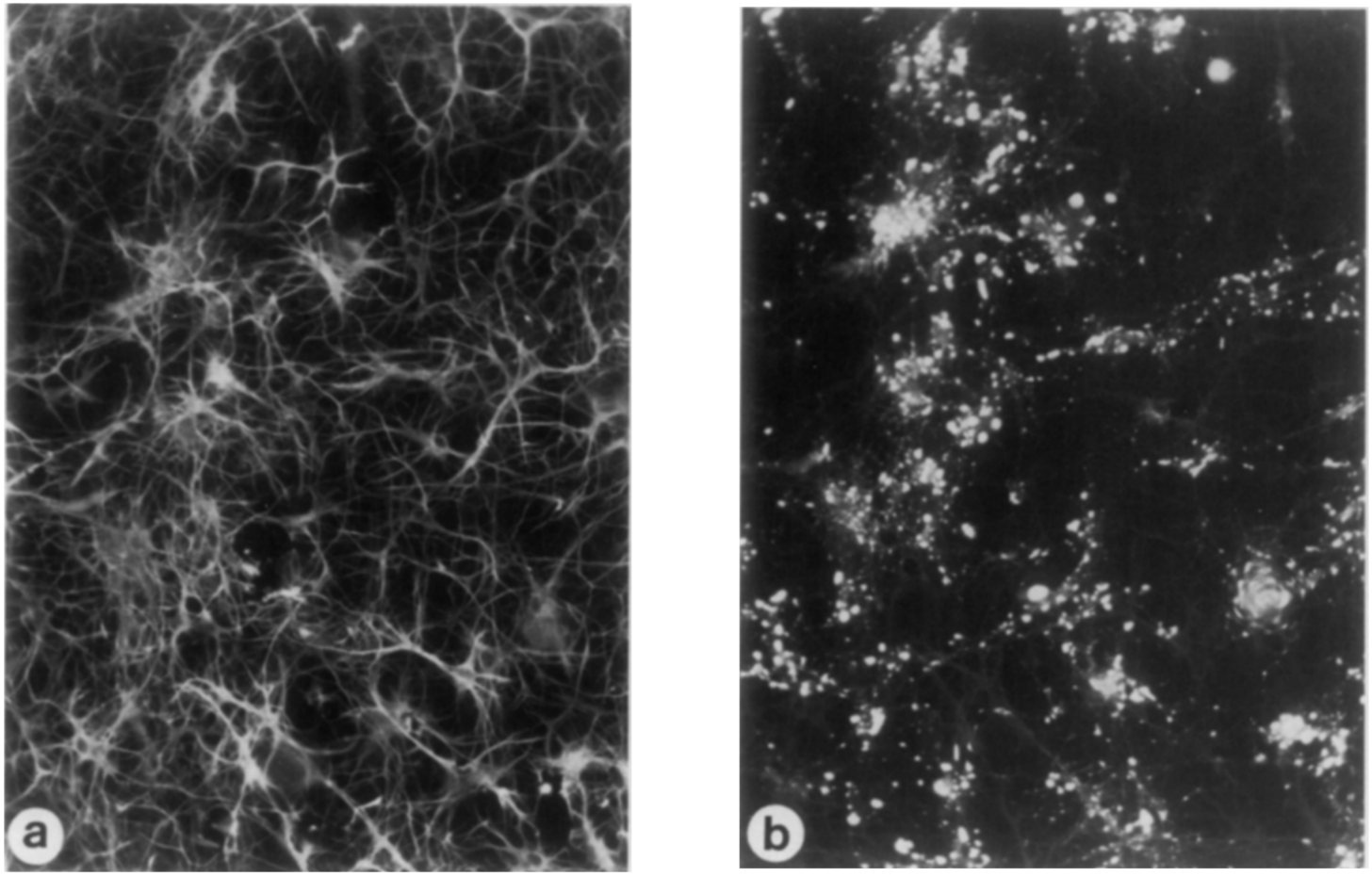


Fig. 2. Double immunofluorescent labelling of astrocytes and CDV-SH antigen in dissociated canine brain cell cultures at 21 days p.i. (a) A field of astrocytes stained with anti-GFAP (1:50) and rhodamine-conjugated secondary antibody; (b) the same field showing the staining with fluorescein-conjugated anti-CDV (1:5). 250×.

