Canine distemper virus (CDV) causes a multifocal demyelinating disease in dogs. The mechanism of acute demyelination in distemper is still poorly understood. The initial demyelinating lesion in distemper is directly virus induced, since there is a clear correlation between the occurrence of demyelination and CDV replication in the cells of the white matter. Yet, there is little evidence for oligodendroglial infection. Changes of these cells have been reported in vitro and in vivo. The in vitro studies showed that – in contrast to other cells such as astrocytes and macrophages – oligodendrocytes hardly express CDV protein. However, we could show that these cells underwent a restricted infection with transcription of CDV RNA and that this phenomenon correlated with down-regulation of myelin gene transcription. The extension of these in vitro findings in vivo was obscured by the lack of reliable oligodendrocyte labelling techniques in canine brain tissue sections. In this study we combined immunohistochemistry with in situ hybridization to examine oligodendrocytes in demyelinating lesions and to investigate the question of oligodendrocyte infection in vivo. We could demonstrate that CDV infection leads to massive down-regulation of myelin gene expression in demyelinating lesions and that this effect correlates in part with a restricted infection of oligodendrocytes.

**Key words** Canine distemper virus · Oligodendrocytes · In situ hybridization

**Introduction**

Canine distemper virus (CDV), a morbillivirus closely related to measles virus, encodes all virus proteins on a single, non-segmented negative-stranded RNA. CDV causes a multifocal demyelinating disease in dogs. The mechanism of demyelination in distemper has been subject of several studies [17]. There is no doubt that the initial demyelinating lesion in distemper is directly virus induced, since there is a clear correlation between the occurrence of demyelination and CDV replication in the cells of the white matter [20]. However, ultrastructural studies generally agree that there is little evidence for oligodendroglial infection, the obvious explanation for the phenomenon of demyelination. Indirect evidence for oligodendroglial impairment in acute lesions is the observation of segmental demyelination and of ultrastructural pathological changes of oligodendrocytes [11, 16]. Such changes in oligodendrocytes had been reported earlier in vitro using primary canine brain cell cultures [24, 25]. These cultures have been extensively used to study viral tropism. These in vitro studies showed that – in contrast to other cells such as astrocytes and macrophages – oligodendrocytes hardly express CDV protein [27]. However, we could show that these cells underwent a restricted infection with transcription of CDV RNA, and that this phenomenon correlated with down-regulation of myelin gene transcription, presumably the underlying reason for degeneration of these cells [9]. Although infection of primary dog brain cell cultures with virulent CDV closely mimics the infection in the central nervous system (CNS) in vivo, extrapolation of in vitro findings to the disease in vivo has to be viewed with caution. The extension of the in vitro findings in vivo has so far been obscured by the lack of reliable oligodendrocyte labelling techniques in canine brain tissue sections, in contrast to cultured canine oligodendrocytes which can be specifically labelled by a wide variety of antibodies [22, 23]. The advent of in situ hybridization (ISH) techniques for demonstrating oligodendrocytes on paraffin sections [1, 4] opened a new avenue to investigate oligodendrocytes in vivo. We have developed canine-specific clones for myelin mRNA and suitable labelling techniques [8]. In the past we have also produced complementary probes, mRNAs corresponding to the different genes of CDV and antibodies to viral proteins [26]. Using a combination of these techniques we set out to study oligodendrocytes in...
demyelinating lesions and to investigate the question of oligodendrocyte infection in vivo.

Material and methods

Animals

Brain tissues were derived from 8 dogs that had been experimentally infected with A75/17-CDV in the frame of another study. The details of these experiments have been reported elsewhere [18, 19]. Brains were removed immediately after death, fixed in 4% buffered paraformaldehyde and processed for paraffin embedding.

Selection of lesions

Tissue sections immunolabelled for CDV were examined by light microscopy. Areas of white matter showing CDV infection were marked. Two types of infected areas were selected: areas in which no structural loss of myelin was found and areas in which overt demyelination was present. Serial sections of blocks containing these areas were made and used for further studies. A total of 30 infected areas was investigated.

Complementary DNA clones

A complementary DNA (cDNA) clone for the identification of oligodendrocytes, containing parts of the coding region of the proteolipidprotein (PLP) gene and a clone complementary to the P coding region of the virulent A75/17-CDV RNA were produced as published earlier [8, 26]. They were subcloned into the pSPT19 plasmid vector (Boehringer Mannheim, Germany) carrying promoters for the DNA-dependent SP6 and T7 RNA polymerases.

