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Canine Distemper Virus Persistence in the Nervous System Is Associated with Noncytolytic Selective Virus Spread

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Canine distemper virus (CDV), a negative-strand RNA morbillivirus, causes a progressive demyelinating disease in which virus persistence plays an essential role. The antiviral immune response leads to virus clearance in the inflammatory lesions. However, CDV can replicate and persist outside these inflammatory lesions within the brain. How CDV is capable of persisting in the presence of an effective antiviral immune response is poorly understood. In the present investigation, we studied several aspects of virus replication in primary dog brain cell cultures (DBCC), comparing an attenuated CDV strain and a virulent CDV strain. Confluent DBCC were infected with either virulent A75/17-CDV or attenuated Onderstepoort-CDV and monitored for 60 days. Persistence was not associated with defective virus production, because all mRNAs and corresponding proteins were continuously expressed in the noncytolytic infection. Quantitative measurements did not detect a difference between the two types of infection in the rate of virus transcription and protein synthesis at the level of the single cell. However, electron microscopy and virus titration experiments showed that in the persistent CDV infection virus budding is strongly limited compared with that of the attenuated virus. Morphometry and immunocytochemistry showed profound differences in the way the two viruses spread in the culture. The attenuated CDV spread randomly to immediately adjacent cells, whereas persistent CDV spread selectively to more-distant cells by way of cell processes. In conclusion, the present study supports a mechanism of CDV persistence through selective spread by way of cell processes, enabling virulent CDV to invade the central nervous system without the need of releasing much virus into the extracellular space.

Canine distemper virus (CDV), a negative-strand RNA morbillivirus related to measles virus, causes in dogs and other carnivores a naturally occurring demyelinating disease which is considered to be an animal model for multiple sclerosis in humans (1). Virus persistence in the central nervous system (CNS) appears to play an essential role in the chronic progression of the disease (3, 8, 10, 22), but not much is known about mechanisms of persistence in distemper. There is an intrathecal antiviral immune response in chronic progressive distemper, with invasion of inflammatory cells in the brain which leads to viral clearance (3, 23). However, inflammation and viral clearance are restricted to certain lesions; simultaneously with this obviously effective antiviral immune response, CDV continues to replicate and spread in astrocytes in other areas of the brain without eliciting an inflammatory response (3, 11, 23). These observations suggest that CDV persists because the immune response lags behind virus replication and spread. It appears that CDV-infected astrocytes in these areas are not readily recognized by the immune response, at least for some time. It is possible that there is reduced expression of the virus in these areas of the CNS, in analogy to human infection with subacute sclerosing panencephalitis (SSPE) virus, a virus closely related to CDV. Persistence in SSPE is indeed associated with incomplete or defective transcription or translation compared with that of regular measles virus (2, 4, 12, 18, 19). All biological parameters of CDV, including viral transcription, translation, and replication, have been well studied in strains grown in cell lines (5, 13–15, 20, 21). Because these laboratory CDV strains have lost virulence and the ability to produce a persistent infection in the CNS, it is questionable whether the results derived from cell lines are also applicable to the in vivo infection. The problem is that virulent CDV will not grow in cell lines unless it is adapted by many weeks of blind passaging, after which it loses its ability to cause persistence and disease in vivo. However, CDV-infected tissues from dogs with distemper establish a persistent, multifocal noncytolytic infection of astrocytes in primary dog brain cell cultures (DBCC) immediately upon inoculation, without the need for adaptation (7, 17, 28). Moreover, when CDV from such infected cultures is inoculated back into dogs, a persistent infection of the CNS with neurologic lesions which does not differ from the disease induced by transmission from dog to dog ensues (7). Thus, primary DBCC would lend themselves well to studies of the biological properties of virulent CDV. Comparison of this information with the established knowledge about attenuated viruses may help to identify virus factors associated with virulence and persistence. Because virus replication is dependent not only on the strain but also on cellular factors, a comparison of virulent and apathogenic strains requires that the strains are propagated in the same system. It has previously been found that attenuated CDV strains, which can be easily propagated in a wide variety of cell cultures, also grow very well in astrocytes in DBCC (7, 28), behaving as in cell lines, producing cytolytic plaques.

In the present study, we compared several aspects of virus replication in DBCC of a previously characterized, adapted, cytolytic Onderstepoort (OP)-CDV propagated in cell lines with those of a virulent, persistent A75/17-CDV strain propagated in dogs. We show that persistence is associated with marked differences in spread between the two viruses. These differences in spread are the result of differences between the two strains in virus release.

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MATERIALS AND METHODS

Virus. A virulent CDV strain, A75/17-CDV (gift from M. Appel, Cornell University, Ithaca, N.Y.), was propagated in specific-pathogen-free dogs (Federal Institute of Virus Diseases and Immune Prophylaxis, Mittelhäusern, Switzerland). Lymphoid tissue from these dogs containing large quantities of virus was homogenized and frozen in aliquots at −70°C until used. The tissue culture-adapted OP-CDV strain (gift of U. Kihm, Federal Institute of Virus Diseases and Immune Prophylaxis) was propagated in Vero cells.

Antibodies. For the demonstration of virus proteins, we used two monoclonal antibodies (MAbs) to the nucleocapsid protein (NP protein) (D110) (3), one MAb to the M protein (XI6), and one MAb to the H protein (XI23). These antibodies have been generated and characterized in our laboratory. MAbs against the F and P proteins were kindly provided by C. Oervell (Karolinska Institute, Stockholm, Sweden). All of these antibodies were shown to bind to attenuated CDV as well as to virulent CDV. Monospecific antibodies to the CDV L protein are not available.