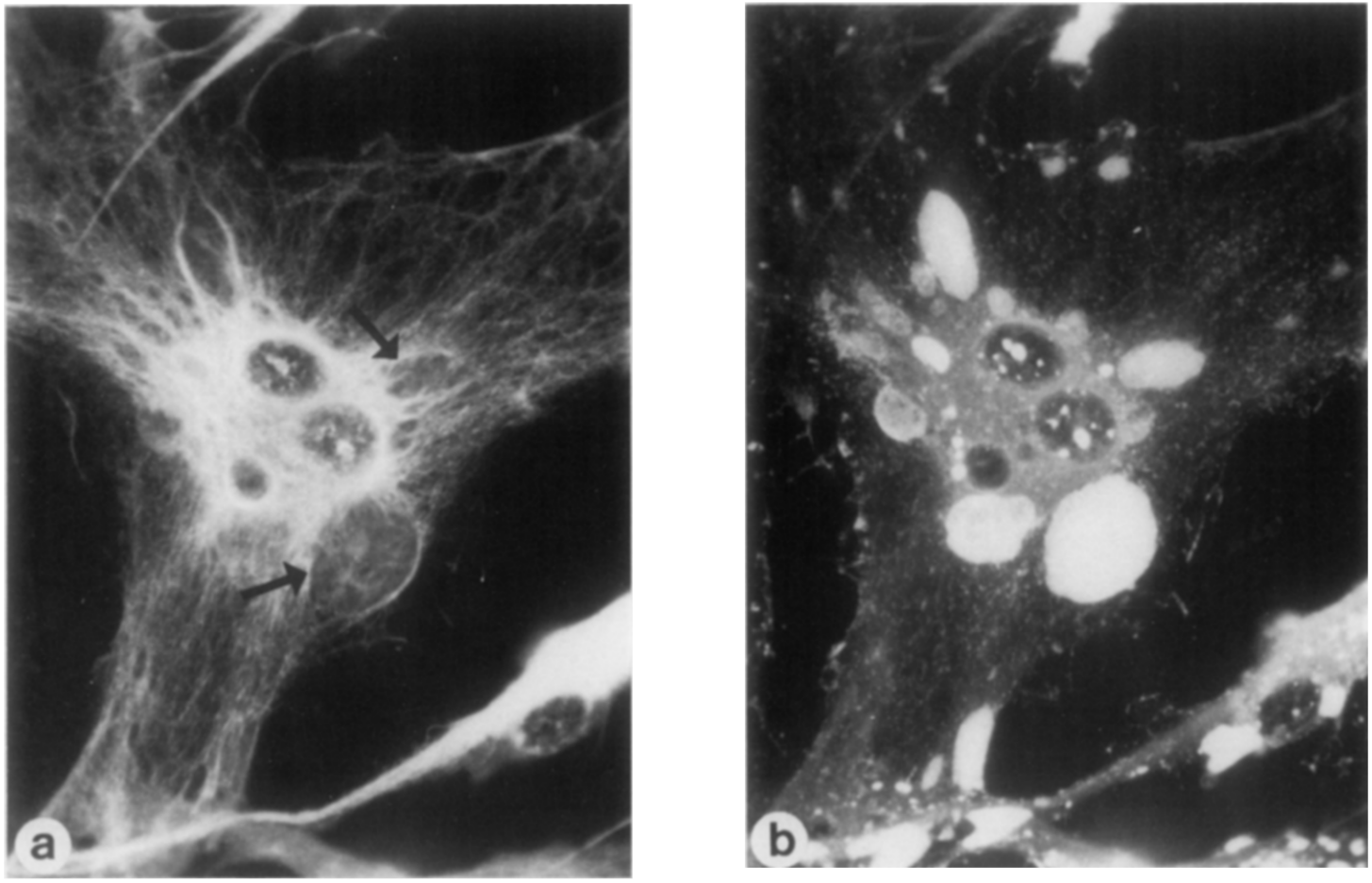


Fig. 3. Double immunofluorescent labelling of astrocytes and CDV-RO antigen in dissociated canine brain cell cultures at 14 days p.i. At weekly time points after inoculation with CDV the cultures were fixed for 5 min in acetone and incubated with anti-GFAP (1:50) and rhodamine-conjugated secondary antibody (a) followed by fluorescein-conjugated anti-CDV (1:5) (b). Arrows point to areas of GFAP exclusion by CDV cytoplasmic inclusions. Note the binucleated syncytium. 500×.

