ELSEVIER

Journal of Neuroimmunology 125 (2002) 30–41

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Increased expression of pro-inflammatory cytokines and lack of up-regulation of anti-inflammatory cytokines in early distemper CNS lesions

S. Markus [a], K. Failing [b], W. Baumgärtner [a,*]

[a] *Institut für Veterinär-Pathologie, Justus-Liebig-Universität Giessen, Frankfurter Str. 96, 35392 Giessen, Germany*
[b] *Institut für Veterinär-Physiologie, Justus-Liebig-Universität Giessen, Frankfurter Str. 95, 35392 Giessen, Germany*

Received 13 June 2001; received in revised form 3 December 2001; accepted 16 January 2002

Abstract

To investigate the pathogenesis of early lesions in canine distemper virus (CDV) leukoencephalomyelitis, the expressions of pro- and anti-inflammatory cytokines such as interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and transforming growth factor (TGF)-β and the housekeeping genes β-actin and GAPDH were studied using semi-quantitative RT-PCR. Relative cytokine values were related to the degree of CDV infection, MHC class II expression and infiltration of CD4-, CD8- and CD3ε-positive lymphocytes. Actin up-regulation, in contrast to GAPDH, was influenced by CDV infection and therefore could not be used as an internal standard to study cytokine expression. In early CDV infection of the cerebellum, either no detectable lesions or mild infiltration of CD8 positive cells or demyelination and up-regulation of MHC class II antigen were observed. IL-6, -8, -12 and TNF-α transcripts were found in 94%, 94%, 78% and 56% of distemper dogs, respectively, compared to 17%, 33%, 0% and 0% in controls, whereas IL-1β, -2 and IFN-γ were not detectable in any of the studied cerebella. Conversely, IL-10 and TGF-β transcripts were present in 83% and 100% of the investigated cerebella of distemper dogs and controls. Relative RT-PCR results, expressed as %GAPDH, revealed a significant up-regulation of IL-6, -8, -12 and TNF-α mRNA in distemper dogs; whereas IL-10 and TGF-β showed only a weak and not significantly increased expression following infection. Relative pro-inflammatory cytokine expression values were highest following CDV infection, indicating that the virus itself directly triggered the up-regulation of the pro-inflammatory cytokines. Succeeding changes, such as lymphocyte infiltration, MHC class II up-regulation and demyelination resulted only in a minor additional increase in cytokine expression, implying a secondary or by-stander mechanism of cytokine activation by these changes. Disease initiation and progression in early distemper leukoencephalomyelitis seemed to be due to a lacking or inappropriate response of the anti-inflammatory cytokines in the presence of a vigorous up-regulation of pro-inflammatory cytokines.

Keywords: Canine distemper virus; Leukoencephalomyelitis; Demyelination; Cytokines; Semi-quantitative RT-PCR; Multiple sclerosis

1. Introduction

Canine distemper virus (CDV), a morbillivirus of the family Paramyxoviridae, causes a systemic, often fatal disease in carnivores, which is commonly associated with nervous disturbances (Krakowka et al., 1985; Alldinger et al., 1996; Frisk et al., 1999). Leukoencephalomyelitis with demyelination is a common finding in distemper dogs, whereas gray matter lesions such as post-vaccinal encephalomyelitis, inclusion body polioencephalitis and old dog encephalitis are only found infrequently (Gaedke et al., 1999; Nesseler et al., 1999). Histologically, changes in distemper leukoencephalomyelitis (DL) can be classified into "early lesions" consisting of acute to subacute non-inflammatory plaques and "late lesions" representing subacute inflammatory and chronic plaques (Gaedke et al., 1999; Wünschmann et al., 1999). Based on the morphological similarities of the lesions, DL is considered to be a spontaneously occurring animal model for multiple sclerosis (MS) and other demyelinating diseases of humans (Koestner, 1975; Krakowka et al., 1985; Sanders et al., 1993; Gaedke et al., 1999).

* Corresponding author. Institut für Pathologie, Tierärztliche Hochschule Hannover, Bünteweg 17, 30559 Hannover, Germany. Tel.: +49-511-953-8620; fax: +49-511-953-8675.

E-mail address: wolfgang.baumgaertner@tiho-hannover.de (W. Baumgärtner).

0165-5728/02/$ - see front matter
PII: S0165-5728(02)00027-9

Dissection of plaque type characteristics in DL indicated a biphasic process in the pathogenesis of myelin loss (Alldinger et al., 1996; Gaedke et al., 1999). Early lesions, developing in the presence of a systemic virus-induced immunosuppression (Wünschmann et al., 1999), are characterized by minimal to strong expression of viral protein and mRNA within the lesions, mild to moderate MHC class II up-regulation and lacking or only mild infiltration of CD3ε- and CD8-positive lymphocytes (Alldinger et al., 1996; Tipold et al., 1999; Wünschmann et al., 1999). In early lesions, myelin loss is considered to be a consequence of restricted CDV replication within oligodendrocytes and down-regulation of myelin gene transcripts (Graber et al., 1995; Zurbriggen et al., 1998). Additionally, CD8-positive lymphocytes and astrocyte-derived TNF-α might exacerbate the progression of lesions (Tipold et al., 1999; Wünschmann et al., 1999; Gröne et al., 2000). Chronic lesions are associated with reduced expression or elimination of CDV antigen and mRNA, strong MHC class II up-regulation and prominent lymphocyte infiltration. While intra-lesional infiltrates are dominated by CD8+ cells, CD4+ and B cells are more prominent in perivascular cuffs (Alldinger et al., 1996; Wünschmann et al., 1999). In addition, pro-inflammatory cytokines including IL-1, -6 and -12 have been detected by immunohistology in resident and infiltrating inflammatory cells in DL, indicating a highly complex possible immune-mediated immune response (Gröne et al., 2000).

Cytokines are often differentiated into pro- and anti-inflammatory cytokines and they can play a beneficial or deleterious role in disease progression (Woodroofe, 1995; Navikas and Link, 1996; Rothwell, 1997; Selmaj and Raine, 1988). The role of cytokines in the pathogenesis of CNS disorders has been investigated in various human diseases including MS and acquired immune deficiency syndrome (McGuinness et al., 1997; Seilhean et al., 1997; Baranzini et al., 2000). Pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-12, TNF-α and IFN-γ are associated with disease progression through attraction and activation of inflammatory cells and induction of nitric oxide, whereas IL-10 and TGF-β play an essential role in disease remission (Brosnan et al., 1995; Calabresi et al., 1998; Bertolotto et al., 1999).

To further elucidate the molecular mechanisms responsible for disease initiation and progression in DL, it was the aim of this study to investigate the expression of pro- and anti-inflammatory cytokines in early distemper lesions of dogs by reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Tissue samples, histology and immunohistology

A total of 22 cerebella, 18 from distemper and 4 from control dogs, were investigated. Distemper dogs, 11 females and 7 males, between 2 months and 3 years of age, represented submissions to the Institut für Veterinär-Pathologie, Justus-Liebig-Universität Giessen, Germany. Vaccination records revealed that three distemper dogs were completely vaccinated, five incompletely and three were not vaccinated. The vaccination records of seven dogs remained undetermined. Control dogs, two females and two males, were 9 months old and completely vaccinated against various canine pathogens including CDV. During necropsy, tissues including CNS were collected and fixed in 10% non-buffered formalin. Following paraffin embedding, sections were cut at 2–4 μm thickness and stained with hematoxylin and eosin (HE). Additionally, OCT-embedded frozen tissue blocks from the CNS were prepared as described (Wünschmann et al., 1999). Distemper lesions were classified into different types of plaques according to the literature and based on experimental data obtained in previous studies (McCullough et al., 1974; Baumgärtner et al., 1989; Gaedke et al., 1999; Gröne et al., 2000).

Table 1
Specificity, clone number, dilution/concentration of antibodies/lectin, and marked cell populations

Specificity	Clone	Dilution/concentration	Marked cell population
CDV-N	3.991 (NP2)	1:6000	CDV infected cells
CDV-NP	rabbit anti-CDV	1:1000	CDV infected cells
caMHC II	dog 26-1	1:500	antigen presenting cells, most likely microglia/macrophages
caCD21(like)	CA.1D6	1:500	B lymphocytes
caCD3ε	dog 12-1	1:1000	T lymphocytes
caCD4	YKIX302.9.3.7	1:100	T helper cells
caCD8	dog 10-1-1	1:1000	cytotoxic T cells
huGFAP	6F2	1:50 or 1:15	astrocytes
GFAP	rabbit anti-cow (polyclonal)	1:500	astrocytes
BS-1	–	2 μg/ml	microglia/macrophages/endothelial cells
von Willebrandt factor	rabbit anti-human (polyclonal)	1:1000	endothelial cells

CDV-N = canine distemper virus nucleoprotein.
ca = canine; hu = human; MHC II = MHC class II; GFAP = glial fibrillary acidic protein; BS-1 = biotinylated lectin from Bandeiraea simplicifolia.

