

Cell-cell Interaction Appears to Represent a Pivotal Factor for Susceptibility of Adult Canine Schwann Cell-like Brain Glia to Canine Distemper Virus Infection *in vitro*

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Abstract

Recently, we reported the preferential infection of a novel glial cell type from adult canine brain, termed Schwann cell-like brain glia (SCBG) with canine distemper virus (CDV). To further characterize virus-cell and cell-cell interactions, SCBG in purified and mixed brain cell cultures was infected with different CDV strains. Cell cultures were infected with the attenuated CDV-Ond, CDV-2544, CDV-OndeGFP, CDV-R252 and the mustelid virulent CDV-5804PeGFP strain. Cytopathic effects (CPEs) and the percentage of infection were monitored daily at 3 and 10 days post-infection (dpi), respectively. At 10dpi, the CDV strains induced mild to moderate and severe CPEs in purified SCBG and fibroblasts, respectively, while both cell types displayed mild to moderate CPE in mixed brain cell cultures. Comparative analysis of CDV infection revealed that the virulent CDV-5804PeGFP strain caused a delayed infection of SCBG in mixed culture. Independent of the virus strain, infection of purified SCBG was significantly compared to mixed brain cell cultures. Contrary to this, fibroblasts were infected to the same extent in both culture conditions. There are two main findings of the present study. Firstly, CDV strains displayed differential infection of SCBG in purified cultures. This is a confirmation and extension of previous findings in mixed culture. Secondly, a dramatic decrease in the extent of CDV infection was observed after purification of SCBG. This was independent of the CDV strain used and implies that undetermined interactions between SCBG and other cells, eliminated during purification, crucially determine the susceptibility of SCBG to CDV infection *in vitro*.

Keywords: canine distemper virus, cell-cell interaction, Schwann cell-like brain glia

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บทคัดย่อ

บทบาทของปฏิกิริยาระหว่างเซลล์ต่อความไวรับต่อเชื้อไวรัสไข้หัดสุนัขของเซลล์เพาะเลี้ยงเกลียที่คล้ายชวานน์เซลล์ในสมองสุนัข

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ศึกษาปฏิกิริยาระหว่างเซลล์ที่แยกได้จากสมองสุนัข ต่อความไวรับต่อเชื้อไวรัสไข้หัดสุนัขสเตรนต่างๆ ทำการติดเชื้อไวรัสลงในเซลล์เพาะเลี้ยงเกลียที่คล้ายชวานน์เซลล์ (Schwann cell-like brain glia; SCBG) เซลล์ไฟโบรบลาสต์และเซลล์ผสมทั้งสองชนิด สเตรนไวรัสที่ใช้ประกอบด้วย ชนิดที่ไม่ก่อโรครุนแรง (CDV-Ond, CDV-2544, CDV-OndeGFP, CDV-R252) และชนิดที่ก่อโรครุนแรง (CDV-5804PeGFP) ฝ้าสังเกตการเกิด cytopathic effect (CPE) ทุกวัน และร้อยละการติดเชื้อในวันที่ 3 และ 10 ผลการศึกษาพบว่าในวันที่ 10 หลังการติดเชื้อไวรัสทุกสเตรนก่อให้เกิด CPE ชนิดรุนแรงน้อยถึงปานกลางในเซลล์ SCBG และชนิดรุนแรงมากในเซลล์ไฟโบรบลาสต์ ในขณะที่พบ CPE ชนิดรุนแรงน้อยถึงปานกลางในเซลล์ผสมทั้งสองชนิด เมื่อเปรียบเทียบอัตราการติดเชื้อ เซลล์ SCBG เพียงอย่างเดียวแสดงร้อยละการติดเชื้อต่ำกว่าเซลล์ SCBG ในเซลล์ผสมทั้งสองชนิดอย่างมีนัยสำคัญ โดยไวรัสสเตรน CDV-5804PeGFP ก่อให้เกิดอัตราการติดเชื้อต่ำที่สุดในเซลล์ SCBG ในเซลล์ผสมทั้งสองชนิด ในขณะที่เซลล์ไฟโบรบลาสต์แสดงร้อยละการติดเชื้อไม่แตกต่างกันในเซลล์เพาะเลี้ยงทั้งสองแบบไม่ว่าจะใช้ไวรัสสเตรนใดก็ตาม มีสองประเด็นหลักที่ได้จากการศึกษา คือ (1) ไวรัสไข้หัดสุนัขสเตรนต่างๆ มีความสามารถในการก่อให้เกิดการติดเชื้อในเซลล์เพาะเลี้ยงที่มี SCBG เพียงอย่างเดียวไม่เท่ากัน และ (2) อัตราการติดเชื้อของเซลล์เพาะเลี้ยงที่มี SCBG เพียงชนิดเดียวลดลงอย่างมีนัยสำคัญ เมื่อทำการแยกเซลล์ชนิดนี้ออกมาจากเซลล์เพาะเลี้ยงที่มีเซลล์หลายชนิดผสมกัน ซึ่งพบลักษณะนี้ในไวรัสทุกสเตรน ดังนั้นจึงอาจกล่าวได้ว่าปฏิกิริยาระหว่างเซลล์ SCBG และเซลล์ชนิดอื่นๆ ในสมองที่ได้ทำการแยกออกไป มีผลต่อความไวรับของเชื้อไวรัสไข้หัดสุนัข

คำสำคัญ: ไวรัสไข้หัดสุนัข เซลล์เพาะเลี้ยงเกลียที่คล้ายชวานน์เซลล์ในสมอง ปฏิกิริยาระหว่างเซลล์

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Introduction

Canine distemper virus (CDV), a single-stranded negative sense RNA morbillivirus of the family Paramyxoviridae, is closely related to measles virus and can cause epidemic infection in a variety of carnivores especially in the dog (Van Moll et al., 1995; Baumgärtner et al., 2003; Wohlsein et al., 2007; Beineke et al., 2009). Demyelinating distemper leukoencephalitis (DL) appears as a main sequel of central nervous system (CNS) infection of dogs and is considered a naturally-occurring translational animal model to study multiple sclerosis (MS) in humans (Summers et al., 1994; Baumgärtner et al., 2005; Vandeveldt et al., 2005). The pathological findings in the CNS can be categorized as acute, subacute non-inflammatory, subacute inflammatory, chronic and sclerotic lesions (Alldinger et al., 2000; Beineke et al., 2009). Factors contributing to lesion development include age of the animal at the time of infection, immune status of the host and the virulence of the

virus strain (Krakowka and Koestner, 1976; Baumgärtner et al., 2005; Beineke et al., 2009).

During natural or experimental CDV infection, up to 30% of the dogs exhibit neurological signs (Rudd et al., 2006). Interestingly, the development and progression of CNS lesions depend among other factors on the CDV strain. While some strains (CDV-A75/17 and CDV-R252) induce a demyelinating leukoencephalomyelitis, others such as the CDV-Snyder Hill (Wohlsein et al., 2007) and the CDV-Lederle strain (Lednický et al., 2004) induce a polioencephalitis or a non-specified encephalitis, respectively. Recently, recombinant CDV clones expressing the enhanced green fluorescent protein (eGFP), e.g. the mustelid neurovirulent CDV-A75/17eGFP and the mustelid virulent CDV-5804PeGFP strain, the rgA75/17-V and the CDV-OndeGFP strains were used to study CDV pathogenesis (Plattet et al., 2004; von Messling et al., 2001, 2004; Rudd et al., 2006). More importantly, some of these constructs such as the CDV-A75/17eGFP strain retained their virulence at least for ferrets.

