# Loss of virulence of canine distemper virus is associated with a structural change recognized by a monoclonal antibody

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*Abstract.* The monoclonal antibody (mAB) L1, which binds to the nucleocapsid protein of canine distemper virus (CDV), was shown to bind to avirulent CDV obtained after serial passages in Vero cells, but not to two different virulent demyelinating CDV-strains propagated in dog glial cell cultures. However, when both virulent CDV-strains were passaged through Vero cells they expressed, after a number of passages, an epitope recognized by mAB L1. The occurrence of the L1 epitope appeared to coincide with loss of virulence in animal inoculation experiments.
*Key words.* Avirulence; canine distemper virus; monoclonal antibody; virulence.

Canine distemper virus (CDV), a morbillivirus, causes a multisystemic disease including severe neurological complications in dogs and other carnivora[1]. After adaptation to cell lines, CDV quickly loses its ability to cause disease[2]. However, when attenuated CDV is passaged in primary host cells, virulence returns[3]. The question of virulence is not only important in connection with vaccine production, since neurological complications following vaccination against distemper have been reported, but also for pathogenesis research involving in vitro experiments. Generally, the virulence or avirulence of CDV is determined by inoculation of susceptible animals which are subsequently observed for the occurrence of clinical and pathological signs of distemper. There are no accepted in vitro methods to distinguish between virulent and attenuated CDV strains.

Differences in binding patterns of monoclonal antibodies (mAB) to viral antigens have been related to virulence[5]. Although a large number of mABs against CDV have been described[6], they have not been used, to our knowledge, in virulence studies. We describe a mAB which binds to established attenuated CDV-strains, but not to virulent CDV. We have shown that the epitope recognized by this antibody is acquired when virulent CDV-strains are adapted to cell lines by repeated passaging.

## Materials and methods

*Tissue cultures.* African green monkey kidney (Vero) cells were grown on glass coverslips mounted with silicongrease in Petri dishes (3003, Falcon). Dog brain cell cultures (DBCC) were prepared as described elsewhere[7]. Both culture systems were maintained under standard tissue culture conditions.

*Canine distemper virus.* Three strains of CDV were used: A75/17-CDV and CH 84-CDV (isolated from a naturally-infected dog during an outbreak of distemper in Switzerland, 1984) are virulent strains whereas Onderstepoort-CDV (OP-CDV) is an attenuated vaccine strain. OP-CDV was propagated in Vero cells. CH 84-CDV and A75/17-CDV were multiplied in CDV-seronegative dogs[8].

*Passaging of virulent CDV in Vero cells.* Vero cell monolayers were inoculated at near-confluence with A75/17-CDV containing dog lymphoid tissue [$10^{2.5}$ tissue culture infectious dose ($TCID_{50}$) as titrated in canine lung macrophages[9]] or CH 84-CDV containing dog lymphoid tissue (titer unknown). Control cultures were inoculated with OP-CDV ($4.4 \times 10^3$ $TCID_{50}$ as assayed in Vero cells). The inoculates were suspended in tissue culture medium, and virus adsorption was allowed to take place for 1 h at 37 °C. To obtain adaptation, cultures were rinsed with PBS 3–4 days after inoculation and treated with 5 ml of trypsin-EDTA solution (0.25%, 043-05300, Gibco BRL) for 5 min at 37 °C. The cells were collected, centrifuged, washed once in medium and transferred into new Petri dishes containing glass coverslips. Trypsinisation was repeated every 3–4 days, up to 20 times. Coverslips were removed at regular intervals and monitored immunocytochemically for the presence of CDV antigen as described below.

*CDV-infection of DBCC.* DBCC were infected upon confluency, 10–14 days after seeding, with a) virulent A75/17-CDV (dog lymphoid tissue, titer as indicated above), b) virulent CH 84-CDV (dog lymphoid tissue, titer unknown), c) A75/17-CDV which had been passaged 16 times through Vero cells ($0.3 \times 10^5$ $TCID_{50}$ as assayed in Vero cells) and d) OP-CDV (titer as indicated above). At regular intervals, CDV infection was monitored immunocytochemically as described below.

*mAB L1 and other anti-CDV antibodies.* Spleen cells of BALB/c mice (immunized with purified OP-CDV) were prepared according to a standard cell fusion protocol[10]. The mAB L1 which was obtained and used in this study was shown to precipitate the nucleocapsid protein (NP) of CDV in a radioimmunoprecipitation assay, and is of the $IgG_{2b}$ subclass[11]. As a control mAB we used an anti-NP mAB 4317, kindly provided by Dr. C. Örvell[6]. CDV antigens were demonstrated by using two additional established mABs, namely D110 and K4. mAB D110 binds to the NP and mAB K4 to the haemagglutinin protein (H) on Western blot analysis of CDV separated by SDS-PAGE[8]. Both mABs are of the $IgG_1$ subclass. In

addition, anti-CDV antiserum (prepared in rabbits) was used[12].

*Immunocytochemistry.* CDV-infected cultures were fixed with ethanol:acetic acid (95:5, v:v). To demonstrate viral antigens, the unlabelled antibody peroxidase-antiperoxidase (PAP) method was applied[13]. mABs L1, D110 and K4, or rabbit-anti-CDV were used as primary antibodies, and followed by the corresponding second antibody layer and PAP complex (P850; Z113, Dakopatts)[8]. Finally, 3,3-diaminobenzidine-tetrahydrochloride (D 5637, Sigma) was added as chromogen. Occasionally, cell nuclei were counterstained with hematoxylin.