Probes. cDNA clones complementary to NP, P, M, F, H, and L proteins of the virulent A75/17-CDV strain were produced in our laboratory as described previously (29). In short, virus RNA was purified from DBCC infected with virulent CDV by extraction with GTC (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauroyl sarcosine, 0.1 mM β-mercaptoethanol). The resulting RNA was reverse transcribed, and the cDNA was used as a template for PCRs and cloned into SP6/T7 transcription vector pSPT19/18 (Boehringer Mannheim). The cloned DNAs were checked by determining part of the DNA sequence with the Sequenase system (U.S. Biochemicals, Cleveland, Ohio). From these clones, we prepared strand-specific digoxigenin (DIG)-labelled RNA probes (29).

DBCC. DBCC were grown on poly-L-lysine-coated glass coverslips as described elsewhere (29). These cultures contain predominantly astrocytes and can be maintained for several months (25, 27). All experiments were performed five times. Part of each batch of confluent 14-day-old DBCC was inoculated with 10^2 PFU of both strains per ml, as determined in preceding titration experiments. At 6, 9, and 12 days and at weekly intervals thereafter up to 10 weeks after infection, the DBCC were harvested and fixed with 95% ethanol–5% acetic acid for 10 min at −20°C. Corresponding noninfected DBCC were treated the same way and served as negative controls for the immunocytochemical staining.

Immunocytochemistry. CDV antigens were demonstrated by the unlabelled-antibody–peroxidase antiperoxidase (PAP) technique as previously described (3). DBCC were harvested at regular intervals from 6 to 63 days postinfection (p.i.) and treated with the MAbs for 2 h at 37°C. The second incubation was performed with goat anti-mouse immunoglobulin G, diluted 1:40, for 30 min at room temperature, and then with PAP complex (1:100) for 30 min. Finally, samples were treated with hydrogen peroxide and diaminobenzidine. OP-CDV- and A75/17-CDV-infected cultures were processed simultaneously at each sampling date with the same reagents under exactly the same conditions.

In situ hybridization. In situ hybridization was performed on primary DBCC for the detection of all virulent canine distemper mRNAs and the virus genome (29). After fixation with 4% paraformaldehyde buffered with phosphate-buffered saline, the cultures were treated with 0.2 M HCl and proteinase K at 0.5 μg/ml, postfixed, washed, and prehybridized. Hybridization was carried out overnight at 52°C with 10 ng of DIG-labelled probe per μl of hybridization solution. Excess labelled RNA probe was removed by several washes with 2× and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and one treatment with RNase. The slides were incubated with an anti-DIG antibody conjugated with phosphatase and then subjected to development of nitroblue tetrazolium and X-phosphate (Boehringer Mannheim).

Protein dot blotting. Virulent CDV proteins NP, P, M, F, and H were screened by a protein dot blot assay as described by the vendor (Bio-Rad, Glattbrugg, Switzerland). Protein samples were prepared from A75/17-CDV-infected and corresponding noninfected DBCC at 14 and 56 days p.i. Ten micrograms of these protein samples was blotted onto a nitrocellulose membrane with a dot blot manifold (Bio-Rad). After being blocked in 5% powdered milk–10% normal goat serum in Tris-buffered saline, the blots were incubated with MAb to either NP, P, M, F, or H. Antibody binding was demonstrated by the PAP method as described above (see "Immunocytochemistry").

RNA dot blotting. Virulent CDV mRNAs coding for all virus proteins and genomic CDV RNA were screened by an RNA dot blot assay according to the instructions of the vendor (Boehringer Mannheim). RNA samples were prepared from A75/17-CDV-infected DBCC at 14 and 56 days p.i. as described above for the cDNA clones. A 1-μl portion of the RNA sample (1.5 μg/μl) was spotted onto a dry nylon membrane and fixed in an oven at 120°C for 30 min. The blot was prehybridized at 68°C for 1 h in 5× SSC–50% formamide–0.02% sodium dodecyl sulfate (SDS)–0.1% *N*-lauroyl sarcosine. Then the prehybridization buffer was replaced by fresh prehybridization solution containing 100 ng of DIG-labelled probe per ml. Hybridization was carried out at 68°C overnight. The blot was washed twice for 5 min each in 2× SSC–0.1% SDS at room temperature and twice for 15 min each in 0.1× SSC–0.1% SDS at 68°C. The colorimetric detection was performed as described above (see "In situ hybridization"). In one additional experiment, RNA expression of OP-CDV was compared with that of A75/17-CDV at 14 days p.i.

Quantitative studies. Cultures harvested at 6, 9, and 12 days were examined with contrast-enhanced video microscopy, using a microscope equipped with differential interference contrast (DIC) optics attached to a high-resolution video camera. The microscopic image is transmitted to an electronic image processor (Argus 10; Hamamatsu, Munich, Germany) and to a personal computer. This setup allowed the measurement of specific areas and determination of the average density of the immunocytochemical labelling and in situ hybridization product within a given area or a single cell. Recently, it has been shown that the immunocytochemical labelling density accurately reflects the antigen concentration (24). MAb D110, which was used for all quantitative studies, recognizes an epitope on the NP protein of all CDV strains in tissues and in cell cultures (3, 7, 28). Furthermore, immunocytochemical labelling corresponds with the results of in situ hybridization for NP mRNA in nearly 100% of infected cells (26). All data were statistically analyzed by using the Systat computer software (Systat Inc., Evanston, Ill.). Data derived from antigen density measurements per cell were depicted graphically by box plots. For the analyses of plaque size, histograms were used. Distributions were tested for normality by probability plot and Lilliefors test. In addition, the mean and the standard deviation were calculated for each variable. Significant differences between two variables were detected by the nonparametric Mann-Whitney U test. The level of significance was $a = 0.05$. A similar approach was used to measure the amount of CDV mRNA at 7, 10, and 14 days, following in situ hybridization with a probe against the P-protein message.