These recombinant strains allow the direct visualization of CDV infected glial cells either *in vivo* or *in vitro* and can be applied to document viral spread, neuroinvasion and viral cell tropism (Plattet et al., 2004; von Messling et al., 2001, 2004; Rudd et al., 2006; Seehusen et al., 2007; Orlando et al., 2008). Moreover, using CDV recombinant strains it is now possible to determine which part of the CDV genome contributes to viral virulence, cell-cell fusion and syncytia formation (Plattet et al., 2004; von Messling et al., 2001, 2004; Rivals et al., 2007).

Thus far, the only known receptor for CDV is the signaling lymphocyte activation molecule (SLAM) receptor, a molecule restricted to activated cells of the mononuclear lineage (Cocks et al., 1995; Aversa et al., 1997; Tatsuo et al., 2001; von Messling et al., 2006; Rivals et al., 2007). Nevertheless, since the wild type CDV-A75/17 strain, a demyelinating strain, was successfully used for infection of cells lacking the SLAM receptor such as canine footpad keratinocytes (Engelhardt et al., 2005; Rivals et al., 2007), it must be assumed that other so far unknown receptors are involved in the infection of cells from diverse lineages. Thus, CDV displays a broad cell tropism and thereby affects various organs including skin, respiratory, gastrointestinal, and urinary tract as well as the CNS, frequently associated with the formation of characteristic inclusion bodies (Rungsipipat et al., 2000; GrÖne et al., 2003; Koutinas et al., 2004).

The cytopathic effect caused by attenuated CDV in permissive cells such as the African green monkey kidney cell line (Vero cells) includes single cell necrosis and multinucleated giant cell formation (von Messling et al., 2001; Singethan et al., 2006; Techangamsuwan et al., 2010). Once CDV is adapted to the *in vitro* conditions, it loses its *in vivo* virulence and the property to proliferate in primary cultures including canine pulmonary macrophages (Appel, 1978; Metzler et al., 1980). However, the virus will maintain its capacity to induce cell-cell fusion, like *in vivo*, and the ability to infect glial cells, e.g. microglia and astrocytes (Zurbriggen and Vandeveld, 1983; Pearce-Kelling et al., 1990; 1991). The possibility of reversion to virulence exists (Appel, 1978; Lednicky et al., 2004). This has been achieved for the attenuated CDV-Rockborn strain after six sequential passages in CDV-susceptible dogs or ten passages in primary canine pulmonary macrophage cultures (Appel, 1978). In addition, spontaneous reversion to virulence must be considered. Similarly, a vaccine escape virus has been proposed as the cause of distemper in a dog in Belfast, Northern Ireland (Harder and Osterhaus, 1997). Moreover, a similar mechanism has been proposed for the CDV-Lederle strain that was used as a vaccine strain in the past (Lednicky et al., 2004). It has been assumed that the 1998 CDV lineage could also be a vaccine escape virus from a dog vaccinated with CDV-Lederle vaccine strain (Lednicky et al., 2004). The exact mechanisms involved in these processes, however, remained undetermined so far. In all cases, the virulence of CDV appears to be associated with the capacity of the virus to replicate in dog macrophages (Appel, 1978) since it is well known that contrary to primary neurotropic viruses, CDV

reaches the CNS only secondarily after widespread infection of lymphatic organs and peripheral blood mononuclear cells (von Messling et al., 2004, 2006; Rudd et al., 2006). Circumstantial evidence indicated that infection of microglia might also be related to virulence (Baumgärtner, personal observation). However, further studies are necessary to determine the possible role of peripheral macrophages and microglial cells as determinants of virulence. Furthermore, whether infection of certain glial cells or the lack of it is related to virus virulence or specific receptors remains to be determined.

Recently, we established glial cell cultures from the adult canine brain as an *in vitro* model to study CDV pathogenesis (Seehusen et al., 2007; Orlando et al., 2008; Techangamsuwan et al., 2008, 2009). Moreover, we identified a novel cell type with Schwann cell-like properties (Orlando et al., 2008), now designated Schwann cell-like brain glia (SCBG) (Beineke et al., 2009). SCBG cells represented the main constituent in the cell cultures obtained from young adult dogs and displayed a high susceptibility to CDV infection (Orlando et al., 2008). The term "aldynoglia" was introduced by Nieto-Sampedro to denominate a group of CNS macroglia with axonal regeneration-promoting properties expressing the typical Schwann cell marker neurotrophin receptor p75 (p75^{NTR}) (Gudiño-Cabrera and Nieto-Sampedro, 1998; Nieto-Sampedro, 2003). Aldynoglia cells are found in diverse brain areas of the adult brain that promote the continuous axon renewal throughout lifetime (Nieto-Sampedro, 2003). Moreover, it was reported that glial cells expressing the Schwann cell prototype marker p75^{NTR} may emerge under pathophysiological conditions, including demyelination, to lesioned areas (Keirstead et al., 1999; Blackmore, 2005).

It has been postulated and demonstrated that lesions in DL are strongly associated with oligodendrocyte damage (Vandeveld et al., 1985; Zurbriggen et al., 1986, 1987, 1998). However, the fact that SCBG shows high susceptibility to CDV infection *in vitro* indicates that damage of these cells during CDV infection *in vivo* might also contribute to the development and progression of DL. At present, cell-cell interactions relevant for CDV infection and virus spread of these cells remain to be determined. The aim of the present study was, therefore, to characterize cell-cell interactions during *in vitro* CDV infection in more detail. For this purpose, SCBG in mixed brain cell cultures and after purification was exposed to different attenuated and a mustelid virulent CDV strain in order to substantiate the hypothesis that SCBG represents a crucial glial cell population within the CNS for CDV infection and virus spread CNS as well as its possible involvement in the development of CDV demyelinating lesions. Here, we report a dramatic reduction in the susceptibility of purified SCBG to CDV infection in contrast to cells maintained in a mixed glial cell environment. This suggests that so far undetermined cell-cell interactions and/or factors of non-SCBG are crucial for CDV susceptibility of SCBG *in vitro*.

Materials and Methods

Mixed cell cultures from adult canine brain: All cultures were obtained from 6-month-old Beagle dogs (n=8) not suffering from nervous systemic disease, as determined by clinical and histopathological examination. For mixed brain cell cultures, brains were collected at necropsy under sterile conditions at the Department of Pathology according to the institutional regulations and ethics of the University of Veterinary Medicine Hannover (Germany). The cells were isolated as described previously (Seehusen et al., 2007; Orlando et al., 2008). Briefly, after careful removal of meninges, olfactory bulb and medulla oblongata, the brain was minced and mechanically dissociated. The cell suspension was then treated for 30 min at 37°C with trypsin-EDTA (final concentration: 0.025%, PAA Laboratories, Marburg, Germany) and DNase I (0.05 mg/ml, Roche, Germany). Mechanical dissociation into single cells was done using a flame-constricted Pasteur pipette. Finally, after a series of centrifugation steps, cells were seeded in DME/F12 medium containing fetal calf serum (FCS, 10%, PAA), gentamycin (500 mg/l, PAA) and penicillin/streptomycin (100 U/ml, 100 mg/ml, Biochrom AG, Germany) at a density of 7×10^4 cells/cm² in poly-L-lysine (PLL, 100 µg/ml, Sigma-Aldrich Chemical, Taufkirchen, Germany) -coated 24-well plates (NuncTM, Nunc GmbH & Co KG, Wiesbaden, Germany) and 25-cm² flasks (NuncTM) for infection and expansion, respectively. Cultures were maintained under standard conditions (37°C, 5% CO₂ incubator, water-saturated atmosphere) and medium was changed twice a week.