Preparation of non-radioactive probes for ISH

Strand-specific digoxigenin-labelled RNA probes were prepared as described previously [8, 13]. After linearization, the DNAs were transcribed using the Ribomax system (Promega, Switzerland). The probes were shortened to a length of about 150 bases according to the procedure of Cox et al. [6] and stored in diethylpyrocarboclate-treated water (DEPC water) at −70°C until further use. For the double ISH, a biotin-labelled RNA probe was prepared from the clone complementary to the P coding region of the virulent A75/17-CDV using the same protocol as for the digoxigenin labelling. The DIG RNA labelling mix (Boehringer Mannheim) was replaced by the biotin RNA labelling mix (Boehringer Mannheim) in the transcription reaction.

Antibodies

Rabbit anti-glial fibrillar acidic protein (GFAP; Dakopats, Copenhagen, Denmark) was used to demonstrate astrocytes. For the detection of CDV antigen monoclonal antibody (mAb) D110 was used. This antibody recognizes an epitope within the nucleocapsid protein of CDV [3, 10].

Double-labelling techniques

Immunohistochemistry for astrocytes with ISH for oligodendrocytes

Oligodendrocytes were demonstrated by ISH using the PLP probe. ISH was performed as described previously [9]. Deparaffinized rehydrated brain sections were incubated in 0.2 M HCl for 20 min. After a quick wash with 2 × SSC (sodium chloride/sodium citrate buffer), the cells were permeabilized with proteinase K (Boehringer Mannheim) at a concentration of 10 µg/ml for 15 min at 37°C. Following a postfixation with 4% paraformaldehyde in PBS (5 min) the sections were boiled in the microwave for 1 min in 6 M urea. The samples were washed in 2 × SSC and prehybridized at 50°C for at least 1 h in 50% formamide (v/v), 4 × SSC, 2 × Denhard’s reagent and 250 µg RNA/ml. Hybridization was performed overnight at 50°C in 50% (v/v) formamide, 4 × SSC, 2 × Denhard’s reagent, 500 µg RNA/ml and 10% dextrane sulfate (w/v). The final concentrations of the labelled probes were approximately 2 ng/µl. After hybridization, excess labelled RNA was removed by washing in 2 × SSC and by RNAse treatment: 100 U/ml RNase T1 (Boehringer Mannheim) and 0.1 µg/ml DNase-free RNase (Boehringer Mannheim) at 37°C for 30 min. After washing in 2 × SSC (55°C, 20 min) and 0.2 × SSC (55°C, 20 min), the samples were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim). For the following color reaction we used 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (NBT; Sigma, Buchs, Switzerland). The ISH procedure was followed by immunohistochemistry for the identification of astrocytes with a rabbit anti-GFAP antibody using the unlabelled peroxidase-antiperoxidase method as described previously [7].

Immunohistochemistry for CDV proteins with ISH for CDV mRNA

The ISH for CDV P-mRNA was combined with immunohistochemistry for the demonstration of CDV proteins with mAb D110 [3]. ISH was performed as described above with the following modifications: the concentration of proteinase K was 5 µg/ml and the urea treatment was omitted.

Immunohistochemistry for CDV proteins with ISH for PLP mRNA

The ISH for the demonstration of PLP mRNA was performed as described above followed by the immunohistochemistry for CDV proteins using mAb D110.

ISH for CDV mRNA with ISH for oligodendrocytes

For the double ISH, the CDV-P probe was labelled with biotin and the PLP probe with DIG. The ISH protocol was similar to the regular procedure as described above for PLP. Both probes were hybridized simultaneously, washed and then incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase and Texas red/streptavidin (Vector Laboratories) 20 µg/ml mM Tris pH 7.5, 150 mM NaCl (TNB) for 2 h at room temperature. The samples were washed with 100 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TNT) three times for 5 min, and then incubated with 5 µg/ml biotinylated anti-streptavidin (Vector Laboratories) in TNB for 1 h at 37°C. After repeated washing with TNT, the slides were treated with Texas red/streptavidin (20 µg/ml TNT) for 30 min at 37°C, washed again with TNT and incubated with 5 µg/ml biotinylated anti-streptavidin. After washing with TNT, the samples were treated again with Texas red/streptavidin (20 µg/ml TNB) for 30 min at 37°C, washed and the color reaction performed using 5-bromo-4-chloro-3-indolyl phosphate and NBT.

Microscopy

Slides were examined with a standard light microscope. Double ISH preparations were evaluated with a laser scan microscope (LSM IV, Zeiss). Laser light excitation resulted in a far stronger signal than conventional UV light. The fluorescent ISH product was visualized simultaneously with the NBT reaction product by superimposing digitalized images of either product using the hard- and software associated with the LSM. Confocal scanning was used to obtain a better resolution of the fluorescence signal.