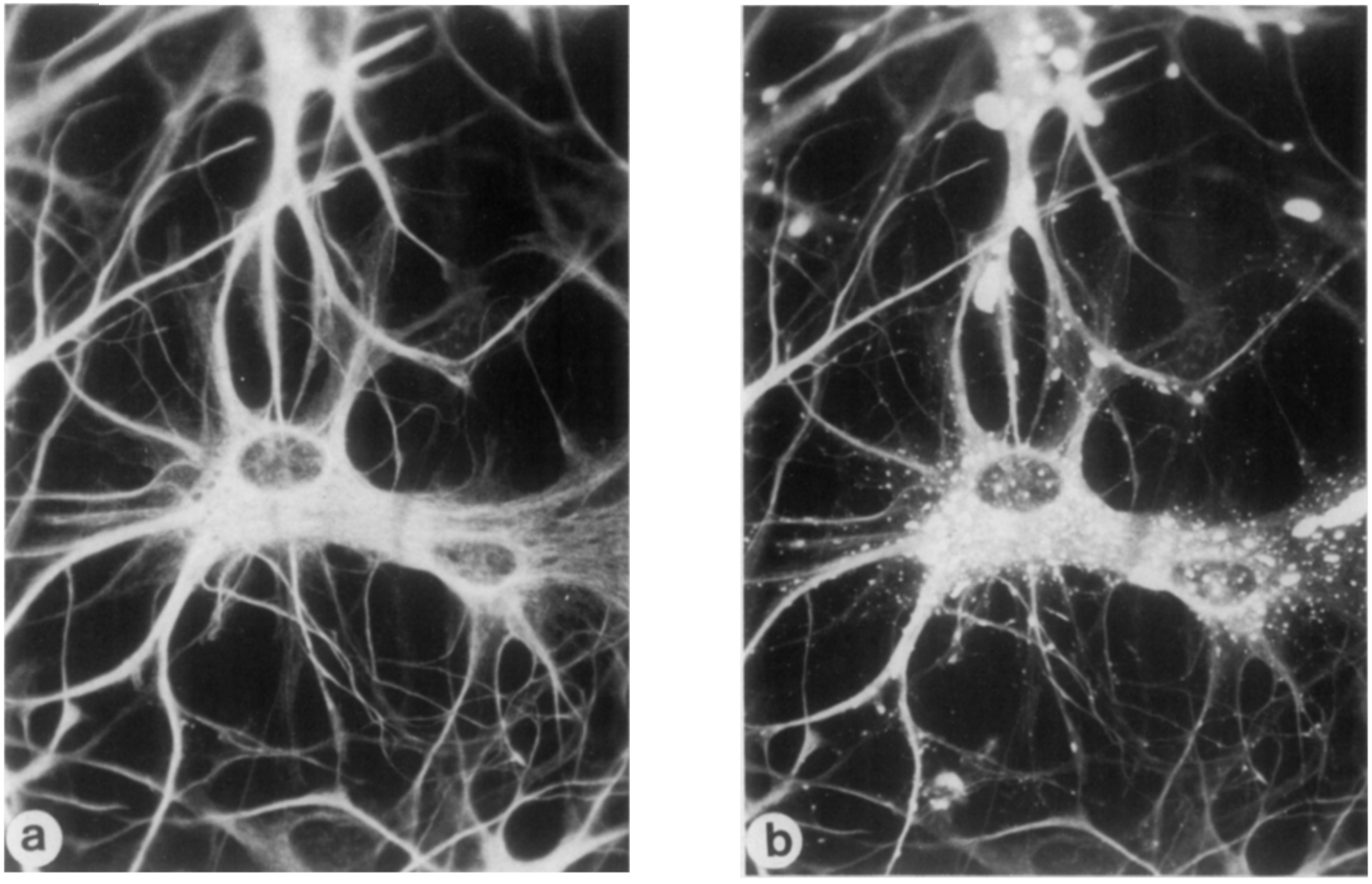


Fig. 4. *Double labelling of astrocytes and CDV-SH at 21 days p.i. by methods described under Fig. 3. A binucleate astrocyte syncytium stains with anti-GFAP and rhodamine (a) and with fluorescein-conjugated anti-CDV (b). 500×.*