Virus titration. To examine the amount of infectious CDV released into the tissue culture medium, the supernatant fluids of the OP-CDV- and A75/17-CDV-infected DBCC were collected at regular intervals from 3 to 52 days p.i., immediately frozen at −70°C, and stored until further processing. At the end of the observation period, all supernatant fluids were titrated on DBCC grown in 24-well plates. The DBCC in each well were incubated with 300 μl of the test medium for 1 h, washed, and then incubated in complete tissue culture medium for 4 days. Then all cultures were washed, fixed with 5% acetic acid–95% ethanol, and stained immunocytochemically for the NP protein of CDV. The amount of DBCC infectious virus was determined by counting the immunocytochemically positive plaques. All samples were tested in quadruplicate. Supernatant fluids from noninfected DBCC served as negative controls.

Electron microscopy. For ultrastructural studies, selected OP-CDV- and A75/17-CDV-infected DBCC were harvested, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, and processed for ultrathin sections as described previously (6). Briefly, fixed cultures were washed in 0.1 M *S*-collidine buffer, pelleted, and treated with 1% osmium tetroxide and 2% manyl acetate. The pellets were embedded in Epon, and ultrathin sections were contrasted with lead citrate.

RESULTS

Spread of CDV in the culture. (i) Persistent infection with A75/17-CDV. Soon after inoculation, virus foci consisted of scattered, single infected cells interspersed with virus antigen-free cells (Fig. 1a). Virus NP protein was diffusely distributed in cytoplasm and cell processes; even the finest ramifications contained virus antigen (Fig. 2a). CDV-infected cells had contact with each other through virus antigen-containing cell processes (Fig. 2a). Often, these cell processes appeared to have merged. During the following weeks, the infected foci gradually increased in size. The spreading infection did not induce any cytopathogenic effect. Virus also spread within the foci so that the initial fine network of infected cells became gradually replaced by a confluent infection, eventually involving 100% of the cells by 3 weeks p.i. Increasing contacts between infected cells led to cell fusion, with the formation of steadily increasing numbers of syncytia. This cytopathogenic effect was generally not associated with cell damage (Fig. 1c). Occasionally, rounding of syncytia with regressive changes of the cells and focal retraction of the cell layer occurred. However, apart from such isolated changes, the cultures infected with virulent CDV remained a continuous cell layer during the whole observation period. Immunostaining for the other proteins of CDV revealed a distribution pattern of infection and progress of virus spread identical to those seen with the antibody against NP protein. In situ hybridization for the virus genome and for the mRNAs complementary to all proteins of CDV essentially corresponded to the immunocytochemical results (Fig. 2b). The finely granular reaction product also outlined the perikarya and major cell processes, but not the finest ramifications, of the noncytolytically infected cells.

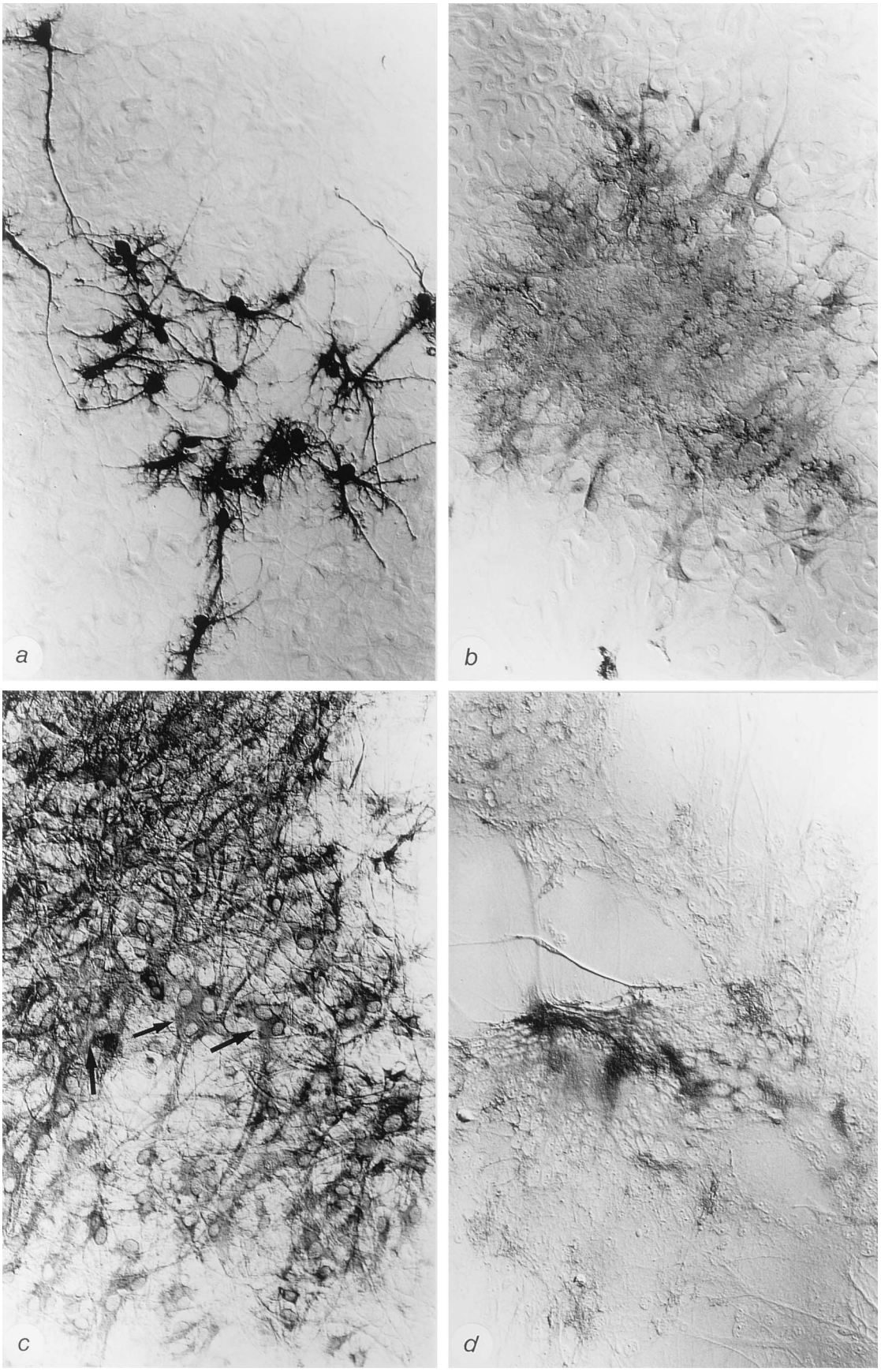
a
b
c
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FIG. 1. Immunoperoxidase staining for CDV NP protein in primary DBCC. DIC microscopy with a magnification of ×225 was used. (a) Persistent infection with A75/17-CDV 7 days p.i. A small plaque with relatively few single infected cells interspersed with uninfected cells is shown. (b) Cytolytic infection with OP-CDV 7 days p.i. The plaque size is similar to that in panel a. Nearly all cells within the perimeter of the plaque contain virus antigen, with loss of individual cell contours. (c) Persistent infection with A75/17-CDV 19 days p.i. Edge of a large plaque. The infection has reached confluency towards the center of the plaque, with formation of fused multinucleated cells (arrows). However, the infected cells and the cell layer remain intact. (d) Cytolytic infection with OP-CDV 14 days p.i. Several large multinucleated syncytia which are undergoing destruction, with retraction and total loss of the cell layer, are apparent.