Serial frozen tissue sections, cut at 10-μm thickness, were used for histology and immunohistology. In addition, 16 following sections were used for mRNA analyses. Routine histology included HE and luxol fast blue cresyl violet (LFB) stain for evaluation of myelin loss. To detect cellular antigens, the avidin–biotin–peroxidase complex (ABC, Vector Laboratories, Burligame, USA) method was used (Alldinger et al., 1996; Wünschmann et al., 1999; Gröne et al., 2000). Briefly, sections were incubated with a canine distemper virus (CDV) nucleoprotein (NP2)-specific monoclonal or a rabbit polyclonal nucleoprotein CDV-specific antibody (#25, rabbit 162, kindly provided by C. Örvell, Stockholm, Sweden) and B- and T cell, as well as MHC class II-specific antibodies (kindly provided by E. Kremmer, GSF-Forschungszentrum, Institute for immunology, Munich, Germany). In addition, GFAP-and von Willebrand factor-specific mono- or polyclonal antibodies (Dako Diagnostics, Hamburg, Germany; Cymbus Biotechnology, UK) and the biotinylated lectin from Bandeiraea simplicifolia (BS)-1 (Sigma, St. Louis, USA; Table 1) were used. Controls included omission of the primary antibody, link antibody and ABC or substitution of the specific antibody with

Table 2
Primer sequences used for the amplification of housekeeping genes, cytokines and CDV RNA with melting point temperature and length of amplicon

Canine cDNA (accession number)[a]	Primer sequences (5′–3′) forward and reverse	Expected amplicon length (bp) and melting point temperature (T_m)
GAPDH	GCC AAA AGG GTC ATC ATC TC	228 bp
U31247[b]	GGG GCC ATC CAC AGT CTT CT	87.8 °C
β-Aktin	CGT TGC TAT CCA GGC TGT GC	435 bp
Z70044[b]	GTA GTT TCG TGG ATG CCA CA	92.0 °C
IL-1β	TCC AAT GTG AAG TGC TGC TGC	323 bp
Z70047[b]	TAT GAG TTA GAC AGC ACC AGG	85.8 °C
AF322078[c]		
IL-2	AGA TGG AGC AAT TAC TGC TGG	120 bp
U28141[b]	ATT CTG TGG CCT TCT TGG GCG TGT	79.6 °C
AF333118[c]		
IL-6	GCA AGG AGG CAC TGG CAG AA	102 bp
U12234[b]	TTC TTG TCA AGC AGG TCT CC	80.8 °C
AF333119[b]		
IL-8	ACT TCC AAG CTG GCT GTT GC	172 bp
U10308[b]	GGC CAC TGT CAA TCA CTC TC	83.2 °C
IL-10	CCT GGG TTG CCA AGC CCT GTC	212 bp
U33843[b]	ATG CGC TCT TCA CCT GCT CC	88.7 °C
AF333120[c]		
IL-12 p 40	CAC CTG CCA TAC CCC TGA AG	452 bp
U49100[b]	CAC TGC CTT CCT GAC ACT CC	85.8 °C
AF333121[c]		
IFN-γ	GCA AGT AAT CCA GAT GTA TCG	283 bp
S41201[b]	TTA TCG CCT TGC GCT GGA CC	83.3 °C
AF333123[c]		
TNF-α	CCA AGT GAC AAG CCA GTA GC	274 bp
Z70046[b]	TCT TGA TGG CAG AGA GTA GG	90.0 °C
TGF-β	TTC CTG CTC CTC ATG GCC AC	393 bp
L34956[b]	GCA GGA GCG CAC GAT CAT GT	93.2 °C
CDV N635	ACA GGA TTG CTG AGG ACC TAT	287 bp
X02000[b]	CAA GAT AAC CAT GTA CGG TGC	83.4 °C

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; IFN = interferon; TNF = tumor necrosis factor; TGF = transforming growth factor; CDV = canine distemper virus; bp = base pairs; T_m = melting temperature, calculated.

[a] GenBank™/EMBL, data bank accession number.

[b] Accession number of cDNA sequences used for designing primers.

[c] Accession number of sequenced amplicon obtained with newly created primer pairs.

ascites from non-immunized Balb/cJ mice. The spleen tissue of a control dog and CDV-infected African green monkey kidney (Vero) cells served as positive controls.

The immunohistological expression of leukocyte differentiation molecules and MHC class II and CDV antigen was evaluated quantitatively at the intralesional and extralesional sites (Wünschmann et al., 1999). The number of cells stained per square unit of tissue within each compartment was counted using an ocular morphometric grid. Values represented the number of cells per 0.25 mm^2. GFAP and BS-1 antigen distribution was scored semiquantitatively (− = negative, + = single positive cells, ++ = moderate number of positive cells, +++ = numerous positive cells).

Sequential double immunohistochemical staining using selected tissue sections was performed in order to identify the cells which demonstrated immunoreactivity for CDV antigen and MHC class II molecules (Gröne et al., 2000). Briefly, after the chromogenic reaction with DAB for localization of CDV or MHC class II antigen, the sections were washed in Tris–HCl buffer for 5 min followed by overnight incubation at 4 °C with cell-type specific antibodies. After washing, the sections were incubated with biotinylated link antibodies (Vector Laboratories, 1:100) for 30 min, followed by ABC-alkaline phosphatase (Vector Laboratories) for 30 min. Positive reactions were visualized by the New Fuchsin substrate system. Co-localization of antigens was identified either by the presence of both colors in one cell (e.g. segmental staining of cellular processes) or by a pinkish brown mixed color.

2.2. Primer design and controls

The primers for PCR were taken from the literature (GAPDH, β-actin, IL-8, TNF-α, TGF-β and CDV; Gröne et al., 1998a,b; Frisk et al., 1999) or designed from canine-specific cDNA sequences (Table 2, GenBank™/EMBL Data bank) using the Primer Designer Program (IL-1, -2, -6, -10, -12 and IFN-γ; Science and Educational Software, State Line, PA). Positive controls for RT-PCR included canine peripheral blood leukocytes stimulated with 5 μg/ml Concanavalin A (Con A) and DH82 cells, a cell line from canine malignant histiocytosis, infected with the Onderstepoort CDV strain (Gröne et al., 1998a). Negative controls included the omission of RT and the use of diethyl pyrocarbonate (DEPC)-treated water instead of RNA.

2.3. RNA isolation and semiquantitative RT-PCR

Total ribonucleic acid (RNA) was isolated from 16 following frozen sections using Trizol (Life Technologies, Eggenstein, Germany; Gröne et al., 1998a). To avoid carry-over-contamination, the microtome blade was cleaned with 70% ethanol before each isolation. RNA was treated by DNase to avoid amplification of genomic sequences and immediately reverse transcribed employing murine reverse transcriptase (RT-PCR Core Kit, Perkin Elmer, Applied Biosystems, Weiterstadt, Germany; Gröne et al., 1998a). The first strand cDNA served as a template for the PCR using the LightCycler rapid thermal cycler system following the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

In order to optimize the PCR conditions, hot-start (LightCycler-FastStart DNA master SYBR green I, Roche Diagnostics, Mannheim, Germany) and touch-down techniques were employed. For the three templates, optimal reaction results were achieved without hot-start technique (β-actin, Il-10 and CDV; LightCycler-DNA master SYBR green I, Roche Diagnostics, Mannheim, Germany). Touch-down PCR was performed by starting the PCR reaction with an annealing temperature of 4 °C to 10 °C above the final annealing temperature and the reduction of the annealing temperature from cycle to cycle until the final annealing temperature was reached. The cycle numbers varied from 40 to 45 depending upon the used cDNA.

Products of newly created primer pairs were visualized on a 2% ethidium bromide-stained agarose gel, sequenced and submitted to the National Center for Biotechnological Information, Washington, DC (GenBank™/EMBL, Table 2). For semi-quantification of cytokine mRNA, data were analyzed with the LightCycler analysis software 3 (Roche Diagnostics, Mannheim, Germany) during the exponential phase of amplicon formation using the "fit point method".