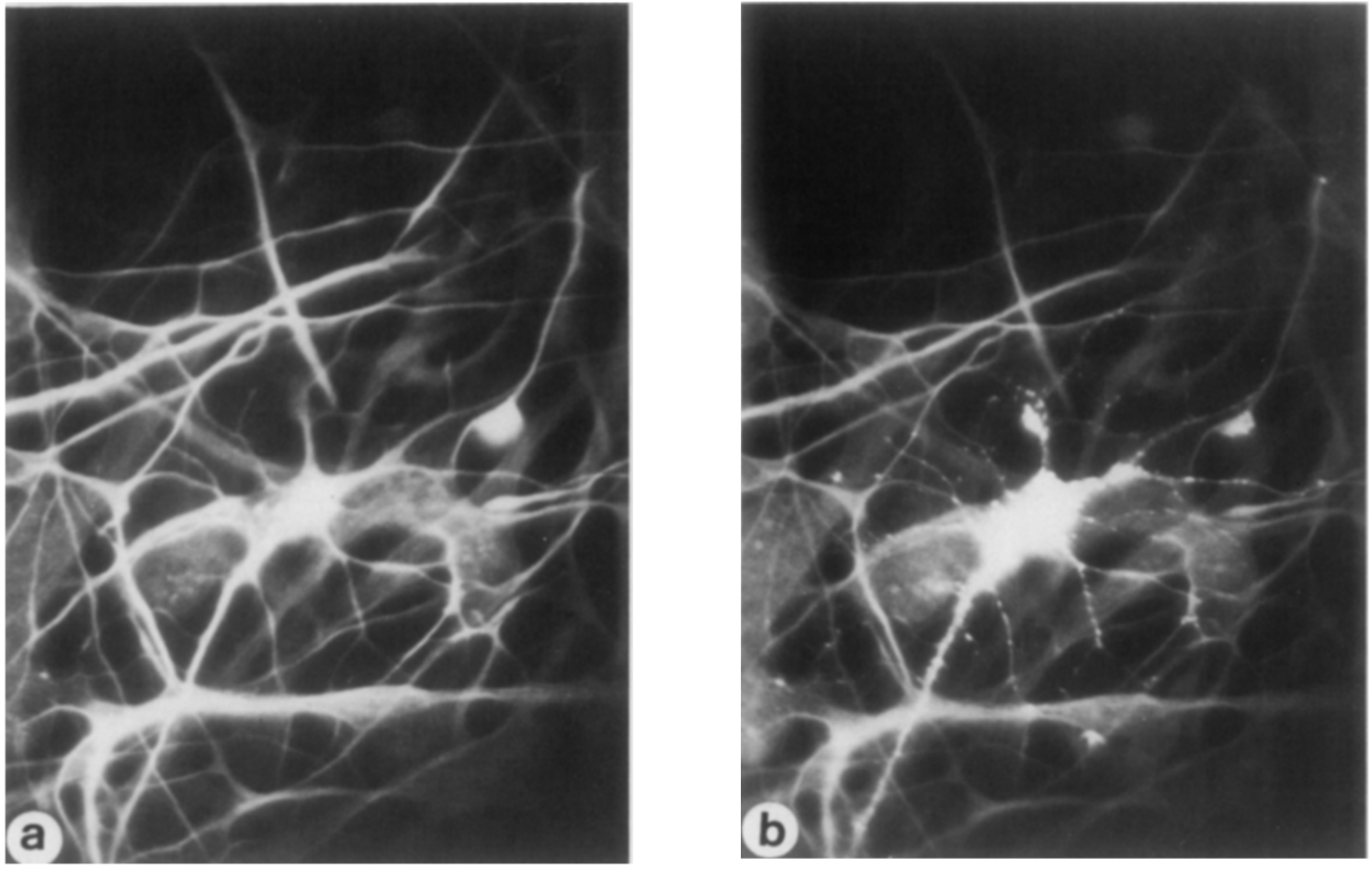


Fig. 5. Double labelling of astrocytes and CDV A75-17 at 7 days p.i. by methods described under Fig. 3. An astrocyte stains with anti-GFAP and rhodamine (a) and with fluorescein-conjugated anti-CDV (b). 500×.

bodies formed, they apparently displaced the glial intermediate filaments, resulting in GFAP-negative areas corresponding with the viral inclusions (Fig. 3). Syncytia, consisting of cells with 2–10 nuclei were stained positively with both GFAP and CDV antisera (Figs 3 and 4) and were present at 7 days p.i., in all infected cultures. The avirulent strain, CDV-RO, produced the largest and most multinucleated syncytia.