(ii) Cytolytic infection with attenuated strain OP-CDV. The pattern of infection of the attenuated, avirulent OP-CDV strain in the DBCC differed markedly from that of the virulent strain. Initial virus foci consisted of a cluster of tightly packed virus antigen-containing cells; nearly all cells within the perimeter of a focus were infected (Fig. 1b). NP protein was also diffusely distributed in the cytoplasm and some larger processes but not in the finer ramifications, as in the persistent CDV infection. Foci gradually increased in size by infection of adjacent perikarya. Initially, cytolysis was moderate at the expanding edge of the plaque, but later, an extensive cytopathogenic effect consisting of fusion involving large numbers of cells rapidly developed. Syncytium formation quickly led to cell destruction, with focal retraction and complete removal of the cell layer (Fig. 1d). Merging of such plaques eventually resulted in severe depletion of cells in large areas of the culture. Immunostaining for the other proteins of CDV revealed a distribution pattern of the infection and progress of virus spread identical to those seen with the antibody against NP protein. Very similar microscopic appearances of virus spread were produced by in situ hybridization for all CDV mRNAs and the virus genome.

(iii) Control cultures. No virus antigens or virus nucleic acid sequences were detected by immunocytochemistry and in situ hybridization in the noninfected corresponding control cultures.

Dot blots. Virulent CDV NP, P, M, F, and H proteins, virus genomic RNA, and all mRNAs coding for the respective virus proteins were screened by dot blotting. All tested proteins (Fig. 3) as well as genomic RNA and all mRNAs (Fig. 4) were