Relative amounts of cytokine mRNA were determined by calculating the ratio between cytokine mRNA and the

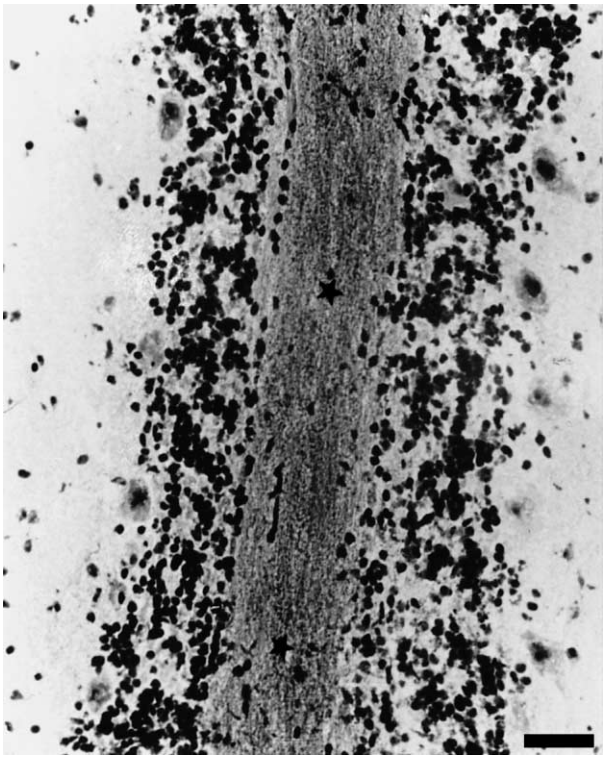

Fig. 1. Demonstration of intact myelin in the white matter of a cerebellar folium of a control. Luxol fast blue cresyl violet stain. * = intact myelin. Bar = 60 μm.

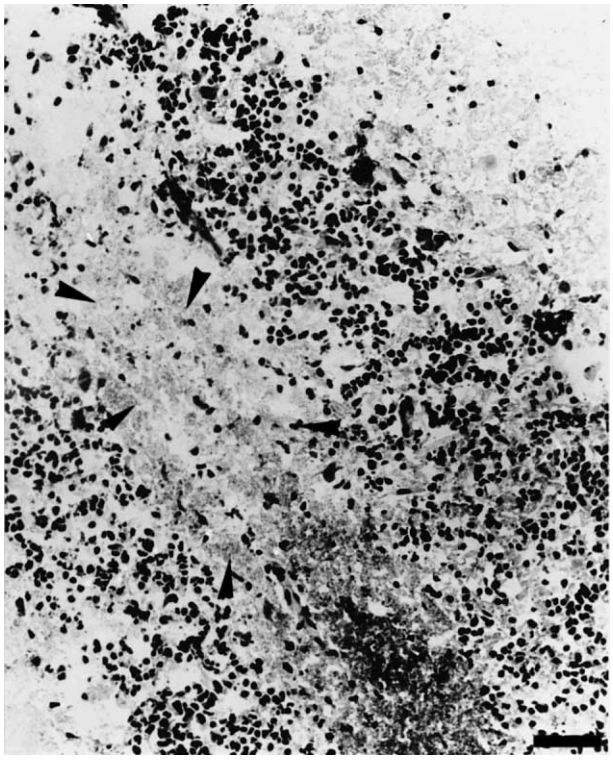

Fig. 2. Myelin loss in the white matter of a cerebellar folium of a group IV animal. Luxol fast blue cresyl violet stain. Arrowheads depict area of myelin loss. Bar = 60 μm.

mRNA of the housekeeping gene GAPDH (units cytokine mRNA/units GAPDH mRNA × 100 = relative amount of cytokine mRNA in %GAPDH). For data presentation, the box and whisker plot was used.

2.4. Statistical analyses

For statistical analyses of the immunohistological and RT-PCR results, the Kruskal–Wallis-test, Spearmans rank-correlation test and the test according to Nemenyi were applied (Dixon, 1993; Wünschmann et al., 1999). A level of $p < 0.05$ was considered significant.

3. Results

3.1. Histopathological findings

Brain tissue from control animals was without significant microscopic findings. Overall classification of the neuropathological changes in distemper dogs, achieved by using formalin-fixed CNS tissue sections from each animal, revealed that two dogs lacked histopathological CNS changes, one showed acute alterations characterized by vacuolization and single cells necrosis, 12 displayed a non-inflammatory subacute distemper leukoencephalomyelitis (DL) consisting of vacuolization, demyelination, malacia, gemistocytes and reactive astrogliosis with few parenchymal inflammatory cells, two dogs showed subacute DL with inflammation consisting of vacuolization, demyelination, reactive astrogliosis, malacia, gemistocytes, and mild perivascular lymphocyte infiltration. One animal displayed the chronic stage of the disease characterized by severe perivascular lymphocyte cuffing in addition to the above-mentioned findings. Eosinophilic intranuclear and intracytoplasmic inclusion bodies were found in astrocytes and macrophages. They were most frequently detected in animals with subacute lesions.

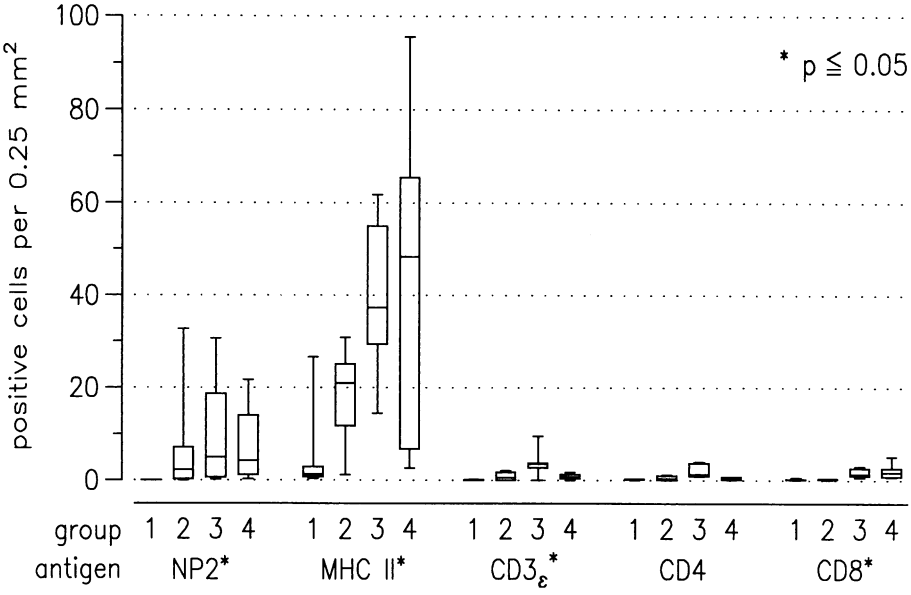

Fig. 3. Number of CDV antigen (NP2), MHC class II, CD3, CD4 and CD8 positive cells in controls (group I = 1) and distemper dogs (groups II–IV = 2–4) with different types of changes in early distemper leukoencephalomyelitis. Box and whisker plot displays values as minimum, maximum, median, and the lower and upper quartile.

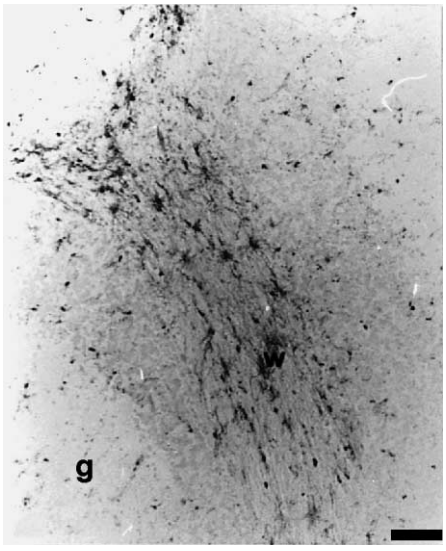

Fig. 4. Distribution of CDV antigen in a cerebellar folium of distemper dogs classified in group II. Few randomly distributed CDV antigen positive astrocytes and endothelial cells in the gray and white matter. g = gray matter; w = white matter. ABC method, CDV (NP2)-specific monoclonal antibody. Bar = 100 μm.