Establishment of pure fibroblast cultures from adult canine skin: For cultivation of canine dermal fibroblasts, small pieces of subcutis were collected under sterile conditions. Tissues were cut into small pieces (1 mm³), transferred to 100-mm petridishes (NuncTM) and fixed to the bottom of the dish using heated forceps. Cultures were maintained in minimum essential medium Eagle's salts (MEME, PAA), medium containing FCS (10%) and penicillin/streptomycin (1%) under standard conditions (see above). Medium was replaced every 3 days. After cultivation of explants for about one week, remaining tissue pieces were removed and the outgrowing fibroblasts were detached from the dish using trypsin. Fibroblasts were seeded onto 25cm² culture flasks for expansion. The purity of the cultures was confirmed using conventional microscopy and immunostaining for vimentin (Clone V9; dilution 1:100; Dako Cytomation GmbH, Hamburg, Germany). Cultures reproducibly consisted of more than 95% fibroblasts (data not shown).

Antibody-based purification of Schwann cell-like brain glia (SCBG): Purification of SCBG from confluent brain cell cultures was performed by using antibody based separation (MACS, Miltenyi Biotec) as previously described (Wewetzer et al., 2005; Krudewig et al., 2006; Techangamsuwan et al., 2008). The anti-human-p75^{NTR} antibody (supernatant hybridoma, dilution 1:5, American Tissue Culture Collection, USA) and goat-anti-mouse IgG

MicroBeads (dilution 1:20, Miltenyi Biotec) were used as primary and secondary antibodies, respectively. Following labeling with anti-p75^{NTR} antibodies and adsorption to antibody-coated magnetic beads, cells were seeded in PLL-coated culture flasks and expanded in DME/F12 medium supplemented with 10% FCS under standard conditions. Immunostaining with anti-p75^{NTR} antibodies after 7-14 days demonstrated that the purity of the cultures exceeded 95% (data not shown).

CDV infection of mixed brain cell cultures and purified SCBG and cutaneous fibroblasts: For virus infection, SCBG (passage 5) and fibroblasts (passage 5) were seeded at a density of 8×10^3 cells/well in 96-well PLL-coated microtiter plates (NuncTM) while infection of mixed brain cultures was performed in 24-well plates as described previously (Orlando et al., 2008). Infection was done at 3 weeks and 3 days after seeding of mixed cultures and the purified cultures, respectively, and 3 days following antibody-based purification for the SCBG. Cultures were infected at a multiplicity of infection (MOI) of 0.1 using different CDV strains, including the CDV Onderstepoort strain (CDV-Ond, TCID₅₀/ml= $10^{6.25}$, kindly provided by Dr. Metzler, Institute of Virology, Veterinary Medical Faculty, University of Zürich, Switzerland), the Ond strain with eGFP expression (CDV-OndeGFP, TCID₅₀/ml= $10^{4.5}$, kindly provided by Dr. von Messling, Institute Armand-Frappier, University of Quebec, Canada), the CDV-R252 strain (TCID₅₀/ml= 10^6 , kindly provided by Dr. Krakowka, Ohio State University, USA), the CDV-2544 strain (TCID₅₀/ml= $10^{6.5}$, kindly provided by Dr. Haas, Institute of Virology, University of Veterinary Medicine Hannover, Germany), and the recombinant mustelid virulent CDV-5804PeGFP strain (TCID₅₀/ml= 10^6 , kindly provided by Dr. von Messling). The fluorescent CDV strains were generated by the introduction of the eGFP gene as an additional transcription unit in the CDV genome (von Messling et al., 2004; Suter et al., 2005). All attenuated CDV strains and the mustelid virulent CDV-5804PeGFP strain were propagated in normal Vero cells and in Vero cells expressing dog SLAM receptor (Vero.DogsSLAM, kindly provided by Dr. von Messling), respectively. The virus containing supernatant (SNT) of infected cell cultures was harvested, aliquoted and stored at -80°C until used. Virus infection was done as described (Seehusen et al., 2007; Orlando et al., 2008). Briefly, mixed brain cultures, purified SCBG and cutaneous fibroblast cultures were washed twice with FCS-free medium and incubated with the virus inoculum in FCS-free medium or FCS-free medium alone (mock-infection) for 2 hours under standard conditions. Thereafter, the cultures were washed twice with FCS-free medium and maintained in DME/F12 (mixed cultures, purified SCBG) or MEME (purified fibroblasts). The cultures were monitored daily for CPE until 10dpi.

Immunofluorescence assays: For the antigenic characterization of the cells and for expression of CDV antigen, the cultures were immunostained with cell type-specific monoclonal antibodies (mAb) such

as p75^{NTR}, (dilution 1:5; ATCC); A2B5 (clone 1520 HB-29, dilution 1:2; ATCC) and O4 (dilution 1:2; kindly provided by Dr. J. Trotter, Mainz, Germany) for SCBG, anti-gial fibrillary acidic protein (clone G-A-5, GFAP; dilution 1:400; SIGMA, Deisenhofen, Germany) for astrocytes and anti-vimentin (clone V9, dilution 1:100; Dako Cytomation GmbH, Hamburg, Germany) for fibroblasts. A polyclonal antibody (pAb) directed against the anti-ionized calcium-binding adapter molecule 1 (Iba1; dilution 1:250; Wako Chemicals GmbH, Neuss, Germany) was used to identify microglia. CDV nucleoprotein (CDV-NP) antigen detection following infection with non-eGFP expressing CDV strains (CDV-Ond, CDV-R252, CDV-2544) was performed by using the pAb #25 (1:2000) and the mAb (clone N° 3.991, dilution 1:6000) (both kindly provided by Dr. C. Örvell, Central Microbiological Laboratory of Stockholm, Sweden). Co-expression of cell type and virus-specific markers was used to determine the percentage of infected cells at 3 and 10 dpi.

Detection of cell surface markers (A2B5, O4, p75^{NTR}) and intracellular antigens (GFAP, vimentin, Iba1) was done using viable and fixed cell cultures, respectively (Orlando et al., 2008). Expression of cell type specific markers was studied both in CDV-infected and mock-infected cultures using an inverted fluorescence microscope (Olympus IX-70, Hamburg, Germany). The different cell types were identified according to their morphological characteristics and immunoreactivity. The percentage of infected cells was determined by double-staining for CDV antigen and specific cell markers and by counting displaying colocalization of two antigens in five different high-power fields per well cells. Each experiment was done in triplicates for each cell preparation.