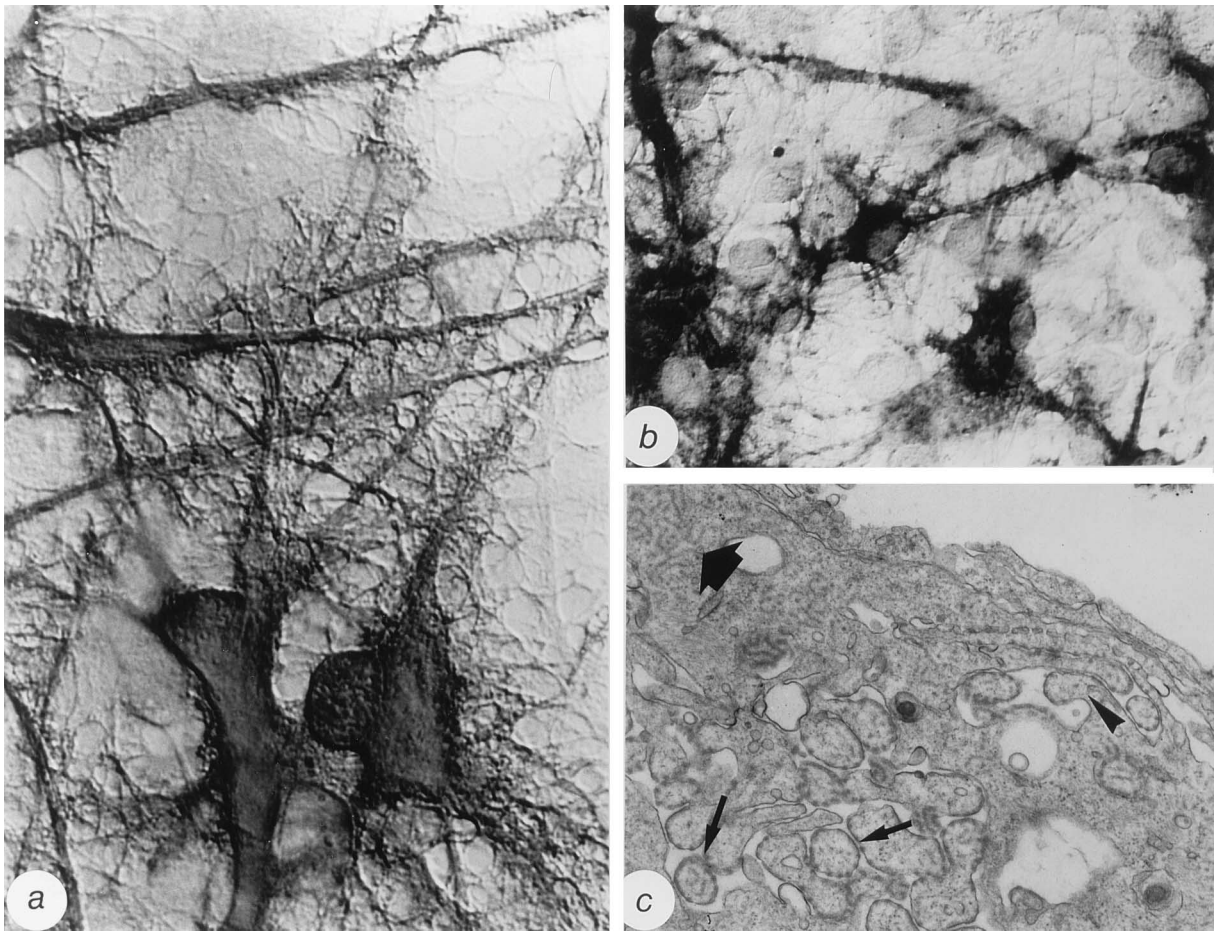

FIG. 2. Primary DBCC infected with CDV. (a) Edge of a plaque in persistent infection with A75/17-CDV 12 days p.i. CDV NP protein is diffusely distributed throughout the cytoplasm and cell processes, including their finest ramifications. The infected cells remain intact. MAb D110-PAP staining and DIC microscopy were used. Magnification, ×1,250. (b) Cluster of noncytolytically infected cells labelled with in situ hybridization for P-protein mRNA of CDV in persistent infection with A75/17-CDV 12 days p.i. Contact between infected cells by way of cell processes is evident. Anti-DIG phosphatase and DIC microscopy were used. Magnification, ×400. (c) Electron microscopical examination of cytolytic infection with OP-CDV 5 days p.i. Many virus particles (arrows) with typical morbillivirus morphology amidst opposing cell processes are seen. Several stages of virus budding (arrowhead) from a cell containing many fuzzy nucleocapsids (large arrow) are visible (arrows). A thin section stained with lead citrate and uranyl acetate is shown. Magnification, ×11,000.

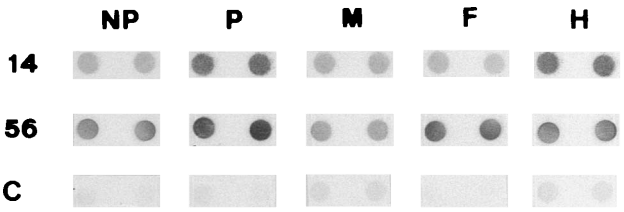

FIG. 3. Dot blots tested (in duplicate) for virulent CDV NP, P, M, F, and H proteins at 14 and 56 days p.i. Monospecific antibodies to the CDV L protein are not available. All tested proteins were expressed at both times. C, corresponding controls derived from noninfected cultures. Labelling intensity increased from 14 to 56 days p.i. because of increasing amounts of virus in the noncytolytic infection.

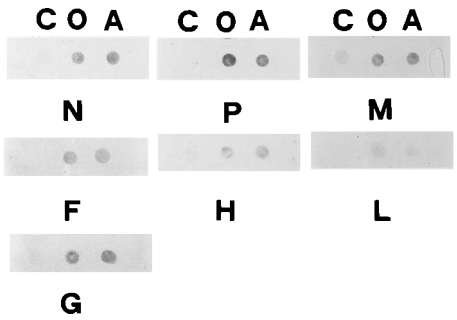

FIG. 5. Comparison of infections with OP-CDV and A75/17-CDV in a different experiment from that whose results are shown in Fig. 4. Dot blots of genomic RNA (G) and all mRNAs of attenuated OP-CDV and virulent A75/17-CDV at 14 days p.i. are shown. All tested virus nucleic acid sequences are expressed similarly in the two infection types. C, corresponding controls derived from noninfected cultures.

expressed at 14 and 56 days p.i. No virus antigens or virus nucleic acid sequences were detected in the noninfected corresponding control cultures. The levels of expression of mRNAs corresponding to all CDV proteins at 14 days p.i. were similar in the two types of infection (Fig. 5).

Quantitative studies. One batch of DBCC was used for quantitative studies. The multiplicities of infection were similar for the two infections, as titrated on day 6 p.i., with 74 PFU for A75/17 and 76 PFU for OP-CDV.

(i) Plaque size. The total size of single plaques was determined at 6 and 9 days p.i. for both infections. The perimeter of a plaque was determined by the most peripherally located infected cells, as seen by immunocytochemical staining for CDV. The results were compiled and evaluated in a histogram (Fig. 6). At 6 and 9 days p.i., the distribution of the plaque sizes of both infection types was positively skew. The standard deviations in the two infections were similar at 6 and 9 days p.i. There was no statistically significant difference in the mean plaque size between the two infections on either sampling day (day 6 p.i., $P > 0.5$; day 9 p.i., $P > 0.6$). Neither was there a statistically significant difference in average plaque size between the two infections on either sampling day. Thus, the average plaque sizes increased similarly in the two infections from days 6 to 9.

(ii) Number of infected cells per plaque. The number of infected cells per plaque was evaluated by counting all nuclei which were associated with positive immunostaining for CDV NP protein of surrounding cytoplasm within the perimeter of a plaque, assuming that one nucleus originally corresponded to one cell. This number was then divided by the size of the plaque. The resulting value was expressed as number of nuclei per area unit. For each infection, all infected cells within five randomly chosen plaques were counted at both times. At both times, i.e., 6 and 9 days after infection, the numbers of infected cells per surface unit were consistently four to seven times higher in OP-CDV-infected cultures than in A75/17-CDV-infected cultures.

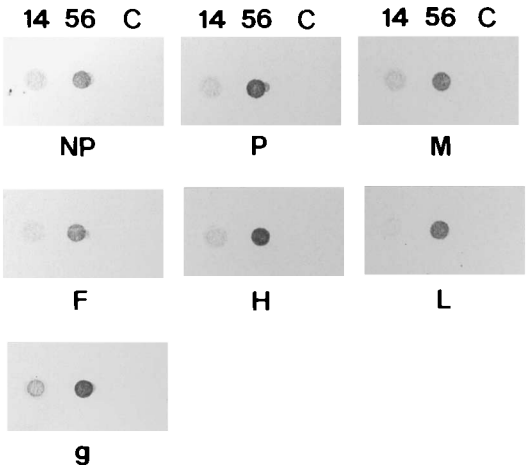

FIG. 4. Dot blots for virulent CDV genomic RNA and all mRNAs coding for the indicated virus proteins at 14 and at 56 days p.i. Virulent genomic RNA (g) and all mRNAs were expressed at both times. No virus nucleic acid sequences were detected in the corresponding noninfected control cultures (C). Labelling intensity increased from 14 to 56 days p.i. because the amount of CDV continuously increases in the noncytolytic infection.