Preparations double labelled for protein and mRNA were systematically documented on color slides. The slides were then projected on white paper and cell counts were made.
Results

Demyelination was mostly located in periventricular and subpial areas in the brain stem or cerebellum. It consisted of clearly delineated plaques with pallor of the tissue, vacuolation and mild astrocytic gliosis. In several lesions there were also activated microglia and macrophages in close apposition to demyelinated fibers. In some there were a few scattered cells with shrunken pyknotic nuclei.

CDV infection

Demyelinated and some intact white matter areas contained large amounts of CDV-infected glial cells, as seen with immunohistochemistry and ISH (see Fig. 2A). CDV infection gradually dissipated away from the edge of the lesion. The outline of the infected cells and their processes was frequently seen. Such cells appeared to be morphologically intact.

ISH for oligodendrocytes

White matter areas contained large numbers of oligodendrocytes (Fig. 1A, B). They were visible as small round cells, evenly distributed throughout the myelinated areas, often in small rows and clusters. Occasionally, they had short thin cell processes. On double labelling with GFAP, there was a clear and absolute separation between PLP-labelled cells and astrocytes (Fig. 2B). A few oligodendrocytes were also seen in the gray matter. The number of oligodendrocytes in the white matter was estimated to be at least as large as that of astrocytes. In demyelinated lesions, there was virtually a complete lack of PLP labelling (Fig. 1A, B, E). The fringes of demyelinated lesions contained cells weakly labelled with PLP (Fig. 1C, D). In CDV-infected but not yet demyelinated white matter areas most oligodendrocytes were apparently infected, although the density of PLP labelling seemed to be diminished in some lesions as compared to the immediately surrounding uninfected tissue.

Discussion

The mechanism of acute demyelination in distemper is still poorly understood. Electron microscopical studies rarely revealed oligodendrocyte infection [2, 11, 16, 21], although segmental demyelination [11] and degenerative oligodendroglial changes in acute foci [16] are strongly suggestive of a primary oligodendroglial lesion. Such damage had been noticed before in oligodendrocytes in vitro [24, 25] and was later correlated with restricted infection of these cells [27]. Restricted CDV infection in vitro leads to marked down-regulation of myelin gene transcription in oligodendrocytes [9] and reduced oligodendrocyte-specific enzyme activity [7].

The present study shows that severe down-regulation of myelin transcription also takes place in vivo. The

Fig. 2 A Periventricular demyelinating lesion (ventricle IV, upper right). ISH for CDV P mRNA. Many infected glial cells. B Brain stem of a normal dog. Double labelling for oligodendrocytes (blue) and astrocytes (brown). Oligodendrocytes are at least as numerous as astrocytes. C CDV-infected intact (no demyelination) white matter in the cerebellum. Double labelling for PLP and CDV as described in Fig. 1. Several oligodendrocytes are in close proximity to CDV-infected cells. One oligodendrocyte (arrow) is sandwiched between infected astrocytes. CDV antigen is rarely found in oligodendrocytes: the inset shows two oligodendrocytes in which CDV antigen (arrows) is located in the oligodendrogial perikaryon. D Infected intact (no demyelination) area in the cerebral white matter. Double ISH: typical presentation of viral mRNA (orange fluorescence) in astrocytes in between interfascicular oligodendrocytes (black). Inset: colocalization of CDV mRNA with PLP mRNA in cell on the right. E–G Acute demyelinating lesions. Double labelling for CDV P mRNA by ISH (blue) and CDV nucleocapsid protein by immunohistochemistry (brown). As expected, most infected cells in the lesions are double labelled, whereby the brown reaction product has a tendency to dominate. Occasionally cells are seen (arrows) which are only blue labelled, indicating restricted infection. A × 100; B, E–G × 200; C, D × 400. Insets × 1000

Double labelling experiments

Several thousand CDV-infected cells and labelled oligodendrocytes were examined. The results of the various experiments are presented in Table 1. Double labelling for CDV nucleoprotein and PLP mRNA revealed only a few oligodendrocytes which appeared to contain CDV protein (Fig. 2C). Double labelling for CDV protein and CDV mRNA revealed double labelling of most cells in infected areas, the brown reaction product (diaminobenzidine) of the immunohistochemistry having a clear tendency to cover the blue product of the ISH reaction (Fig. 2E–G). A few cells were noted in which only ISH product was visible (Fig. 2E–G). Double ISH for CDV mRNA and PLP mRNA using fluorescence to demonstrate viral RNA yielded an excellent separation of the reaction products (orange versus black; Fig. 2D). This examination revealed that by far most of the viral RNA was associated with astrocytes (Fig. 2D); colocalization of both products was found in ca. 8% of the oligodendrocytes.
strongly diminished expression of PLP mRNA correlated well with the presence of demyelination, which in turn was closely associated with expression of viral protein and nucleic acids in the glial cells of the demyelinated areas. The massive reduction of the PLP mRNA signal did not appear to be the result of simple removal of oligodendrocytes from the area. Considering the large number of oligodendrocytes in the canine white matter, systematic
Table 1  Double labelling (oligo oligodendrocytes)