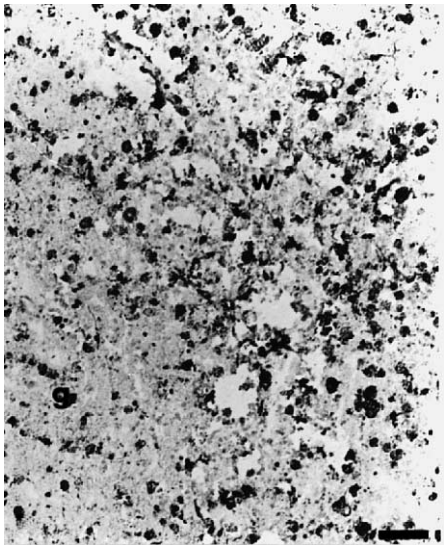

Fig. 6. Distribution of CDV antigen in a cerebellar folium of distemper dogs classified in group IV. Numerous CDV antigen positive gitter cells and astrocytes. g = gray matter; w = white matter. ABC method, CDV (NP2)-specific monoclonal antibody. Bar = 100 μm.

Using immunohistochemistry for the evaluation of CDV antigen distribution and HE and myelin staining, the selected frozen tissue sections of the investigated animals were classified into four groups. Group I, four dogs, consisted of CDV antigen negative control animals. Groups II to IV animals, six in each group, displayed different stages of early changes in DL. Group II animals exhibited virus antigen expression in the white matter without associated histopathological changes, group III dogs were characterized by virus antigen expression and a mild parenchymal lymphocyte infiltration, and group IV distemper dogs displayed parenchymal lymphocyte infiltration and demyelination (Figs. 1 and 2).

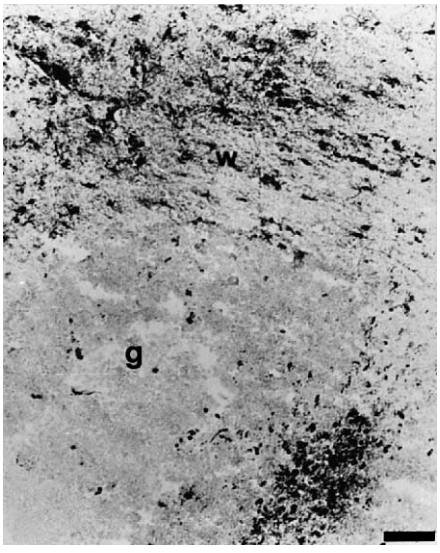

Fig. 5. Distribution of CDV antigen in a cerebellar folium of distemper dogs classified in group III. Increased CDV antigen immunoreactivity in early white matter lesions. g = gray matter; w = white matter. ABC method, CDV (NP2)-specific monoclonal antibody. Bar = 100 μm.

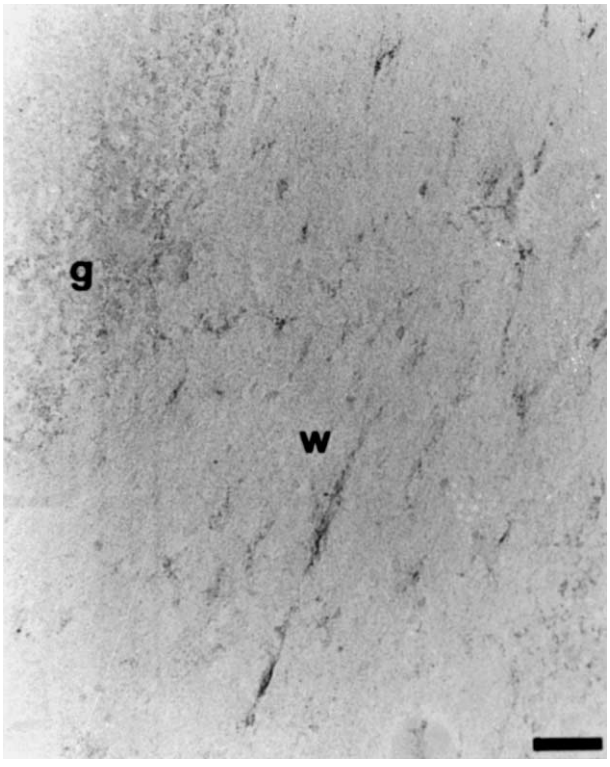

Fig. 7. Distribution and immunoreactivity of MHC class II antigen in group I (controls) dogs. Few weakly positive microglia and endothelial cells in the white matter. g = gray matter; w = white matter. ABC method, MHC class II specific monoclonal antibody. Bar = 60 μm.

3.2. Immunohistological findings

The controls lacked immunoreactivity for CDV nucleoprotein, whereas in distemper dogs nucleoprotein antigen was demonstrated in all groups to varying degree (Figs. 3–6). CDV antigen, located in the cytoplasm and nucleus, was frequently found in astrocytes, microglia/macrophages, ependymal and endothelial cells and occasionally in neurons. The number of CDV antigen positive cells increased significantly ($p = 0.0037$) from groups II to IV (Fig. 3). Some group II animals showed few randomly distributed CDV antigen positive cells within the white matter and in the adjacent granular layer (Fig. 4). Group III animals displayed a moderate number of CDV antigen positive cells within the white matter lesions and the adjacent granular layer (Fig. 5). In group IV dogs, a high number of intra- and extra-lesional cells including gitter cells were stained for CDV nucleoprotein antigen (Fig. 6).

In controls, only sparse expression of MHC class II antigen on microglial cells was present, whereas CDV-infected dogs displayed a significant ($p = 0.0029$), mild to severe, plaque-associated MHC class II up-regulation on activated microglial cells increasing from groups II to IV (Figs. 3, 7–10). MHC class II expression was observed on endothelial cells, T cells, and gitter cells (Fig. 10). There was a significant correlation between MHC class II expression and the increasing number of CDV antigen positive cells ($p < 0.001$).

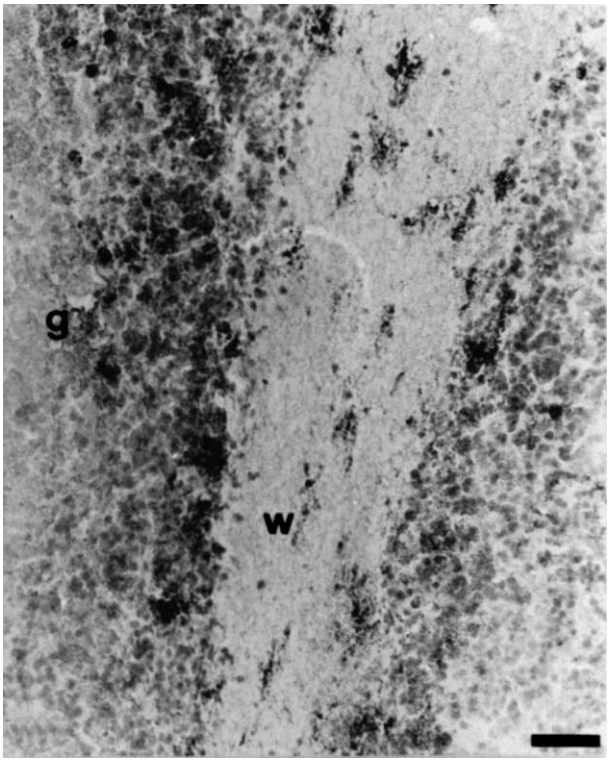

Fig. 8. Distribution and immunoreactivity of MHC class II antigen in group II distemper dogs. Increased immunoreactivity of endothelial/perivascular cells and microglia in the white matter and adjacent gray matter. g = gray matter; w = white matter. ABC method, MHC class II specific monoclonal antibody. Bar = 60 μm.

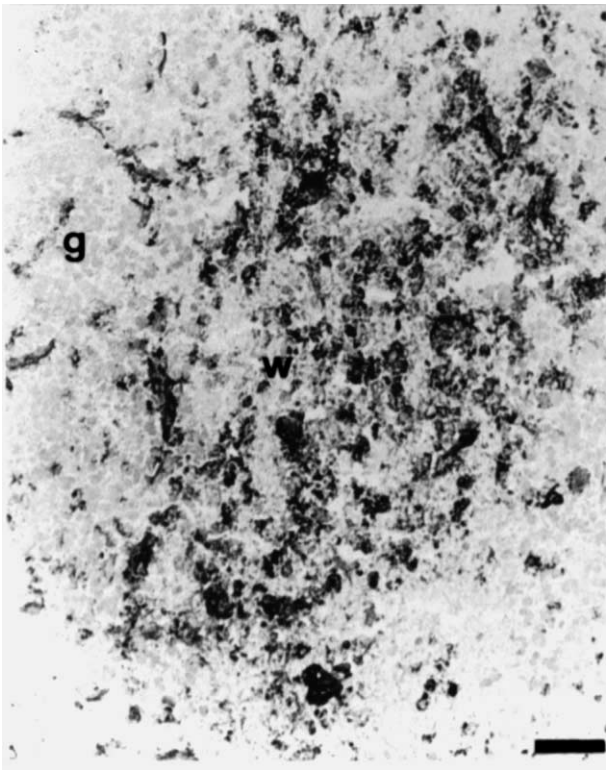

Fig. 9. Distribution and immunoreactivity of MHC class II antigen in group III distemper dogs. Focal, moderate to severe immunoreactivity in a white matter lesion and adjacent gray matter. g = gray matter; w = white matter. ABC method, MHC class II specific monoclonal antibody. Bar = 60 μm.