(iii) Expression of CDV RNA and protein per individual cell. To determine the amount of virus protein in single cells, we evaluated the intensity of immunocytochemical labelling for CDV NP protein in OP-CDV- and A75/17-CDV-infected cells at 6, 9, and 12 days p.i. For the immunolabelling, we used MAb D110, which had been raised against OP-CDV but recognizes an epitope which occurs in all CDV strains tested so far. On each day, 50 individual CDV antigen-positive cells at the growing edge of randomly selected plaques were evaluated, with the assumption that these were the cells in which the most recent and active virus replication had occurred. These cells were viewed on the video monitor at high magnification. A large section of the cytoplasm immediately adjacent to the nucleus was electronically delineated, and the average density of the immunocytochemical product in this area was measured. The data were statistically evaluated by using a box plot (Fig. 7). The virus protein concentration per cell varied little among the majority of the OP-CDV-infected cells at all three measuring times. The situation was very similar in A75/17-CDV-infected cells. Likewise, the overall distributions of the density data were similar for the two infection types. When the average virus protein contents per cell were compared for the two infection types, the amount of NP protein per cell was significantly larger in the persistent CDV infection at 6 days p.i. and much larger still at 9 and 12 days p.i. The same approach was used to evaluate the amount of virus mRNA in single cells by in situ hybridization against the P-protein mRNA (Fig. 8). The medians for the A75/17-CDV and OP-CDV infections were always very similar.

Virus titration. CDV release into the culture medium was determined by a titration assay in which primary DBCC freshly grown in microtiter plates were inoculated with supernatants derived from the infected cultures. Low levels of virulent A75/17-CDV could be detected in the supernatant fluid starting at

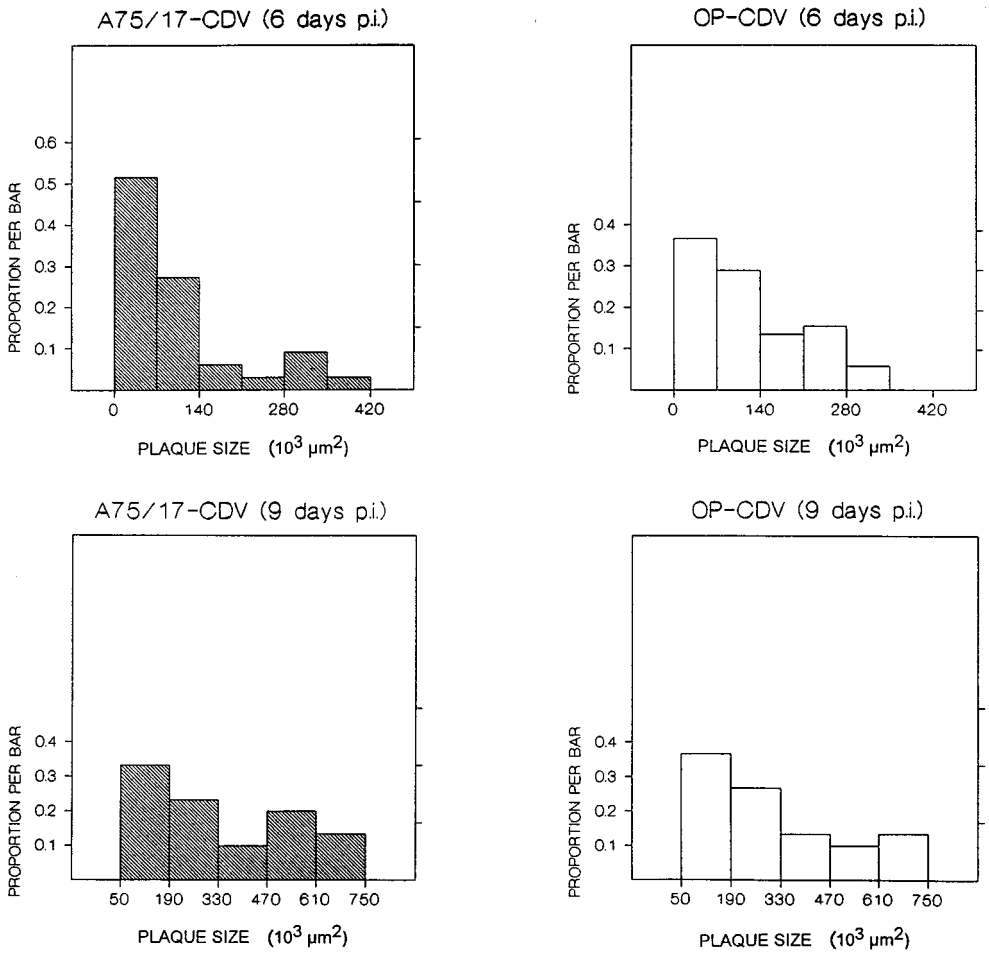

FIG. 6. Histograms of plaque size. The perimeter of the plaque was determined by the most peripherally located infected cells as seen with immunohistochemistry. The ranges in plaque size at 6 and 9 days p.i. are similar in the two infection types. Thus, the distances covered by the two types of infection within this interval are approximately the same.

15 days p.i. (Fig. 9). This value remained very low throughout the observation period, although the infection had reached confluency by day 20, with nearly all cells in the culture containing virus antigen. Tissue culture-adapted OP-CDV was detectable in the culture medium already at 3 days p.i. The amount of infectious culture-adapted OP-CDV steadily increased up to 700 DBCC infectious doses per ml (Fig. 9). The plaques in the titration cultures inoculated with supernatant derived from the OP-CDV infection at 5 weeks p.i. merged, and single plaques could not be counted any more. Therefore, the highest value of this titration assay is 700 DBCC infectious doses per ml. No infectious CDV was found in the supernatant fluid of the control cultures.