CDV-RO produced a rapidly cytolytic CPE in astrocytes which was observed within 7 days p.i. By 21 days p.i., the number of astrocytes was markedly reduced. Despite prominent cytolysis, some astrocytes survived in the CDV-RO infected cultures up to the last time point sampled (63 days p.i.). In contrast, very little cytolysis was produced in the cultures infected with either of the two virulent strains. Despite the formation of syncytia in the virulent virus-infected cultures, the density of the astrocyte populations remained equal to that observed in the control cultures up to 63 days p.i.

Kinetics of astrocyte infection

The percentage of astrocytes that expressed CDV antigen increased with time after inoculation in a pattern that varied with the virus strain [Fig. 6(a)]. The vaccinal strain, CDV-RO, produced a rapid infection which reached 100% by 14 days p.i. CDV-SH resulted in a slightly more delayed rate of infection, reaching 75% of infected astrocytes by 14 days p.i. and 100% by 35 days p.i. In contrast, CDV A75-17 infected fewer than 25% of astrocytes for the first 28 days p.i. (within ± 7 days in replicate experiments). At 35 days p.i. (Fig. 6a) a rapid rise in the percentage of infected astrocytes was observed and 100% of astrocytes were infected by 49 days p.i.

Production of infectious virus by canine dissociated brain cell cultures

Infectious virus was released into the supernatants of the infected cultures. The virus titers, as determined in the Materials and methods section, varied with viral strain and were directly proportional to the percentage of astrocyte infection [Fig. 6(b)].

Kinetics of astrocyte infection with brain cell-passaged virus

We investigated whether the rapid rise in astrocyte infection, which was observed in the CDV A75-17 infected cultures beyond 35 days p.i., represented a change in the virus population. Accordingly, we removed supernatant fluid from cultures at 42 days p.i. for inoculation of newly dissociated canine brain cell cultures. This inoculum was designated CDV A75-17 passage one (CDV A75-17 P1). For comparison, supernatant fluid from 42 days p.i. CDV-SH infected cultures was also inoculated into newly derived brain cell cultures. As controls, stock CDV A75-17 and CDV-SH were again examined. Titers of all inoculum viruses were adjusted so that equal multiplicities of infection were used in comparison experiments.

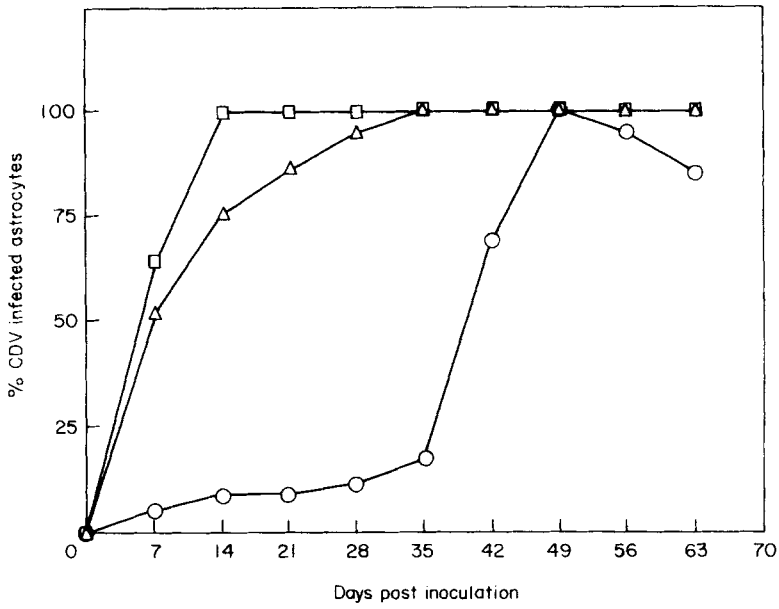
The CDV A75-17 P1 produced a rapid infection of astrocytes after 7 days p.i. and released high titers of infectious virus into the supernatant (Fig. 7). In contrast, CDV-SH P1 did not show enhanced infection but resulted in an infection rate comparable to that of stock CDV-SH of similar titer [Fig. 8(a)]. Virus titer released by CDV-SH P1 also was not enhanced [Fig. 8(b)].

Discussion and conclusions

Astrocytes have been shown to be an important target of CDV infection *in vivo*[6-8] and *in vitro*.[9-10] The possible role of astrocytes in CDV induced encephalitis, viral persistence, and demyelination has been discussed. The rapid and cytolytic infection of astrocytes *in vitro* produced by a vaccinal strain of CDV (the Onderstepoort strain) has been described,[10] but comparative studies of the rate of astrocyte infection by virulent and attenuated CDV strains has not previously been reported.