In controls, only few randomly distributed CD3ε, CD4 and CD8 antigen-positive cells were detected. The cerebella of the groups II to IV animals were characterized by a low but significant number of infiltrating CD3ε- ($p = 0.011$) and CD8-positive cells ($p = 0.0369$), whereas the number of CD4-positive cells remained unchanged ($p = 0.8562$) compared to controls (Fig. 3). The CD21-positive B lymphocytes were only rarely observed in all four groups. It appeared that the number of CD3ε- and CD4-positive cells was higher in dogs of group III, and there seemed to be a slight increase of CD8 antigen-positive cells from groups II to IV animals. However, both observations were not significant. In group II dogs, T-lymphocytes were randomly distributed in the white matter. In addition, a focal accumulation of lymphocytes in plaque-like regions with and without demyelination was observed in animals of groups III and IV.

Staining for GFAP revealed only minor differences between group II distemper dogs and controls. There was an increase of GFAP-positive cells within the white matter lesions in group III dogs. In group IV dogs, staining intensity appeared to be enhanced adjacent to demyelinated lesions and reduced in the center of the plaques. BS-1

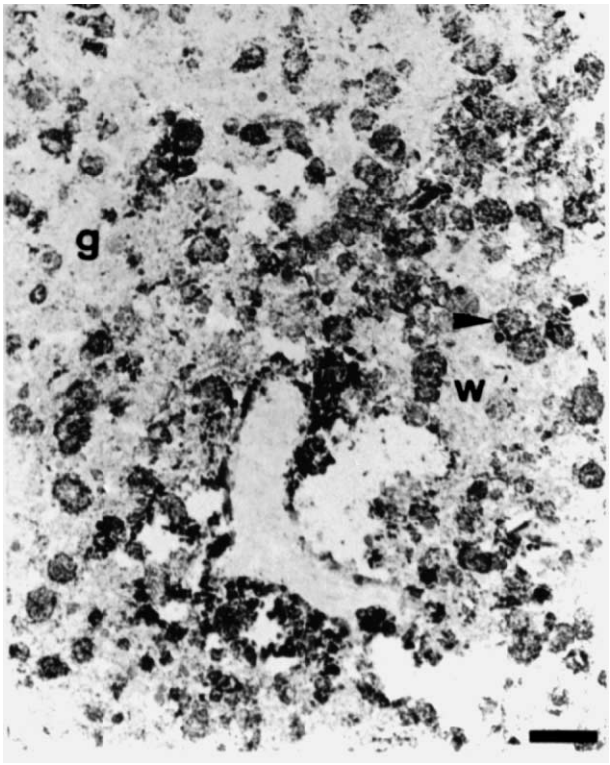

Fig. 10. Distribution and immunoreactivity of MHC class II antigen in group IV distemper dogs. Prominent immunoreactivity of gitter cells (arrowhead) in a group IV lesion with malacia. g = gray matter; w = white matter. ABC method, MHC class II specific monoclonal antibody. Bar = 60 μm.

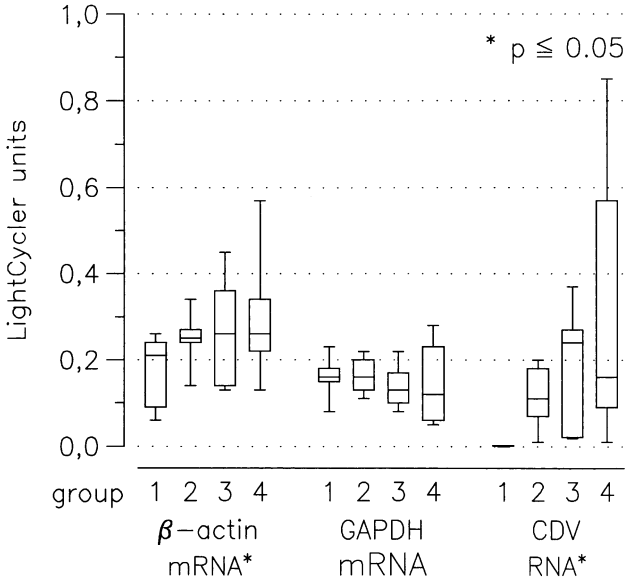

Fig. 11. Detection of β-actin, GAPDH and CDV RNA in controls (group I = 1) and distemper dogs (groups II–IV = 2–4), expressed in units as obtained by real-time PCR. Box and whisker plot displays values as minimum, maximum, median, and the lower and upper quartile.

immunoreactivity was found on microglial cells/macrophages and endothelial cells. In groups I and II dogs, the reaction was comparable and restricted to vascular walls and individual round cells in the meninges and brain parenchyma. In group III dogs, a slightly increased immunoreactivity was found on the microglia within the white matter lesions. In lesions with malacia, the gitter cells were BS-1 positive in the group IV dogs.

3.3. Housekeeping gene, CDV and cytokine expression

The integrity of isolated RNA was demonstrated by amplification of β-actin and GAPDH mRNA in all isolates (Fig. 11). The amount of detectable GAPDH transcripts revealed no significant differences between controls and infected animals ($p = 0.7149$), whereas β-actin mRNA displayed a significant correlation to the amount of virus antigen positive cells in the cerebella of distemper dogs ($p < 0.05$). The latter indicated that the housekeeping gene β-actin in contrast to GAPDH did not represent a useful internal standard to correlate the cytokine expression to.

CDV nucleoprotein RNA was only present in the cerebella of dogs with immunohistochemically confirmed CDV infection. Statistical analysis revealed a positive correlation between the number of antigen positive cells and the amount of detectable viral RNA expressed in units ($p < 0.001$).

All cytokine transcripts were successfully amplified from Con A-stimulated lymphocytes and DH82 cells confirming the suitability of the canine-specific primers. However, no transcripts for IL-1, IL-2 and IFN-γ were detected in controls and distemper dogs (Fig. 12).

Transcripts for the pro-inflammatory cytokines IL-6, IL-8 IL-12 and TNF-α were found in 17%, 33%, 0% and 0% of control animals, respectively (Fig. 12). In contrast, the mRNA for IL-6, IL-8, IL-12 and TNF-α was detected in 94%, 94%, 78% and 56% of distemper dogs, respectively (Fig. 12). The mRNA for both anti-inflammatory cytokines, IL-10 and TGF-β, was present in 83% and 100% of controls and infected animals, respectively (Fig. 12).

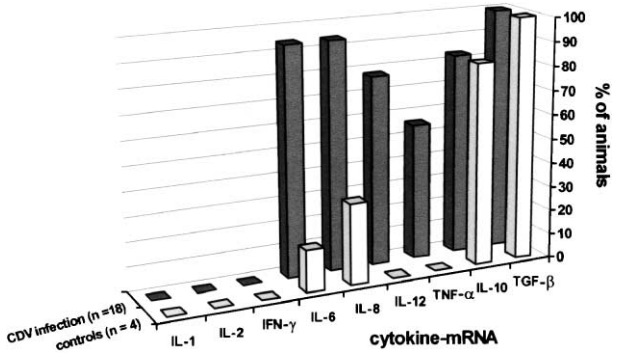

Fig. 12. Percentage of controls (group I) and distemper dogs (group II–IV, combined) expressing the investigated cytokines in the CNS.