Virus titration assay: To calculate the tissue cultures infectious dose (TCID₅₀) of released virus of CDV-

infected culture, the SNT was harvested at 2 hours, 3 and 10 dpi and titrated in quadruplicates in Vero.DogsSLAM (1.5x10⁴/well) (Orlando et al., 2008). After 5 days of incubation under standard conditions, the cells were examined for CPE and the TCID₅₀/ml was calculated according to the Reed and Muench method (Frisk et al., 1999^{a,b}).

Statistical analysis: Statistic analyses were carried out using the statistical software SAS, version 9.1 (SAS Institute, Cary, NC) and significance was defined as $p \leq 0.05$. The percentage of 'infected cells' revealed a right skewed distribution and a test of lognormal distribution was not rejected. Therefore, logarithmic transformation was performed prior to the analysis; however results are presented after retransformation to the original scale. The percentage of CDV infection of SCBG and fibroblasts in mixed brain cell cultures, and purified SCBG and cutaneous fibroblasts were compared to each other by using two-way analysis of variance with 'virus-strain' as independent effect and 'time post infection' as repeated measures with the Tukey's post-hoc test for multiple pair wise comparisons. For the analysis of the linear model, the procedure MIXED was used. In figures, data were diagrammed as median, maximum and minimum. The virus titration data were included into a descriptive analysis and the normal distribution of the data was confirmed by visual assessment of normal probability plots. Finally, these virus titration data were compared using three-way analysis of variance with 'virus strain' as independent effect and 'time post infection' and 'cell type' as repeated measures. Analysis of this linear model was also calculated with the MIXED procedure with the Tukey's post-hoc test for multiple pair wise comparisons. For these parameters the arithmetic means (x) and standard deviation (s) were calculated.

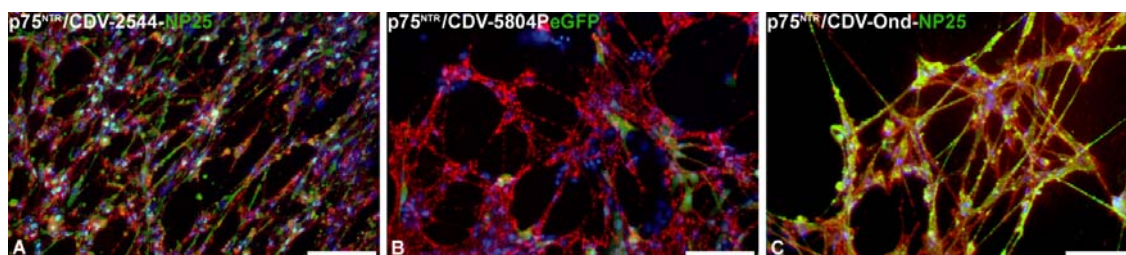


Figure 1 Mixed brain cell cultures infected with CDV-2544 A: CDV-5804PeGFP B: and CDV-Ond C: Immunostained for p75^{NTR} (red) and CDV-NP (green) using a CDV specific polyclonal antibody (#25) at 3 dpi. At this time point, a lower number of SCBG expressing CDV antigen was detected following infection with the mustelid virulent CDV-5804PeGFP strain (B) compared with the attenuated CDV strains (A,C). Bar represents in A-C ~ 200 μm.

Table 1 Cytopathic effect (CPE) in canine Schwann cell-like brain glia (SCBG) and fibroblasts from mixed brain cell cultures and purified Schwann cell-like brain glia and cutaneous fibroblasts^a

Virus strain	SCBG _{mixed}		Fibroblast _{mixed}		SCBG _{pure}		Fibroblast _{pure}	
	3 dpi	10 dpi	3 dpi	10 dpi	3 dpi	10 dpi	3 dpi	10 dpi
CDV-R252	- ¹	Mild ¹	Mild ¹	Moderate ¹	Mild	Moderate	Mild	Severe
CDV-OndeGFP	- ¹	Mild ¹	Mild ¹	Moderate ¹	-	Moderate	Mild	Severe
CDV-Ond	Mild	Moderate	Moderate	Moderate	Mild	Moderate	Mild	Severe
CDV-2544	-	Mild	Mild	Moderate	-	Mild	Mild	n.d.
CDV-5804PeGFP	-	Mild	-	-	-	Mild	-	n.d.

^a Classification of CPE: Mild (5-20% dead cells, single cell necrosis); Moderate (20-60% dead cells, cellular detachment); Severe (more than 60% of dead cells, cellular detachment), _{Mixed}: Cells in mixed brain cell cultures, _{Pure}: Cells in purified cultures, dpi: Days post infection, ¹: Extracted from Orlando et al., 2008, -: No CPE was observed, n.d.: not determined due to a complete cellular detachment at 7dpi, presumable due to advanced CPE

Results

Infection of mixed brain cell cultures with different CDV strains: Brain cell cultures reached 60% confluency 3 weeks after seeding. This time point was defined as day 0 of the infection experiments. The mock-infected and CDV infected cultures were immunostained at 3 and 10 dpi by using cell type-specific antibodies. Infection with CDV-Ond and CDV-2544 strains was visualized using mono- and polyclonal antibodies against CDV-NP while the CDV-5804PeGFP strain could be directly observed due to its genetic modification that allowed the virus to induce eGFP expression. CDV antigen was found both in the cytoplasm and nucleus of infected cells (Fig.1). Infectious virus (TCID₅₀/ml) was present throughout the experiments in all CDV-infected cultures, as determined by virus titration of cell culture SNTs (Fig 2F). The CDV strains induced different degrees of CPE, ranging from mild to severe, consisting of single necrotic cells and cell loss of more than 60% of the monolayer (Table 1). In contrast, infection of fibroblasts with CDV-Ond and CDV-2544 resulted in an increased number of multinucleated

cells at 10dpi. The CDV-Ond-induced CPE increased from mild to moderate at 3 and 10 dpi, respectively. Compared to this, a mild CPE was not observed until 10dpi in CDV5804PeGFP- and CDV-2544-infected cultures. Virus detection using virus-specific poly- and monoclonal anti CDV-NP antibodies or eGFP expression and cell type-specific antibodies (p75^{NTR}, O4, A2B5, GFAP, Iba1, vimentin) revealed no substantial morphological alterations of SCBG and its O4⁺ and A2B5⁺ subpopulations, astrocytes and microglial cells following infection.