| Experiment          | CDV mRNA/CDV protein | CDV mRNA/PLP mRNA | CDV protein/PLP mRNA |
|---------------------|-----------------------|-------------------|----------------------|
| CDV mRNA + cells    | 3721                  | 1516              | –                    |
| CDV protein + cells | 3605                  | –                 | 3077                 |
| Restrictively infected cells | 116          | –                 | –                    |
| % restrictively infected cells | 3.11      | –                 | –                    |
| PLP mRNA + oligo    | –                     | 1888              | 2343                 |
| CDV mRNA + oligo    | –                     | 151               | –                    |
| % CDV mRNA + oligo  | –                     | –                 | 7.99                 |
| CDV protein + oligo | –                     | –                 | 9                    |
| % CDV protein + oligo| –                    | –                 | 0.38                 |

lysis of these cells could not have escaped our attention. We also know from electron microscopical studies that there is no obvious loss of oligodendrocytes in demyelinating lesions, although discrete ultrastructural changes do occur in these cells [16]. In vitro, we have noticed that metabolic changes in oligodendrocytes occur long before morphological alterations become visible [7].

Our findings leave no doubt that demyelination in distemper, at least in the acute stage of the disease, is the result of oligodendrocyte impairment. In the present study we applied extensive double-labelling techniques to establish a direct correlation between demyelination and oligodendrocyte infection. Because of the marked down-regulation of myelin gene transcription in the lesions, the usefulness of the ISH technique to identify oligodendrocytes on the basis of PLP mRNA expression in double-labelling experiments was reduced in demyelinating lesions. On the other hand, the demonstration of small quantities of CDV mRNA may be difficult in the very early stages of infection, preceding demyelination. Despite this rather narrow “window”, by systematically screening the periphery of demyelinating plaques and investigating infected white matter areas in which demyelination and PLP down-regulation had not yet occurred, we did find infected oligodendrocytes. The number of oligodendrocytes expressing CDV protein was exceedingly low. This was not unexpected in view of the above-mentioned electron microscopical studies [7, 16] and CDV infection experiments in brain cell cultures [24]. However, the number of oligodendrocytes expressing viral RNA was much higher than CDV protein-expressing oligodendrocytes. This finding confirms that restricted infection of oligodendrocytes also occurs in vivo, as already described for cultured oligodendrocytes [27]. This is not surprising, since our previous studies showed evidence for restricted infection in neurons in distemper in vivo [13]. This finding has been confirmed by others [14] and restricted infection in CNS cells has been reported since in other viral systems [5]. It is possible that cell-specific factors such as MxA proteins control such events [15]. While this study confirms restricted oligodendrocyte infection in vivo, which we previously found in vitro, its causal relationship to down-regulation of myelin gene transcription and demyelination is not as apparent in vivo as it was in vitro [9]. Demyelination in distemper lesions is complete and all oligodendrocytes in a focus are affected, as shown in the present study. Yet the number of CDV mRNA-expressing oligodendrocytes found in infected but not yet demyelinated areas seems – with 8% of all oligodendrocytes in the infected area – rather small to explain ensuing demyelination. We found that the number of oligodendrocytes in canine white matter at least matches the number of astrocytes. Therefore, if myelin gene down-regulation were to be due to restricted infection, one would also expect a significant number of cells expressing only viral mRNA but no CDV protein in fully demyelinated lesions, in which oligodendrocytes are no longer detected by ISH techniques. However, the total number of cells in demyelinated areas showing restricted infection, as seen on double labelling for CDV protein and mRNA, was of the same order of magnitude as the number of restrictively infected oligodendrocytes found in infected but not yet demyelinated areas. It appears, therefore, that oligodendrocytes do not easily support CDV infection, in contrast to astrocytes, in which very strong signals for transcription and translation of CDV were obtained.

In conclusion, we have shown that CDV infection leads to massive down-regulation of myelin gene expression in demyelinating lesions and that this effect correlates in part with restricted infection of oligodendrocytes. However, we could not establish a full correlation between demyelination and oligodendrocyte infection in the quantitative sense. It is possible that minimal CDV transcription, which is below the limit of detection of the methods used, already suffices to shut down the oligodendrogial metabolic machinery. Alternatively, an indirect mechanism could play a role in oligodendrocyte dysfunction. It is known that these cells are dependent on other cell types for their survival [12]. Even though CDV is a non-cytopathic virus, the massive viral load in myelinated areas could perhaps lead to impairment of supportive functions of these other cell types.

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