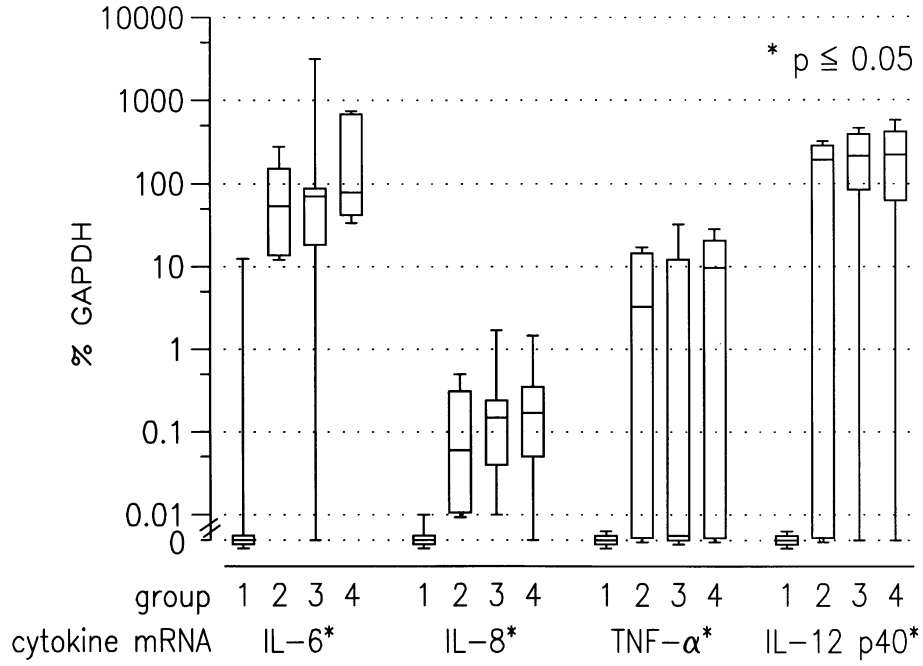

Fig. 13. Relative pro-inflammatory cytokine mRNA values expressed as %GAPDH in controls and distemper dogs (groups I–IV = 1–4). Changes correlated significantly with the amount of detectable CDV antigen. Box and whisker plot displays values as minimum, maximum, median, and the lower and upper quartile.

Semi-quantitative data analyses revealed a differential up-regulation of the detectable cytokines. Pro-inflammatory cytokine values, low or not detectable in controls, were prominently up-regulated in distemper dogs and correlated significantly to the amount of CDV antigen ($p < 0.05$). The classification of CDV lesions into three groups showed that the relative cytokine values were prominently up-regulated as soon as CDV antigen and/or RNA was detectable in the CNS (Fig. 13). Infiltrating lymphocytes, increasing MHC class II up-regulation and demyelination resulted only in minor additional changes in the relative expression values for IL-6, IL-8, IL-12 and TNF-α, defining that CDV infection represented the main triggering event leading to increased expression of these cytokines. Few individuals exhibited extremely high relative cytokine expression values especially for IL-6 supporting the role of this cytokine for disease initiation. Furthermore, up-regulation of IL-6, IL-8, IL-12 and TNF-α in distemper dogs correlated positively to the number of virus antigen positive cells and MHC class II expressing cells ($p < 0.01$). Though both anti-inflammatory cytokines, IL-10 and TGF-β, displayed a slight increase in their relative expression values especially in group IV animals, the changes were not significant and did not correlate to the number of CDV antigen positive cells (Fig. 14; IL-10: $p = 0.5682$ and TGF-β: $p = 0.3367$).

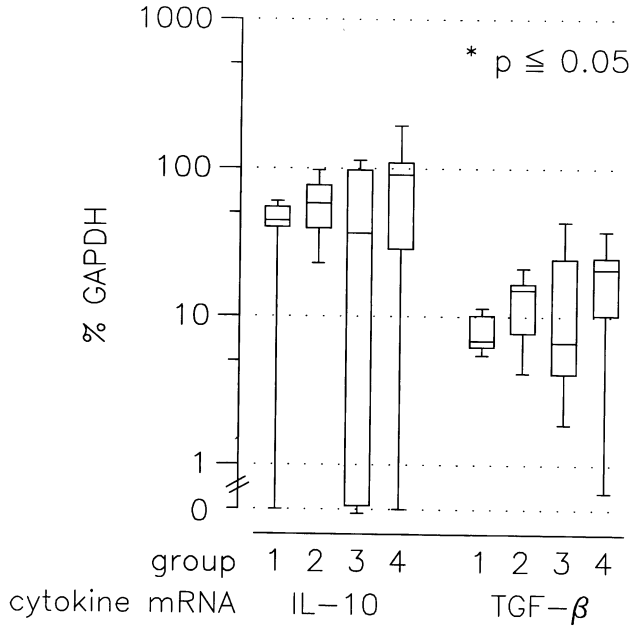

Fig. 14. Relative anti-inflammatory cytokine mRNA values expressed as %GAPDH in controls and distemper dogs (groups I–IV = 1–4). Changes lacked significant correlation with the amount of detectable CDV antigen. Box and whisker plot displays values as minimum, maximum, median, and the lower and upper quartile.

4. Discussion

The present investigation revealed the up-regulation of MHC class II antigen and mild CD8-dominated T cell infiltration in early distemper lesions, associated with a strong up-regulation of IL-6, IL-8, IL-12 and TNF-α

mRNA and a lacking IL-1, IL-2 and IFN-γ mRNA response. Both anti-inflammatory cytokines, IL-10 and TGF-β, exhibited no major changes at the mRNA level following CDV infection. The latter indicated that the dysregulation of the cytokine response, most likely due to a lacking or inappropriate activation of the anti-inflammatory cytokines, might represent the molecular events leading to disease initiation and progression in demyelinating DL.

Immunohistochemical findings confirmed the previous observations in early CNS lesions of distemper (Alldinger et al., 1996; Wünschmann et al., 1999). The rationale for the subclassification of the early phase of DL in three different categories was based on the hypothesis that particular changes such as CDV infection, microglial cell activation, lymphocyte infiltration and demyelination, can be correlated to a phase-specific up-regulation of certain cytokines. However, it seemed that CDV infection represents the main trigger initiating a cascade of events leading to the activation of the pro-inflammatory cytokines, IL-6, IL-8, IL-12 and TNF-α. Following the initial activation, subsequent changes such as lymphocyte infiltration, MHC class II up-regulation and demyelination, resulted in no major additional increase in the relative cytokine values.

Data analysis of the expression values of the housekeeping genes revealed that β-actin, in contrast to GAPDH, correlated significantly to cytokine expression and CDV antigen. This finding indicated that only GAPDH represented a suitable housekeeping gene for the normalization of cytokine expression values in cerebellar distemper lesions. This observation is probably related to previous findings, which showed that actin plays an active role during paramyxovirus morphogenesis and replication (Örvell, 1980; Moyer et al., 1990).

IL-6 mRNA was present in 17% of controls compared to 94% of distemper dogs. More importantly, a dramatic increase of IL-6 mRNA was noticed in individual lesions following CDV infection underlining the noteworthy role of IL-6 in early distemper CNS lesions. IL-6 possesses pro- and anti-inflammatory effects and may induce myelin loss or reduction of demyelination (Campbell et al., 1993; Banks et al., 1994; Woodroofe, 1995; Rothwell, 1997). The contributing role of IL-6 in nervous distemper has been suspected in previous studies, in which IL-6 mRNA was only detectable in the blood cells of animals with early distemper CNS lesions (Gröne et al., 1998b). In addition, IL-6 was found in the CSF cells and perivascular lymphocytes of dogs with DL (Frisk et al., 1999; Gröne et al., 2000). Similarly, in active MS lesions, up-regulation of IL-6 mRNA was detected and IL-6 protein was found in the cells of perivascular cuffs and on the edge of demyelinated plaques by immunohistochemistry (Baranzini et al., 2000; Brosnan et al., 1995; Maimone et al., 1997). Accordingly, in experimental allergic encephalitis (EAE), IL-6 seemed to be important for disease induction (Okuda et al., 1999). Although RT-PCR, as performed in the present investigation, allows no identification of the cell of origin of the cytokine amplicon, immunohistochemistry displayed activation of astrocytes and microglial cells suggesting that these cells represent potential candidates for the IL-6 production in DL (Gröne et al., 2000).

Transcripts for IL-8 were found in 33% of controls and 94% of distemper dogs and relative IL-8 mRNA amounts were several-fold increased in distemper dogs. Both neutrophils and T cells are attracted by IL-8 and microglia cells and astrocytes are potential sources of IL-8 (Van Meir et al., 1992; Tipold et al., 1999). IL-8 activity and mRNA transcripts have also been detected in the CSF and blood of distemper dogs (Gröne et al., 1998b; Tipold et al., 1999). Despite the lack of an overt inflammatory response in early DL lesions, the prominent up-regulation of IL-8 mRNA in areas with mild CD8-positive cell infiltration supports its role as chemokine in DL.