The percentage of each cell type susceptible to CDV infection differed substantially between CDV strains and dpi (Fig 3A-C). Differences in the percentage of infected cells were monitored already at 3dpi. The mustelid virulent CDV-5804PeGFP strain was detected only in microglial cells (Fig 3B). In contrast, both attenuated CDV strains (CDV-Ond, CDV-2544) infected a significantly higher number of SCBG and its O4⁺ and A2B5⁺ subpopulations compared to the CDV-5804PeGFP strain (Fig 3A-C), while no significant differences were detected among

Table 2 Percentage of CDV infection of canine Schwann cell-like brain glia (SCBG) and fibroblasts from mixed brain cell cultures and purified Schwann cell-like brain glia and cutaneous fibroblasts

Virus strain	SCBG _{mixed}		SCBG _{pure}		Fibroblast _{mixed}		Fibroblast _{pure}	
	3 dpi	10 dpi	3 dpi	10 dpi	3 dpi	10 dpi	3 dpi	10 dpi
CDV-R252	100 [†]	100 [†]	5.6 (5.6-5.2)	22.4 (23.5-18.6)	7.5 [†] (8.0-7.4)	45.8 [†] (92.5-11.1)	7.2 (8.0-6.7)	100 (100-92.8)
CDV-OndeGFP	34.4 [†] (47.2-14.8)	98.0 [†] (100-81.7)	0.5 (0.6-0.2)	2.2 (3.8-0.5)	8.0 [†] (2.8-2.1)	15.1 [†] (17.8-11.0)	2.4 (2.6-2.2)	n.d
CDV-Ond	96.5 [*] (100-38.0)	100 [*]	1.6 (1.8-0)	0.4 (0.6-0.2)	11.5 (27.0-	33.5 (100-25.0)	15.6 (16.1-	56.0 (71.9-40.2)
CDV-2544	32.2 [*] (38.0-18.0)	100 [*] (100-95.0)	2.3 (2.7-1.1)	67.1 (80.5-55.6)	6.9 (16.0-0)	58.2 (90.0-48.0)	1.4 (2.3-0)	46.2 (46.3-37.7)
CDV-5804PeGFP	0.4 (1.6-0)	82.0 [*] (100-0)	1.6 (2.1-0.4)	2.9 (3.8-2.2)	0	2.77 (7.0-0)	1.0 (1.04-	n.d.

Mixed; Cells in mixed brain cell cultures, Pure; Cells in purified cultures, *: statistical significant ($p < 0.05$) differences between mixed and purified cultures, †: Extracted from Orlando et al., 2008, n.d.: not determined due to the complete cell loss, presumable due to advanced CPE, The data are expressed as median, maximum and minimum.

the CDV strains with regard to the infection of microglial cells, astrocytes and fibroblasts. Until 10dpi, the percentage of infected SCBG and its O4⁺ and A2B5⁺ subpopulations increased independently of the virus strain used and reached about 50-100%. Despite the increased number of infected cells, their morphology remained unaltered. Significant differences in the percentage of infected cells between the virulent CDV-5804PeGFP and the attenuated CDV-Ond and CDV-2544 strains were only observed for astrocytes and fibroblasts. The CDV-Ond and the CDV-2544 strain infected less than 1% and approximately 65% of astrocytes, respectively (Fig 3; A-C), whereas the virulent CDV-5804PeGFP strain infected an intermediate percentage of about 35% of this macroglial cell population (Fig 3B). The attenuated CDV-Ond and CDV-2544 strains exhibited a significantly ($p < 0.0001$) higher percentage of

infection of fibroblasts with 33 and 58%, respectively, (Fig 3A, C) compared to the low percentage (2%) observed by the CDV-5804PeGFP strain (Fig 3B).

Infection of purified cultures from adult canine Schwann cell-like brain glia (SCBG) and cutaneous fibroblasts with different CDV strains: Three dpi, purified SCBG and fibroblasts displayed a mild CPE characterized by a single cell necrosis in SCBG cultures independently of the used virus strain and, additionally, multinucleated syncytial cells in fibroblasts (Table 1). The degree of severity of the CPE was augmented during the observation period. SCBGs showed a moderate CPE when infected with CDV-R252, CDV-OndeGFP, CDV-Ond and a mild CPE when inoculated with CDV-2544 and CDV-5804PeGFP. In contrast, infected fibroblasts demonstrated a severe CPE and were associated with

syncytial cell formation regardless of CDV strain applied (Table 1). Moreover, a total cell loss was observed in CDV-OndeGFP- and CDV-5804PeGFP-infected fibroblasts at 10dpi.

The cultures were characterized by immunostaining for p75^{NTR} and vimentin to visualize SCBG and fibroblasts, respectively. Expression of both antigens was above 95% and remained unaltered after CDV infection compared to mock-infected controls. Furthermore, infectious progeny virions were detected in both culture systems throughout the observation period with the exception of fibroblasts

cultures infected with CDV-OndeGFP and CDV-5804PeGFP at 10dpi (Fig 2;D,E). Overall, virus titers of infected SCBG were higher compared to CDV-2544- and CDV5804PeGFP-infected fibroblast cultures at 3 dpi and CDV-R252-infected cells at each time point ($p<0.01$).

At 3 dpi, the percentage of SCBG and fibroblasts infected by the different virus strains were similar and below 10% (Fig 4A) except for the CDV-Ond strain, which exhibited statistically and significantly increased infection rate of 17% of

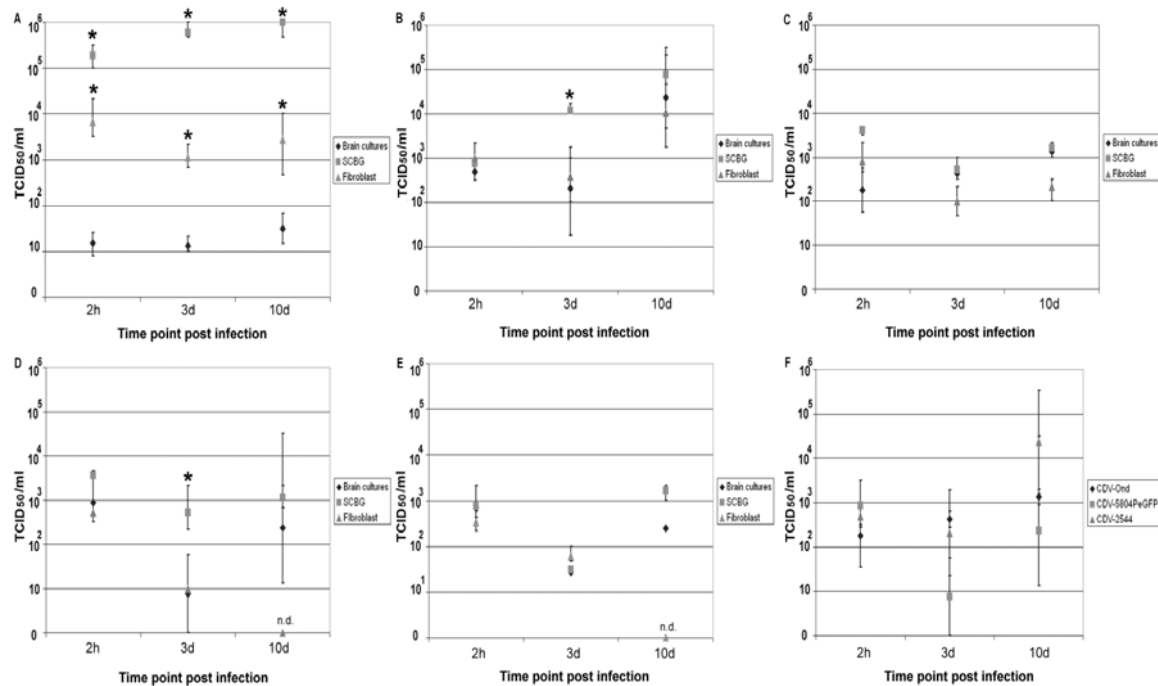


Figure 2 TCID50 in the supernatants from adult canine brain cell cultures, purified Schwann cell-like brain glia (SCBG) and cutaneous fibroblasts infected with the CDV-R252 (A), CDV-2544 (B), CDV-Ond (C), CDV-5804PeGFP (D), CDV-OndeGFP (E) as well as CDV-2544, CDV-Ond and CDV-5804PeGFP in mixed brain cultures (F) at 2h, 3, and 10dpi. At 3dpi, the TCID50 was significantly higher in the SNT from CDV-R252- (A; $p<0.01$), CDV-2544- (B; $p<0.05$) and CDV-5804PeGFP- infected (D; $p<0.01$) purified SCBG compared to mixed brain cell cultures. The same time point, TCID50 in the SNT from CDV-R252-infected fibroblasts was statistically significantly higher (A; $p<0.01$) compared to mixed brain cell cultures. At 10dpi, the TCID50 of purified cultures infected by CDV-R252 was significantly higher (A;SCBG; $p<0.01$ and fibroblast; $p<0.05$, respectively) compared to mixed brain cell cultures. There was no statistically significant difference between the TCID50 from CDV-infected mixed brain cell cultures (F), Data are expressed as means () \pm standard deviation (s). n.d.= not determined.