Both CDV-SH and CDV A75-17 produce a non-cytolytic infection of astrocytes *in*

(a)



(b)

	Days post-infection								
CDV Strains	0	7	14	21	28	35	42	49	56
CDV A75-17	0	<1.5	<1.5	1.5	1.7	1.7	2.7	2.7	2.4
CDV-SH	0	2.9	2.9	2.9	2.9	2.4	2.7	2.0	2.9

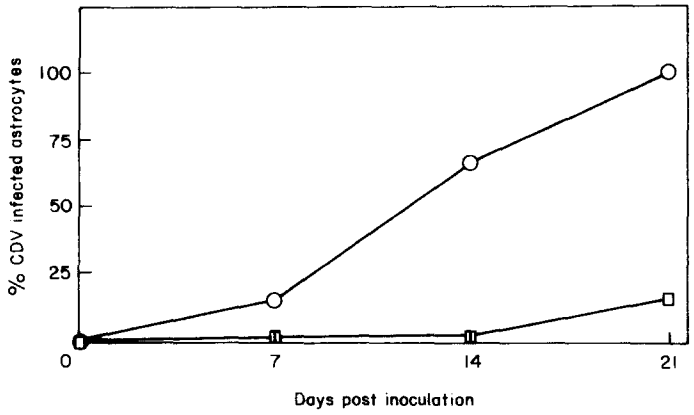
Fig. 6. (a) Percent of astrocytes infected with CDV-RO (□), CDV-SH (△) and CDV A75-17 (○) vs. days post-inoculation in dissociated brain cell cultures. CDV infected astrocytes were labelled by double immunofluorescence and the percent of infected astrocytes was determined at weekly time points. This graph represents one of multiple experiments each showing a similar pattern of kinetics. (b) Infectious titers (in dog lung macrophages) of supernatant fluids removed from CDV-SH and CDV A75-17 infected cultures graphed in (a). CDV-SH and CDV A75-17 inocula both had a $TCID_{50}$ titer of 10^3 in these experiments.

vitro, similar to what is observed in a persistent CNS infection with white matter disease. It may be speculated that if a vaccinal strain of CDV gains access to the CNS in the dog, it may produce a necrotizing effect similar to the astrocyte necrosis observed *in vitro*. In rare case reports, post vaccinal encephalitis in the dog is characterized by a rapidly fulminating course with focal CNS necrosis.[11]

How the astrocytes are able to survive for many weeks in culture while persistently infected with the virulent CDV strains is intriguing. Some CPE, such as cell fusion, leading to the formation of syncytia are noted and passive displacement of the astroglial filaments by viral inclusions is observed. Zurbriggen and co-workers[12] have also noted a rearrangement in the pattern of GFAP staining in CDV infected astrocytes, describing a concentration of fibrils in the perinuclear area and a loss of fibril staining in the rest of the cell body.

The differences in the kinetics of astrocyte infection between the two virulent strains of CDV appears to parallel the course of CNS infection which is seen in dogs. CDV A75-17, which produces more delayed subacute to chronic CNS infection, adapted more slowly to astroglial growth. CDV-SH which produces a more rapid disease course of disease *in vivo* also infected astrocytes much more rapidly in culture.

(a)

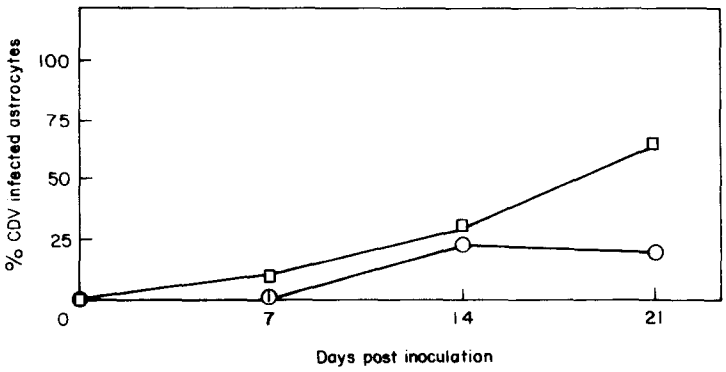


(b)

CDV Strains	Days post-infection		
	7	14	21
CDV-A75P$_1$	0.8	2.9	3.6
Stock CDV-A75-17	<1.5	<1.5	<1.5

Fig. 7. (a) Percent of astrocytes infected with brain cell passaged CDV A75-17 P1 (○) [CDV A75-17 (□)] vs. days post-inoculation. The inoculum for this experiment was derived by removing supernatant at 42 days p.i. from the CDV A75-17 infected cultures described in Fig. 6. This was placed on fresh brain cell cultures and had a $TCID_{50}$ titer in DLM of $10^{2.7}$. (b) Infectious DLM titers of supernatant fluids removed from the cultures graphed in (a).