In controls, no transcripts for IL-12p40 (IL-12) were found, whereas 78% of distemper dogs were positive for IL-12 mRNA. These findings support and extend the previous observations upon the role of IL-12 in the pathogenesis of distemper (Gröne et al., 1998b, 2000; Frisk et al., 1999). The disease-promoting effect of IL-12 has also been demonstrated in MS, EAE and in in vitro studies (Leonard et al., 1995; Vartanian et al., 1995; Becher et al., 1996; Lucchinetti and Rodriguez, 1997; Karp, 1999; Van Boxel-Dezaire et al., 1999).

TNF-α transcripts were not detected in controls but were present in 56% of distemper dogs, supporting the previous findings upon a direct CDV-induced up-regulation of TNF-α by astrocytes, microglia/macrophages and endothelial cells in early DL lesions as well as blood and CSF cells (Gröne et al., 1998b; Frisk et al., 1999; Gröne et al., 2000). Infection of human glial cells with measles virus resulted also in an up-regulation of TNF-α (Schneider-Schaulies et al., 1993). The capacity of TNF-α to cause oligodendrocyte damage and myelin loss has been shown by in vitro studies, in acute MS lesions, and in TNF-α over-expressing transgenic mice (Butt and Jenkins, 1994; Brosnan et al., 1995; Probert et al., 1995; Andrews et al., 1996; Bitsch et al., 2000). The CDV-induced up-regulation of TNF-α in early lesions supports the observation of a previous immunohistological investigation that demyelination in early DL may also be attributed to local TNF-α production (Gröne et al., 2000). In addition, TNF-α might promote the disease process by stimulating lymphocyte trafficking into the CNS in concert with IL-6, increase of MHC classes I and II expression, and activation of iNOS (Merrill and Benveniste, 1996; Lucchinetti and Rodriguez, 1997; Chabrier et al., 1999; Kallmann et al., 2000).

Transcripts of IL-1β, IL-2 and IFN-γ were neither found in controls nor in distemper dogs, indicating that these pro-inflammatory cytokines play no or only an inferior role in the early phase of DL as suggested in previous studies (Gröne et al., 1998b; Frisk et al., 1999). Mechanisms of MHC class II up-regulation in early distemper CNS lesions remain a matter of debate (Alldinger et al., 1996; Gaedke et

al., 1999; Vilafranca et al., 1966). Low MHC class II expression in early lesions could be a consequence of virus-mediated immunosuppression, weak MHC class II induction by CDV or lack of IFN-γ expression (Alldinger et al., 1996; Gaedke et al., 1999). In addition, in vitro studies showed an isotype-specific regulation of MHC class II expression by TNF-α in human monocytes. Whether a similar pathogenesis operates in DL remains to be determined (Jasinski et al., 1995).

Transcripts of both anti-inflammatory cytokines, IL-10 and TGF-β, were present in 83% and 100% of control and distemper animals, respectively, and the relative expression values did not differ significantly between groups. Both cytokines were also frequently found in the blood and CSF of distemper dogs; however, nothing is known about their relative expression values in these body fluids (Gröne et al., 1998b; Frisk et al., 1999). IL-10 and TGF-β inhibit a variety of pro-inflammatory cytokine activities including TNF-α production, MHC class II up-regulation, and autoantigen-mediated inflammatory changes (Merrill and Zimmerman, 1991; Bogdan et al., 1992; Samoilova et al., 1998; Pazmany et al., 2000). Therefore, a potent anti-inflammatory immune response mediated by anti-inflammatory cytokines might be beneficial for the prevention of disease initiation or induction of a remission phase. In MS patients, IL-10 production by CSF cells appeared to be increased during the stable phases of the disease; and in blood cells, TFG-β is increased in the stages of disease remission (Calabresi et al., 1998; Bertolotto et al., 1999).

In summary, a prominent up-regulation of the pro-inflammatory cytokines IL-6, IL-8, IL-12 and TNF-α in early distemper CNS lesions was observed, whereas other pro-inflammatory cytokines such as IL-1, IL-2 and IFN-γ were not detectable. Conversely, IL-10 and TGF-β, though detectable in most distemper cases, experienced no significant up-regulation following CDV infection and lesion development. The lack of increased expression of anti-inflammatory cytokines might represent an early derailment of the immune response in demyelinating distemper leukoencephalomyelitis.

Acknowledgements

The authors wish to thank Annette Artelt for excellent technical assistance, Ute Zeller and Udo Hetzel for photographic support, H. Heiter for help and advice for the statistical analyses, and Dr. Kipar for editorial support with the manuscript. The generous support and help of Dr. J. Borlak and T. Thum with the LightCycler system and the outstanding support of J. Wilhelm for solving the LightCycler software problems is acknowledged. Stefanie Markus received a scholarship from the Graduiertenkolleg ''Molekulare Veterinärmedizin'', Giessen, Germany, and the study was supported by grants of the Deutsche Forschungsgemeinschaft (Ba 815/4-1 and 4-2).

References

Alldinger, S., Wünschmann, A., Baumgärtner, W., Voss, C., Kremmer, E., 1996. Up-regulation of major histocompatibility complex class II antigen expression in the central nervous system of dogs with spontaneous canine distemper virus encephalitis. Acta Neuropathol. 92, 273–280.

Andrews, T., Zhang, P., Bhat, N.R., 1996. Cytokines, ceramide and oligodendrocyte cytotoxicity. J. Neurochem. 66, 89.

Banks, W.A., Kastin, A.J., Gutierrez, E.G., 1994. Penetration of interleukin-6 across the murine blood–brain barrier. Neurosci. Lett. 179, 53–56.

Baranzini, S.E., Elfstrom, C., Chang, S.Y., Tutunoi, C., Burtunoi, C., Murray, R., Higuchi, R., Oksenberg, J.R., 2000. Transcriptional analysis of multiple sclerosis brain lesions reveals a complex pattern of cytokine expression. J. Immunol. 165, 6576–6582.

Baumgärtner, W., Örvell, C., Reinacher, M., 1989. Naturally occurring canine distemper virus encephalitis: distribution and expression of viral polypeptides in nervous tissues. Acta Neuropathol. 78, 504–512.

Becher, B., Dodelet, V., Fedorowicz, V., Antel, J.P., 1996. Soluble tumor necrosis factor receptor inhibits interleukin 12 production by stimulated human adult microglial cells in vitro. J. Clin. Invest. 98, 1539–1543.

Bertolotto, A., Capobianco, M., Malucchi, S., Manzardo, E., Audano, L., Bergui, M., Bradac, G.B., Mutani, R., 1999. Transforming growth factor beta1 (TGFbeta1) mRNA level correlates with magnetic resonance imaging disease activity in multiple sclerosis patients. Neurosci. Lett. 263, 21–24.

Bitsch, A., Kuhlmann, T., Da Costa, C., Bunkowski, S., Polak, T., Brück, W., 2000. Tumor necrosis factor alpha mRNA expression in early multiple sclerosis lesions, correlation with demyelination activity and oligodendrocyte pathology. Glia 29, 366–375.

Bogdan, C., Paik, J., Vodovotz, Y., Nathan, C., 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-β and interleukin-10. J. Biol. Chem. 267, 23301–23308.

Brosnan, C.F., Cannella, B., Battistini, L., Raine, C.S., 1995. Cytokine localisation in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species. Neurology 45, 16–21.

Butt, A.M., Jenkins, H.G., 1994. Morphological changes in oligodendrocytes in the intact mouse optic nerve following intravitreal injection of tumor necrosis factor. J. Neuroimmunol. 51, 27–33.

Calabresi, P.A., Tranquill, L.R., McFarland, H.F., Cowan, E.P., 1998. Cytokine gene expression in cells derived from CSF of multiple sclerosis patients. J. Neuroimmunol. 89, 198–205.

Campbell, I.L., Abraham, C.R., Masliah, E., Kemper, P., Inglis, J.D., Oldstone, M.B., Mucke, L., 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc. Natl. Acad. Sci. U. S. A. 90, 10061–10065.

Chabrier, P.E., Demerle-Pallardy, C., Auguet, M., 1999. Nitric oxide synthases: targets for therapeutic strategies in neurological disease. Cell. Mol. Life Sci. 55, 1029–1035.

Dixon, W.J., 1993. BMDP Statistical Software Manual, vols. 1 and 2, University of California Press, Berkeley.

Frisk, A.L., Baumgärtner, W., Gröne, A., 1999. Dominating interleukin-10 mRNA expression induction in cerebrospinal fluid cells of dogs with natural canine distemper virus induced demyelinating and non-demyelinating CNS lesions. J. Neuroimmunol. 97, 102–109.