Electron microscopy. Ultrastructural characteristics of persistent CDV infection in DBCC have been described previously (6). In persistent CDV infection, accumulation of nucleocapsids in the cytoplasm was frequent. Cell fusion was equally widespread but evidence of virus maturation and release at the cell surface was very difficult to find with A75/17-CDV-infected cells. In contrast, signs of virus maturation with subplasmalemmal alignment of nucleocapsids, spiking of glycoproteins, and budding of virus particles at the cell surface were easily and frequently found with OP-CDV-infected cells (Fig. 2c).

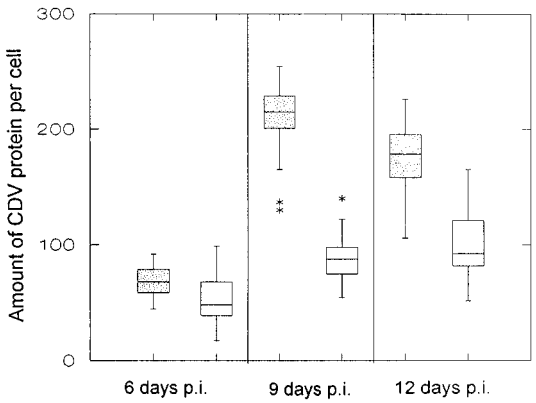

FIG. 7. Box plots of the amount of CDV NP protein per individual cell. The amounts of protein in 50 infected cells at the growing edge of randomly chosen plaques were measured by densitometry at the indicated times. Values for 50% of the cells (boxes) and the medians (horizontal lines within the boxes) are indicated. The ranges in viral protein content among the cells are very similar for the two infection types at all three times. The average amount of viral protein per cell is consistently higher in the persistent infections. Values for different sampling days cannot be compared, because labelling and densitometry procedures were done immediately after sampling, thus on three different dates. ▨, A75/17-CDV; □, OP-CDV; ∗, extreme value.

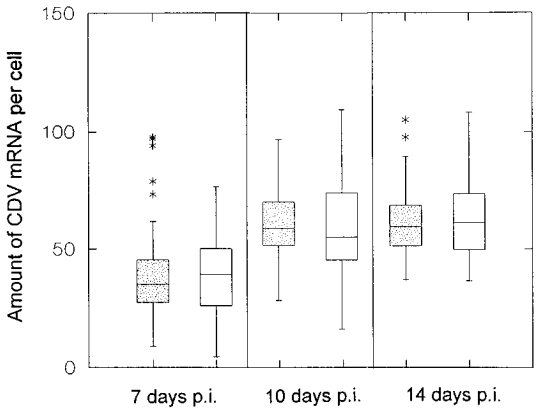

FIG. 8. Box plots of the amount of CDV mRNA per individual cell as mean density after in situ hybridization for the CDV P-protein mRNA. A total of 50 infected cells at the growing edge of the plaques were randomly selected, and the amounts of mRNA were measured by densitometry at the indicated times. At each time, the medians for the A75/17-CDV and OP-CDV infections were very similar. ▨, A75/17-CDV; □, OP-CDV; ∗, extreme value.

DISCUSSION

Although virus persistence is obviously a key event in the pathogenesis of the chronic demyelinating disease in distemper, its mechanism is poorly understood. It has been shown that in human infection caused by SSPE virus, a virus closely related to CDV, persistence is associated with downregulation of genes coding for membrane and/or surface proteins compared with the regulation in regular measles virus (2, 12). Comparison of transcription of all CDV genes in attenuated versus persistent virulent CDV infections by RNA dot blotting did not support a similar phenomenon in CDV infection. We found no evidence that persistence of virulent CDV in DBCC is due to production of defective viruses, since the dot assays revealed that all proteins of A75/17-CDV and all corresponding mRNAs were continuously expressed. In addition, immunocytochemistry and in situ hybridization confirmed that all these viral products are continuously present in nearly all infected cells.

Persistence could be related to the fact that noncytolytic virulent virus replicates less efficiently than attenuated cell culture-adapted strains. Indeed, when the initial development of virus plaques in the cultures was monitored, it was immediately apparent that the number of cells per area unit which became infected during a given interval was much larger in the attenuated than in the persistent infection. One possible explanation for this finding is that the intracellular production of virus proceeds at a higher rate in cells infected with attenuated CDV. We could not show that cells recently infected with virulent virus contained smaller amounts of CDV RNA or protein than cells with the attenuated infection. Rather, the statistical distributions of virus contents among recently infected cells were very similar in the two infection types at three consecutive sampling dates. The higher virus protein concentrations in persistent CDV infection were expected because persistently infected cells continued to survive, in contrast to cytolytically infected cells. These findings indicate that CDV protein synthesis in DBCC proceeds at comparable rates in the two types of infection, once the virus has entered the cell. Thus, the higher infection rate of attenuated CDV in the initial stages of the infection cannot be explained simply by more efficient transcription and translation at the single-cell level.