(a)



(b)

CDV Strains	Days post-infection		
	7	14	21
CDV-SH P$_1$	2.0	1.5	1.5
Stock CDV-SH	2.0	1.7	2.2

Fig. 8. (a) Percent of astrocytes infected with brain cell passaged CDV-SH P1 (○) [CDV-SH (□)] vs. days post-inoculation. The inoculum for this experiment had a DLM $TCID_{50}$ titer of $10^{2.7}$ and was derived by removing supernatant fluid at 42 days p.i. from the CDV-SH infected cultures described in Fig. 6. This was placed on fresh brain cell cultures. (b) Infectious DLM titers of supernatant fluids removed from the cultures graphed in (a).

However, *in vivo*, CDV also infects cells other than astrocytes (leptomeningeal cells, neurons, ependymal cells, pericytes and microglia). Therefore the CNS disease pattern cannot be explained on the basis of astrocyte infection alone. However, the capacity to infect and spread between astrocytic cells *in vitro* without causing cell death, may be an indicator of the capacity of a virus strain to persist in the white matter of the central nervous system.

The mechanism of demyelination associated with certain strains of CDV, such as A75-17, remains unknown. Oligodendrocyte infection by CDV *in vivo* has been documented[13] but appears to be rare and is of uncertain importance. The role of astrocytic infection in CDE has mostly been studied in dogs with the subacute to chronic demyelinating form of encephalitis caused by the A75-17 strain. Since type 2 astrocytes have been proposed to play a role in the myelination[14] infection of this cell type could be related to myelin breakdown. Zurbriggen and co-workers[12] have suggested that toxic factors, released by astrocytes, may result in oligodendrocyte degeneration and thus demyelination.

Our observed differences between the kinetics of the two virulent CDV infections in astrocytes *in vitro* could suggest a mechanism by which the CNS disease varies in the dog. CDV-SH may produce a rapid infection of a variety of cell types in the CNS, including neurons, this causing an acute death. CDV A75-17, in contrast, may slowly spread and persist in a primarily astrocytic population allowing a production of a subacute to chronic disease. It has been suggested[4] that both virulent CDV strains follow a similar sequence of CNS events, namely viral entry, then gray matter disease progressing to white matter disease; CDV-SH may result in the dog's death before demyelination can occur.

The adaptation of CDV A75-17, which occurred during replication in cultured brain cells (astrocytes), may occur *in vivo*. CDV A75-17 may be initially slow to infect and spread between brain cells until it adapts to growth in this environment. In contrast to CDV A75-17 P1, we did not observe an adaptation of wild-type CDV-SH, perhaps because it already rapidly infects these cells.

In other studies the attenuated strain, CDV Onderstepoort (CDV-Ond) has been adapted to different human neural cell lines.[15,16] CDV-Ond typically produces a neuronal and leptomeningeal infection when inoculated intracerebrally into the mouse. Adaptation of CDV-Ond to a glioblastoma cell line resulted in widspread murine glial cell infection *in vivo* as well as an increased rate of glioblastoma cell infection *in vitro*.[15,16] After further adaptation to growth in an oligodendroglioma cell line, CDV-Ond producing demyelinating lesions in mice was reported. These findings suggest that CDV is able to infect a wide variety of CNS cell types and that this ability can be enhanced by passaging the virus through different cell populations.

Other neurotropic viruses, including reovirus[17] and mouse corona virus,[18] in which different strains exhibit different *in vivo* behavior, have been studied in CNS cultures *in vitro*. A distinct neural cell tropism *in vitro* has been shown in the mouse corona virus system where the neurotropic JHM strain infected rate oligodendrocytes while the viscerotropic MHV-3 strain was able to infect rat astrocytes but not oligodendrocytes in culture.

The permissiveness of astrocytes to infection by different strains of CDV may play a role in CDV pathogenesis *in vivo*. Our observed differences in CDV strain behavior in culture may reflect some aspects of the disease in the dog.