Gaedke, K., Zurbriggen, A., Baumgärtner, W., 1999. Lack of correlation between virus nucleoprotein and mRNA expression and the inflammatory response in demyelinating distemper encephalitis indicates a biphasic disease process. Eur. J. Vet. Pathol. 5, 9–20.

Graber, H.U., Müller, C.F., Vandevelde, M., Zurbriggen, A., 1995. Restricted infection with canine distemper virus leads to down-regulation of myelin gene transcription in cultured oligodendrocytes. Acta Neuropathol. 90, 312–318.

Gröne, A., Fonfara, S., Markus, S., Baumgärtner, W., 1998a. RT-PCR amplification of various canine cytokines and so-called house-keeping genes in a species-specific macrophage cell line (DH82) and canine peripheral blood leukocytes. J. Vet. Med. 46, 301–310.

Gröne, A., Frisk, A.L., Baumgärtner, W., 1998b. Cytokine mRNA expression in whole blood samples from dogs with natural canine distemper virus infection. Vet. Immunol. Immunopathol. 65, 11–27.

Gröne, A., Alldinger, S., Baumgärtner, W., 2000. Interleukin-1β, -6, -12 and tumor necrosis factor-α expression in brains of dogs with canine distemper virus infection. J. Neuroimmunol. 110, 20–30.

Jasinski, M., Wieckiewicz, J., Ruggiero, I., Pituch-Noworolska, A., Zembala, M., 1995. Isotype-specific regulation of MHC class II gene expression in human monocytes by exogenous and endogenous tumor necrosis factor. J. Clin. Immunol. 15, 185–193.

Kallmann, B.A., Hummel, V., Lindenlaub, T., Ruprecht, K., Toyka, K.V., Rieckmann, P., 2000. Cytokine-induced modulation of cellular adhesion to human cerebral endothelial cells is mediated by soluble vascular cell adhesion molecule-1. Brain 123, 687–697.

Karp, C.L., 1999. Measles: immunosuppression, interleukin-12 and complement receptors. Immunol. Rev. 168, 91–101.

Koestner, A., 1975. Animal model of human disease: distemper-associated demyelinating encephalomyelitis. Am. J. Pathol. 78, 361–364.

Krakowka, S., Axthelm, M.K., Johnson, G.C., 1985. Canine distemper virus. In: Olsen, R.G., Krakowka, S., Bakeslee, J.R. (Eds.), Comp. Pathobiol. Viral Dis., vol. 2. CRC Press, Boca Raton, FL, pp. 137–164.

Leonard, J.P., Waldburger, K.R., Goldman, S.J., 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. J. Exp. Med. 181, 381–386.

Lucchinetti, C.F., Rodriguez, M., 1997. The controversy surrounding the pathogenesis of the multiple sclerosis lesion. Mayo Clin. Proc. 72, 665–678.

Maimone, D., Guazzi, G.C., Annunziata, P., 1997. IL-6 detection in multiple sclerosis brain. J. Neurol. Sci. 146, 59–65.

McCullough, B., Krakowka, S., Koestner, A., 1974. Experimental canine distemper virus-induced demyelination. Lab. Invest. 31, 216–222.

McGuinness, M.C., Powers, J.M., Bias, W.B., Schmeckpeper, B.J., Segal, A.H., Gowda, V.C., Wesselingh, S.L., Berger, J., Griffin, D.E., Smith, K.D., 1997. Human leukocyte antigens and cytokine expression in cerebral inflammatory demyelinative lesions of X-linked adrenoleukodystrophy and multiple sclerosis. J. Neuroimmunol. 75, 174–182.

Merrill, J.E., Benveniste, E.N., 1996. Cytokines in inflammatory brain lesions: helpful and harmful. Glia 19, 331–338.

Merrill, J.E., Zimmerman, R.P., 1991. Natural and induced cytotoxicity of oligodendrocytes by microglia is inhibitable by TGF beta. Glia 4, 327–331.

Moyer, S.A., Baker, S.C., Horikami, S.M., 1990. Host cell proteins required for measles virus reproduction. J. Gen. Virol. 71, 775–783.

Navikas, V., Link, H., 1996. Review: cytokines and the pathogenesis of multiple sclerosis. J. Neurosci. Res. 45, 322–333.

Nesseler, A., Baumgärtner, W., Zurbriggen, A., Örvell, C., 1999. Restricted virus protein translation in canine distemper virus inclusion body polioencephalitis. Vet. Microbiol. 69, 23–28.

Okuda, Y., Sakoda, S., Fujimura, H., Saeki, Y., Kishimoto, T., Yanagihara, T., 1999. IL-6 plays a crucial role in the induction phase of myelin oligodendrocyte glucoprotein 35–55 induced experimental autoimmune encephalomyelitis. J. Neuroimmunol. 101, 188–196.

Örvell, C., 1980. Structural polypeptides of canine distemper virus. Arch. Virol. 66, 193–206.

Pazmany, T., Kosa, J.P., Tomasi, T.B., Mechtler, L., Turoczi, A., Lehotzky, A., 2000. Effect of transforming growth factor-beta 1 on microglial MHC-class II expression. J. Neuroimmunol. 103, 122–130.

Probert, L., Akassoglou, K., Pasparakis, M., Kontogeorgos, G., Kollias, G., 1995. Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor α. Proc. Natl. Acad. Sci. U. S. A. 92, 11294–11298.

Rothwell, N.J., 1997. Neuroimmune interactions, the role of cytokines. Br. J. Pharmacol. 121, 841–847.

Samoilova, E.B., Horton, J.L., Chen, Y., 1998. Acceleration of experimental autoimmune encephalomyelitis in interleukin-10-deficient mice: roles of interleukin-10 in disease progression and recovery. Cell. Immunol. 188, 118–124.

Sanders, V., Conrad, A.J., Tourtellotte, W.W., 1993. On classification of post-mortem multiple sclerosis plaques for neuroscientists. J. Neuroimmunol. 46, 207–216.

Schneider-Schaulies, J., Schneider-Schaulies, S., ter Meulen, V., 1993. Differential induction of cytokines by primary and persistent measles virus infections in human glial cells. Virology 195, 219–228.

Seilhean, D., Kobayashi, K., He, Y., Uchihara, T., Rosenblum, O., Katlama, C., Briscaire, F., Duyckaerts, C., Hauw, J.J., 1997. Tumor necrosis factor-α, microglia and astrocytes in AIDS dementia complex. Acta Neuropathol. 93, 508–517.

Selmaj, K.W., Raine, C.S., 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann. Neurol. 23, 339–346.

Tipold, A., Moore, P., Zurbriggen, A., Burgener, I., Barben, G., Vandervelde, M., 1999. Early T cell response in the central nervous system in canine distemper virus infection. Acta Neuropathol. 97, 45–56.

Van Boxel-Dezaire, A.H., Hoff, S.C., van Oosten, B.W., Verweij, C.L., Drager, A.M., Ader, H.J., van Houwelingen, J.C., Barkhof, F., Polman, C.H., Nagelkerken, L., 1999. Decreased interleukin-10 and increased interleukin-12p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. Ann. Neurol. 45, 695–703.

Van Meir, E., Ceska, M., Effenberger, F., Walz, A., Grouzmann, E., Desbaillets, I., Frei, K., Fontana, A., de Tribolet, N., 1992. Interleukin-8 is produced in neoplastic and infectious diseases of the human central nervous system. Cancer Res. 52, 4297–4305.

Vartanian, T., Li, Y., Zhao, M., Stefansson, K., 1995. Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis. Mol. Med. 1, 732–743.

Vilafranca, M., Tello, M., Pumarola, M., Domingo, M., 1966. Neural cells from dogs with spontaneous distemper encephalitis express class II major histocompatibility complex molecules. J. Comp. Pathol. 114, 43–50.

Woodroofe, M.N., 1995. Cytokine production in the central nervous system. Neurology 45, 6–10.

Wünschmann, A., Alldinger, S., Kremmer, E., Baumgärtner, W., 1999. Identification of CD4+ and CD8+ T cell subsets and B cells in the brain of dogs with spontaneous acute-, subacute- and chronic-demyelinating distemper encephalitis. Vet. Immunol. Immunopathol. 67, 101–116.

Zurbriggen, A., Schmid, I., Graber, H.U., Vandervelde, M., 1998. Oligodendroglial pathology in canine distemper. Acta Neuropathol. 95, 71–77.