fibroblasts compared to less than 5% of SCBG. At 10 dpi, the percentage of CDV infected SCBG and fibroblasts increased in cultures infected by the CDV-2544, CDV-Ond and CDV-R252 strain, in contrast, there was no infection in the virulent CDV-5804PeGFP and the CDV-OndeGFP strain (Fig 4B). Fibroblasts infected by the CDV-5805PeGFP and the CDV-OndeGFP could not be evaluated numerically because of complete cellular detachment. Both CDV-Ond and CDV-R252 significantly infected more fibroblasts than SCBG at 10 dpi, while there was no difference with respect to the infection rate using the CDV-2544 strain (Fig 4B).

Comparative analysis of CDV infection between mixed and pure glial cultures: Comparing the number of infected SCBG and fibroblasts in mixed brain cell and purified cultures, several important aspects became evident (Table 2; Fig 5A-D). In mixed brain

cell culture, there was a significant difference between the extent of SCBG infection at 3 dpi but not at 10 dpi between the different CDV strains. CDV-Ond and CDV-R252 infected almost 100% of SCBG as early as 3 dpi. At 10 dpi, all viruses infected between 80-100% of the SCBG. Contrary to this was the infection rate of purified SCBG (Table 2). Here, about 22 and 67% of SCBG were infected by CDV-R252 and CDV-2544, respectively, at 3 dpi (Fig 4B). The percentages of infected SCBG in purified and mixed brain cell cultures were similar following CDV-2544 infection. Generally, the percentage of infection of the fibroblasts in mixed and purified cultures was lower compared to SCBG in mixed brain cell cultures. Contrary to SCBG, no significant difference in the percentage of infected fibroblasts was noted between mixed brain cells and purified cutaneous fibroblast cultures. However, the percentage of infection of the fibroblasts in purified cultures could not be

determined due to advanced CPE at 10dpi. Interestingly, the pattern of virus spread differed between both viruses expressing eGFP but not between mixed and purified cultures for each virus. CDV-OndeGFP infected individual cells displaying a random distribution, whereas CDV-5804PeGFP infected patches of cells that continuously increased in size from 3 to 10 dpi.

SNTs from mixed brain cell cultures as well as purified SCBG and cutaneous fibroblasts were collected at 2 hours, 3 and 10 dpi. In general, the obtained TCID₅₀ differed between CDV strains from the mixed brain cell cultures and purified SCBG and cutaneous fibroblast cultures (Fig 2A-E). At 3 dpi, the TCID₅₀ was significantly higher in the SNT from CDV-R252- ($p<0.01$) CDV-2544- ($p<0.05$) and CDV-5804PeGFP-infected ($p<0.01$) SCBG cultures compared to the SNT of the mixed brain cell cultures (Fig 5A, B, E). At the same time point, the TCID₅₀ from CDV-R252-infected fibroblasts was statistically significantly higher ($p<0.01$) compared to mixed brain cell cultures (Fig 5A). Finally, at 10 dpi the TCID₅₀ from the CDV-R252-infected purified SCBG and fibroblast cultures was significantly higher ($p<0.01$ and $p<0.05$, respectively) compared to mixed brain cell cultures (Fig 5A).

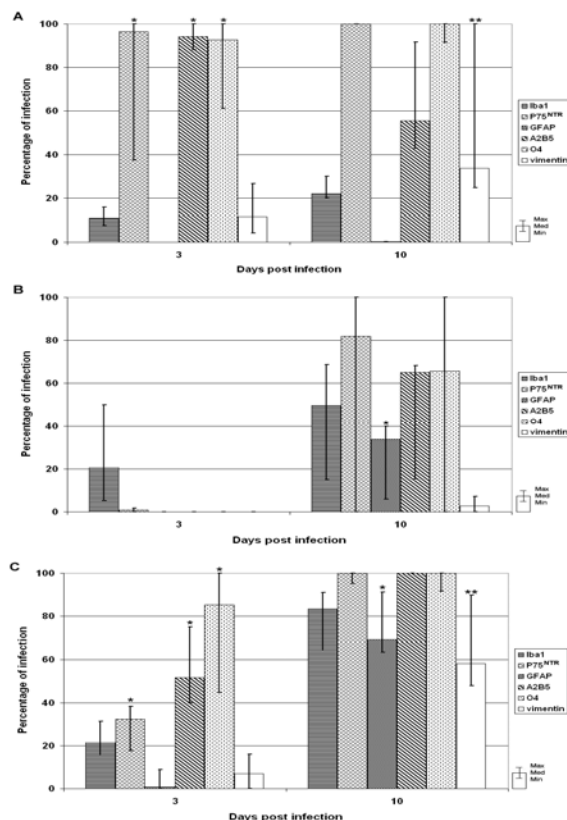


Figure 3 Percentage of CDV-infection of adult canine mixed brain cultures infected with CDV-Onde (A), CDV-5804PeGFP (B) and CDV-2544 (C) at 3 and 10 dpi. There was a statistically significant difference between the percentage of p75NTR⁺, O4⁺ and A2B5⁺ cells infected with the CDV-Onde and the CDV-2544 strain compared to the CDV-5804PeGFP strain at 3 dpi (A, C, *, $p<0.0001$) while there was no statistical significant difference between the percentage of infected microglial cells by the

different CDV strains (A, B, C). However, at this time point the mustelid CDV-5804PeGFP infected only these cells to a considerable amount (B). At 10 dpi, the percentage of infected p75NTR⁺ and its O4⁺, A2B5⁺ subpopulation cells increased in all cultures (A-C). Additionally, only few GFAP⁺ astrocytes were found following CDV-Onde infection compared to CDV-5804PeGFP (~35%) and CDV-2544 (~65%) strain (B, C, *, $p<0.0001$). Furthermore, at this time point both attenuated strains (A, C) infected significantly (A, C, **, $p<0.0001$) more fibroblast compared to CDV-5804PeGFP strain (B). Data are expressed as median, maximum and minimum.