Materials and methods

Canine glial cell cultures. Dissociated brain cell cultures were established from 1- to 2-day-old specific-pathogen-free beagle dogs by a modification of the McCarthy and DeVellis[19]

method. The animals were killed with ether and the brain aseptically removed. The cerebral hemispheres and midbrain were retained and the meninges were removed. This tissue was mechanically dissociated by gently triturating 15 times through a wide bore pipette. The brain tissue was suspended in 15 ml of Eagle's Basal Medium and shaken on a rotary shaker for 15 min at 150 rpm at 37°C followed by addition of trypsin (0.1% final concentration). The tissue then continued incubation for 20 min. The cell suspension in Eagle's Basal Medium containing 15% fetal calf serum (FCS) was filtered through cheesecloth and then nylon filters (Nitex 130 and 35). The cells were seeded at a concentration of 5×10^5 live cells per ml in 1.0 ml Leighton tubes containing 35 × 11 cm glass coverslips. After 4 days *in vitro* culture medium was replaced. After 7 days *in vitro* the FCS concentration was reduced to 10% and the medium was replaced three times a week. The cultures were incubated at 37° in the presence of 5% Co_2. Confluency was reached at 7–10 days *in vitro* and the cultures were maintained up to 70 days.

Infection of cultures with CDV strains SH, A75-17, and RO. Virulent CDV strains, SH and A75-17, from suspended lymphatic tissues of infected dogs were grown in dog thymocyte cultures[20] and stored at −70° in 7% DMSO, 20% FCS and RPMI 1640 medium. CDV-SH and CDV A75-17 inocula were titrated in dog lung macrophages (DLM).[21] Avirulent CDV-RO was propagated and titrated in dog kidney cells.[22] For inoculation of cultures, 10^3 $TCID_{50}$ of CDV-SH, CDV A75-17 and CDV-RO were adsorbed to the cells for 2 h followed by replacement of the media. Cultures were maintained for up to 63 days post inoculation. During this period, CPE caused by the virus was noted in live cultures and at weekly time points coverslips were removed, fixed and stained for immunofluorescent evaluation.

Brain cell passaged virus: CDV-SH P1 and CDV A75-17 P1. CDV A75-17 P1 and CDV-SH P1 were derived from the culture media of CDV infected brain cells after 42 days post-inoculation. These viruses were titrated on DLM cells as were the parent viruses. In experiments to compare CDV A75-17 P1 and CDV-SH P1 with parental viruses, brain cell cultures were inoculated with $10^{2.7}$ $TCID_{50}$ of each passaged virus and parental virus.

Detection of viral antigen in astrocytes. CDV antigen was localized within astrocytes by double immunofluorescence. Coverslips were fixed in acetone for 5 min at room temperature, rinsed in phosphate buffered saline (PBS) and incubated in rabbit anti-glial fibrillary acidic protein (GFAP) (Dako Corp.) diluted 1:50 in PBS. This was followed by a PBS rinse and a second incubation in rhodamine-conjugated goat anti-rabbit IgG (Cappel) diluted 1:20. Viral antigen within the astrocytes was then identified with anti-CDV hyperimmune dog serum directly conjugated to fluorescein.[23] Cells were viewed on a Leitz epifluorescent microscope equipped with rhodamine (530–560 nm) and fluorescein (470–490 nm) filters.

The percentage of infected astrocytes was determined weekly from 7 to 63 days p.i. Using a 50 × water immersion objective, (Leitz Corp.) the total number of astrocytes in a field, identified by GFAP staining under rhodamine fluorescence, was counted. Changing the filter from rhodamine to fluorescein then allowed the number of CDV infected astrocytes in the same field to be counted (Fig. 2). Ten random fields, containing an average of 20 astrocytes per field, were examined on each coverslip. The percentage of infected astrocytes in each field was determined and an average of these 10 fields was calculated. At each time point, three coverslips from CDV-SH, CDV A75-17 and CDV-RO infected cultures were evaluated and results averaged. Thus infection at each time point for the three viral strains is based on an examination of approximately 600 astrocytes. The data reported are from one representative experiment; each experiment was repeated at least three times.

In addition to detection of CDV antigen, viral inclusion bodies were visualized by staining with Shorr stain. Cultures were fixed for 1 min in 50% ether/50% ethanol and rehydrated in decreasing concentrations of ethanol. They were then stained for 20 seconds in Harris's Hematoxylin, rinsed, and stained in Shorr stain (Harleco Corp.) for 1 min, dehydrated in ethanol and mounted in permount for light microscope viewing.

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