Surprisingly, in view of these quantitative results, we found striking differences in virus production in the extracellular compartment. In the supernatant fluids from cultures infected with attenuated CDV, much larger amounts of infectious virus were found than in the supernatant fluids from virulent-CDV-infected cells. Of course, such differences were expected in the early stages of the infection because attenuated CDV initially infected a much larger number of cells. However, the noncy-

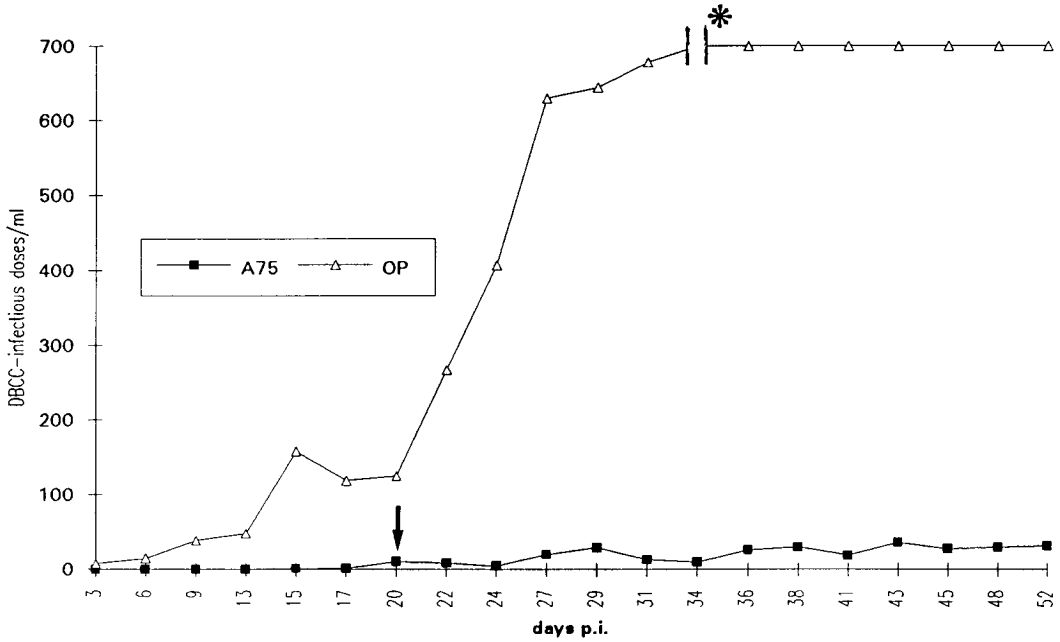

FIG. 9. CDV titration of infectious virus in supernatants derived from the two types of infection. Supernatants were tested on DBCC freshly grown in 24-well plates, by counting plaques following immunohistochemistry for CDV NP protein. Confluency of such plaques in the 24-well plates was reached by ca. 700 DBCC infectious doses per ml, after which (∗) individual plaques could no longer be counted. Persistent infection reached confluency at 20 days p.i. (arrow), with nearly all cells of the source culture containing CDV.

tolytic persistent infection continued to invade not only more and more areas of the culture but also ever-increasing numbers of cells within the initial plaques. Since the persistent infection leaves the cells intact, the number of cells supporting the infection kept steadily accumulating in the culture. Thus, the infection progressed in a logarithmic manner, reaching confluency, with almost all cells in the culture expressing CDV proteins, by 3 weeks p.i. Since by that time attenuated CDV had already destroyed vast parts of the culture, the total number of CDV-containing cells soon became much larger in the persistent than in the attenuated infection. Nevertheless, very little infectious virus was found in the supernatant fluids of persistently infected cells at all stages of the experiment. Ultrastructural examination clearly confirmed that release of infectious virus particles by budding is very widespread in attenuated-CDV-infected cultures but a rare event in cultures infected with virulent virus, even though most cells contained virus nucleocapsids. We must conclude that there are marked differences between these two strains in the way that virus is transmitted from cell to cell.

Indeed, monitoring the infection by immunocytochemistry for viral proteins confirmed that these two strains exhibit completely different patterns of spread. Evidently, attenuated virus spreads randomly in a concentric manner to immediately adjacent perikarya, resulting in a very compact pattern of infection, with nearly all cells within the perimeter of the plaque containing CDV antigen. In view of the titration and ultrastructural findings, showing extensive release of infectious particles, such a pattern of spread is expected. In contrast, immunocytochemistry of persistent CDV showed a loose pattern of infection, with many uninfected cells interspersed with CDV-containing cells. Furthermore, the immunostaining revealed virus protein in the cell processes, including their finest ramifications, and frequent contacts between cells by way of such processes, bypassing immediately adjacent perikarya. As a result, although initially a much lower number of cells within a given plaque was infected with persistent CDV, the measured increase in plaque size within a given period—the distance covered by the infection in the tissue—was equal to that in the infection with the attenuated virus strain. Combining these quantitative and immunocytochemical data, we must conclude that the persistent virus strain spreads preferentially to more-distant cells by way of the cell processes. This could be based on very limited selective budding in certain areas of the cell membrane.

We have shown that in DBCC, virulent CDV behaves differently from attenuated CDV, which showed characteristics in DBCC very much the same as those described previously for several other culture systems. There is no incomplete expression of virulent CDV as in SSPE but, rather, a noncytolytic infection, selective virus spread through cell processes, and very limited budding. No attenuation of virulent CDV takes place in these cultures. Immunocytochemical and morphological studies of sections of brains from dogs with distemper show a loose pattern of infected astrocytes in the white matter, with localization of viral antigen in cell processes and little evidence of cytolysis (28). It has also been very difficult to find viral budding in ultrastructural studies of the lesions (9). The findings in primary DBCC with the same virus, which not only induces disease in vivo but also infects the same target cells, are complementary to what has been seen in vivo. Therefore, it is not unreasonable to conclude that CDV in the brain spreads in a noncytolytic fashion by way of cell processes and very limited budding. We believe that the findings in vivo and in vitro are relevant to the phenomenon of viral persistence. Previous findings in vivo suggest that persistence occurs because the intrathecal immune response keeps lagging behind viral replication and spread. Our observations help to explain why astrocytic CDV infection in the CNS does not readily elicit an antiviral inflammatory reaction: by limiting viral budding and avoiding cell destruction, extracellular release of virus protein and cell debris is strongly reduced. It appears, therefore, that persistence in canine distemper is favored by a strategy of virus spread by which immune recognition of the virus in the CNS is avoided or delayed, as has been proposed by Oldstone and Rall for other virus systems (16).

It is clear that assembly, release, and, consequently, spread of virus depend on specific molecular interactions. Our ongoing studies focus on such molecular aspects of virus maturation and assembly.

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