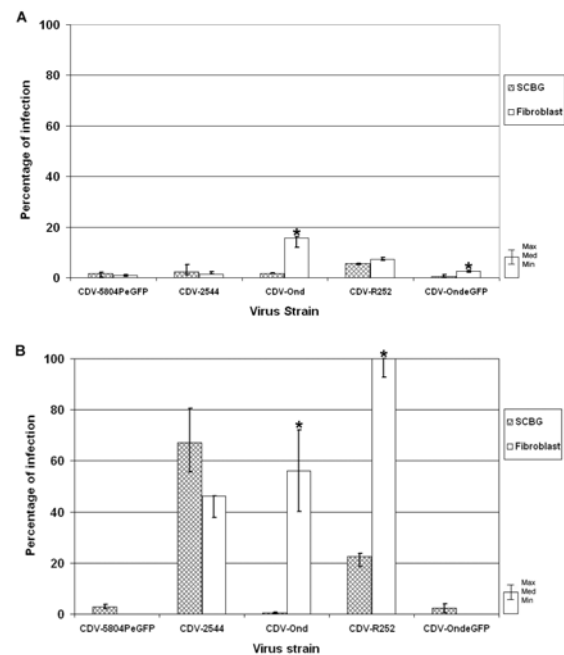


Figure 4 Percentage of adult purified canine Schwann cell-like brain glia (SCBG) and cutaneous fibroblasts infected with CDV-5804PeGFP, CDV-2544, CDV-Onde, CDV-R252 and CDV-OndeGFP at 3 (A) and 10 dpi (B). At 3 dpi, less than 5% of SCBG were infected by any virus strain. The CDV-Onde and the CDV-OndeGFP induced a higher percentage of infection in the fibroblast cultures compared to SCBG cultures (*, $p<0.01$). At 10 dpi, the percentage of infection of SCBG remained unaltered for the mustelid virulent CDV-5804PeGFP, CDV-Onde and CDV-OndeGFP strain, while following infection with CDV-2544 and CDV-R252 the percentage of SCBG infected cells increased up to about 64% and 22%, respectively. In contrary, fibroblasts were substantially infected by the CDV-2544, CDV-Onde, and CDV-R252 strain. In addition, at this time point, the CDV-Onde and CDV-R252 reached a higher percentage of infection in fibroblast cultures compared to SCBG cultures (*, $p<0.01$). For fibroblast cultures, no data of CDV-5804PeGFP and the CDV-OndeGFP could be determined due to the total cell loss at 7 dpi. Data are expressed as median, maximum and minimum.

Discussion

The two major findings of the present study are (i) that adult canine glial cells, including SCBG, astrocytes, and microglia display a differential susceptibility to different CDV strains and (ii) that after purification the susceptibility of SCBG to CDV infection *in vitro* is drastically reduced. The first observation confirms and extends previous findings on the susceptibility of SCBG for attenuated CDV strains (CDV-R252, CDV-OndeGFP) (Orlando et al., 2008). The infection of SCBG with two other attenuated CDV strains used in the present study (CDV 2544, CDV-Ond) reveals a similar mode of infection of this glial cell population in mixed adult canine brain cell cultures of SCBG. The mustelid virulent CDV-5804PeGFP strain caused a delayed infection of SCBG, but not of microglia. The second observation, that infection of SCBG is dependent upon the presence of other cell populations, was unexpected and represents a novel aspect of CDV infection. It points towards the relevance of cell-cell interactions as a crucial factor of CDV infection, susceptibility and virus spread. Whether these microenvironmentally related factors also enhance or contribute to virus virulence needs to be investigated in futures studies.

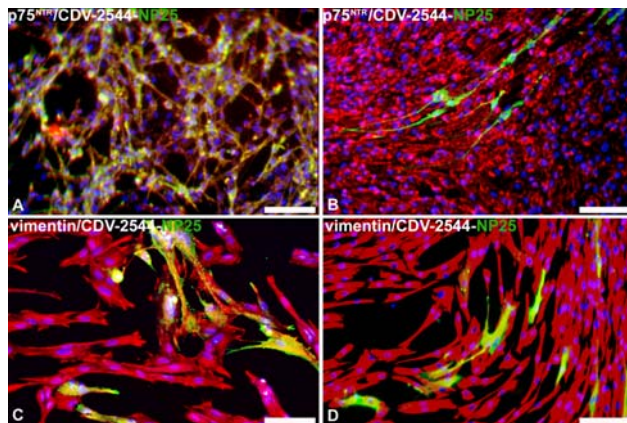


Figure 5 Schwann cell-like brain glia (SCBG) (A,B) and fibroblasts (C,D) in mixed (A,C) and purified cultures (B,D) infected with CDV-2544 strain at 3 dpi. The number of p75NTR⁺ SCBG (red) expressing CDV antigen determined by using the polyclonal antibody directed against CDV nucleoprotein (25NP, green) in mixed cultures was higher compared to SCBG in purified cultures (A, B). In contrast there was no difference between the number of CDV-infected vimentin⁺ fibroblasts observed (vimentin; red, CDV; 25NP, green) in mixed and purified cultures (C,D). Bar represents in A-D ~140µm.

During recent years it has become apparent that the central nervous system contains precursor cells capable of differentiating into glial cells with Schwann cell-like properties under appropriate conditions (Gudiño-Cabrera and Nieto-Sampedro, 1998; Nieto-Sampedro, 2003). There is growing evidence, for example, that remyelination of the experimentally demyelinated spinal cord is not only due to Schwann cells invading the CNS from the periphery across a leaky blood-brain barrier, as

previously thought, but presumably due Schwann cell-like glia arising from central precursors (Keirstead et al., 1999; Blakemore, 2005). Moreover, it was reported that glial cells expressing the Schwann cell prototype marker p75NTR may emerge under pathophysiological conditions, including demyelination, to lesioned areas (Petratos et al., 2004).

Recently, we reported a preferential infection of p75NTR⁺-precursor glia from the adult canine brain, which we now term SCBG, with CDV (Orlando et al., 2008; Beineke et al., 2009). SCBG was not only a major constituent of adult canine cultures, but was also the cell type being most rapidly infected with CDV. After 10 days *in vitro*, CDV was detected in almost 100% of SCBG. Furthermore, we reported CDV strain-specific differential susceptibility among glial cell populations such as astrocytes and microglia (Orlando et al., 2008). Moreover, *in vivo* studies revealed phenotypical changes of glial cells characterized by increased vimentin expression in nervous distemper lesions (Seehusen et al., 2007). Although the role of SCBG in CDV brain lesions has not been investigated yet, the occurrence of vimentin expression in astrocytic cells indicates a disease-stage dependent phenotypical change of glial cells or recruitment of progenitor cells of unknown origin or a transient reversion to an immature phenotype. The increased expression of CDV in these cells indicated either a change of cell tropism of CDV or an alteration in virus replication, transcription and translation during disease progression (Seehusen et al., 2007). Taken together, these results indicated that CDV tropism and spread might be influenced by diverse virus properties as well as cellular factors.

These novel results prompted us to extend our *in vitro* studies by using other CDV strains and to infect mixed and purified brain cell cultures. Interestingly, a dramatic reduction in the susceptibility of SCBG to CDV infection was observed in the virtual absence of other glial cells indicating that probably interactions of SCBG with not yet identified cells or factors are required for increased CDV infection. It is well known that cellular mechanisms such as contact-mediated transfer of virus are an important mode of virus transmission and dissemination *in vivo*. Cell to cell viral transmission has been documented for many different viruses, e.g Human Immunodeficiency Virus (HIV) (Phillips, 1994; Carr et al., 1999).

Moreover, the potential role of macrophages as a source for viral infection and systemic spread in cell to cell virus transmission has been documented in Simian Immunodeficiency Virus (SIV) (Hirsch et al., 1998; Carr et al., 1999). It was concluded that this macrophage-dependent mechanism might be a prerequisite for efficient dissemination of the infection and pathogenic consequences (Hirsch et al., 1998). Moreover, recent studies revealed striking similarities between the basic mechanism of CDV host invasion and those sustaining acute phase of HIV and SIV (von Messling et al., 2006). Thus, it is reasonable to postulate that during CDV infection of mononuclear cell lineages the virus acquires activation or

amplification of its neurovirulence since CDV reaches the CNS only after infection of these cells (Rudd et al., 2006) and a reversion to virulence has been achieved through virus passages in these cells *in vitro* (Appel, 1978). Additionally, this hypothesis may explain the observation that the mustelid virulent CDV-5804PeGFP strain infected microglial cells and subsequently the other glial cell populations, while the attenuated CDV strains that are tissue culture adapted infected the same cells more efficiently. However, whether SCBG infection is related to virus virulence or not remains undetermined. Similarly, the role of p75NTR as a potential virus receptor needs to be addressed in future studies.

The differential susceptibility of astrocytes and microglia to CDV strains in vitro may help to explain the course of demyelinating CDV lesions in vivo: Adult canine brain cell cultures were infected with the attenuated CDV-Ond, CDV-2544 and the virulent CDV-5804PeGFP. The virus strains displayed a different degree of CPE during the experimental period. Moreover, the virulent CDV-5804PeGFP strain exhibited a delayed infection of the cells compared to the attenuated CDV strains and induced only a mild non-cytolytic CPE at 10 dpi. These characteristics appear to be a determinant for CDV persistence, since in the CNS, the virus is found in the periphery of demyelinating lesions indicating that CDV persistence in the CNS depends on a non-cytolytic spread of the virus and a restricted infection of certain cells (Müller et al., 1995; Zurbriggen et al., 1995). This particularity of virulent CDV strains has been described in experiments using brain cell cultures from neonatal dogs (Pearce-Kelling et al., 1990, 1991) as well as in cell lines such as Vero cells where virulent CDV strains are not able to replicate efficiently due to their lack of adaptation *in vitro* (Plattet et al., 2004; Rivals et al., 2007). Contrary to the *in vitro* findings in neonatal cultures (Pearce-Kelling et al., 1990) where infection of astrocytes and microglia occurs independently of the virus strain, the present study reveals a strain-dependent infection of both cell types. At 3 dpi, the mustelid virulent CDV-5804PeGFP strain infected microglial cells at considerable extent, whereas at 10dpi the attenuated CDV-2544 and the mustelid virulent CDV-5804PeGFP infected significantly more astrocytes than the attenuated CDV-Ond. These results are in agreement with our previous study and substantiate the conclusion that the differential susceptibility *in vitro* depends on cellular determinants rather than on methodological problems caused by genetic modifications of GFP-expressing CDV clones (Orlando et al., 2008). Whether the higher percentage of microglia infection by CDV is related to a higher virulence of some of the used strains remains speculative and will require animal experiments.

There is consensus on the fact that astrocytes and microglia are the main CDV targets *in vivo* and *in vitro* (Mutinelli et al., 1989; Pearce-Kelling et al., 1990; Seehusen et al., 2007; Beineke et al., 2009). Consequently, an important role of these cells in the pathogenesis of demyelinating lesions has been assumed (Beineke et al., 2009). Although demyelinating distemper lesions depend upon the

CDV strain, some CDV strains induce a demyelinating leukoencephalomyelitis (CDV-A75/17 and CDV-R252) while other induce either a polioencephalitis (CDV-Snyder Hill) (Summer and Appel, 1994). The fact that astrocytes and microglia were differently infected *in vitro* in the present study indicated that, in addition, cellular factors seemed to contribute substantially to the course of lesion development and myelin loss. In addition, other cells including SCBG represent key players in this already highly complex system of cell-cell and virus-cell interactions.

The reduction in the susceptibility to CDV appears to be a particular property of purified SCBG: The isolation of SCBG from confluent mixed brain cell cultures and cutaneous fibroblasts allowed us to perform comparatively virus infection assays and to investigate cell susceptibility, virus tropism and spread of different CDV strains in both purified cell populations. Until 3 dpi, a minimal to mild CPE was observed regardless of the CDV strain used, while at 10 dpi the degree of CPE varied between different CDV strains and cell types. Interestingly, cutaneous fibroblasts were more susceptible to the different CDV strains. Moreover, purified SCBG showed less pronounced morphological changes than the purified fibroblasts. The latter displayed severe CPE at 10 dpi after infection with CDV-5804PeGFP and CDV-OndeGFP with complete cell loss, whereas the other CDV strains induced only moderated changes in purified SCBG. These results indicate a cell type specific CPE and can explain organ or tissue specific alterations following CDV infection. Additionally, although fibroblast reached a higher percentage of infection, the number of infectious viral particle was higher in the SNTs of purified SCBG cultures, as determined by virus titration, indicating that in these glial cells a selective non-cytolytic virus spread and release similar to persistent infection might have taken place (Zurbriggen et al., 1995) suggesting an important role of SCBG for viral persistence.

The parallel infection of mixed brain cell cultures and purified SCBG and cutaneous fibroblasts allowed us to determine the possible role of cell-cell interactions during CDV infection. The fact that purified SCBG cultures did not reach a high percentage of infection is in contrast to the high percentage of susceptibility of these glial cells in CDV-infected mixed brain cell cultures in this and previous studies (Orlando et al., 2008).

The differential susceptibility argues for the presence of so far undetermined cell-cell interactions or cell-released factors that may influence individual cell susceptibility to CDV infection *in vitro*. It is well known that during CDV infection of mixed brain cell cultures from neonatal dogs, cells are able to interact with each other. Oligodendrocytes are refractory to CDV infection and only a few of these cells contain CDV antigen due to a restricted infection characterized by viral transcription without translation and a marked down-regulation of myelin gene transcription (Vandeveldt et al., 1985; Zurbriggen et al., 1987, 1998; Vandeveldt and

Zurbriggen, 2005). Moreover, oligodendrocytes suffer morphological changes probably due to a secondary process following infection of other glial cells (Zurbriggen and Vandevelde, 1983; Zurbriggen et al., 1986, 1987). Similar secondary cell-mediated mechanisms may occur within SCBG when exposed to CDV in mixed brain cell cultures. Non-SCBG might activate so far undetermined mechanisms that induce morphological and molecular changes that increase the susceptibility of SCBG to CDV. At present, this process seems to be applicable to SCBG only and not to fibroblasts in mixed brain cultures.

In summary, the provided data indicate that SCBG, like astrocytes and microglia, represent an important target cell for CDV. Moreover, SCBG may play a crucial role for viral infection initiation, maintenance and persistence during the course of demyelinating encephalomyelitis. However, so far there is no *in situ* evidence for the presence of SCBG in nervous distemper lesions, therefore, further studies are needed to elucidate the pathogenic role of this glial cell population during nervous distemper.

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