*Animal inoculation.* For in vivo experiments, CDV-seronegative two-month-old SPF dogs were used (Institute for Immunoprophylaxis and Vaccine, Basel). Two animals were inoculated intravenously with 1 ml of a DBCC-homogenate containing A75/17-CDV ($10^2$ $TCID_{50}$), and two further animals with 1 ml of a DBCC-homogenate containing A75/17-CDV passaged 16 times in Vero cells ($0.3 \times 10^5$ $TCID_{50}$). The four dogs were examined clinically for signs of canine distemper until they were euthanized 30 days p.i. Necropsies were carried out on all animals and representative samples of all major tissues were fixed in formalin, embedded in paraffin and examined histopathologically. Paraffin sections from brain, lung, urinary bladder and thymus were examined immunocytochemically for the presence of CDV antigens using mAB D110 and a rabbit anti-CDV antibody.

*Results*

*Characterization of mAB L1.* mAB L1 was shown to precipitate the NP protein of OP-CDV in a radioimmunoprecipitation assay (fig. 1). The precipitate of mAB L1 comigrated with the one from mAB 4317, a well-characterized anti-NP anti-CDV antibody, and with the precipitate of mAB D110. Therefore, mAB L1 and mAB D110 recognize an epitope localized within the NP protein of CDV.

*Immunocytochemistry of OP-CDV in Vero cells.* OP-CDV spread rapidly throughout the Vero cell culture, with formation of syncytia and cytolytic plaques, as previously described[8,14]. CPE reached its maximum 48 h after inoculation with massive destruction of the cell layer. All four antibody preparations bound to CDV-infected Vero cells. Repeated passaging of OP-CDV in Vero cells as a control for the passaging of the virulent virus did not alter the initially-observed binding pattern.

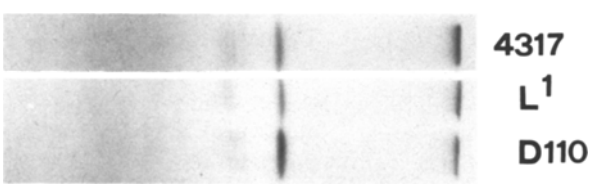


Figure 1. Radioimmunoprecipitation characterizing mAB L1 and mAB D110 as anti-NP antibodies. mAB 4317 is a control anti-NP antibody. As viral antigens we used OP-CDV. All antibodies were undiluted cell supernatant fluid.

*Immunocytochemistry of virulent CDV passaged in Vero cells.* A very few scattered infected cells were found with rabbit-anti-CDV in the first passage of virulent A75/17-CDV in Vero cells. The number of cells detected immunocytochemically with this antibody increased steadily during the following passages. From passage 11 upwards, mAB D110 was employed as an additional antibody. At passage 11, CDV was detected in many cells and at passage 13 in all cells of the culture. Cytolysis or plaque formation was not observed, but some syncytia were found. There was no apparent difference between the two different antibody preparations in the number of detected cells. No infected cells were detected with mAB L1 in the first passages. At passage 11, single clusters of cells with inclusion bodies were positive for mAB L1, and from passage 13 on, no apparent difference was seen in the number of cells recognized by mAB L1 and the two other antibody preparations (fig. 2C, D). The results of the passaging experiments with CH 84-CDV were similar, although mAB K4 was employed in addition to mAB D110 and rabbit-anti-CDV. In one experiment, abundant L1-positive cells were found at passage 8; in the other at passage 13. A few single sporadic L1-positive cells were found in two to three prior passages.

*Immunocytochemistry in DBCC.* Both virulent A75/17-CDV and CH 84-CDV strains produced a slowly spreading non-cytolytic infection in DBCC as described previously[14]. At 4 days p.i., single scattered infected cells or small cell clusters were detected immunocytochemically with rabbit-anti-CDV and both mAB D110 and mAB K4. The number of infected cells steadily increased and by day 20 most cells of the culture were labelled. At all intervals examined, comparable numbers of cells were detected with these 3 antibodies. In contrast, mAB L1 did not label any cells infected with virulent CDV-strains during the first 3 weeks of infection. Later on, only very few positive cells were detected. OP-CDV caused a rapidly-spreading infection with plaque formation and destruction of the entire culture. All antibody-preparations, including mAB L1, showed a similar binding pattern in OP-CDV-infected DBCC as far as the number of cells was concerned (fig. 2B). A75/17-CDV, passaged 16 times through Vero cells, spread rapidly in DBCC, causing a confluent infection within 10–14 days. Initially cytolysis was moderate, but there was considerable destruction of the culture later on. All four antibody preparations, including mAB L1, showed a similar staining-pattern in these cultures.

*Animal inoculation.* The two dogs inoculated with virulent A75/17-CDV propagated in DBCC developed fever, lymphopenia, diarrhea and respiratory signs within the first 2 weeks after inoculation. During the third and fourth week, both dogs became anorectic and lost weight. One dog developed neurological signs at 27 days

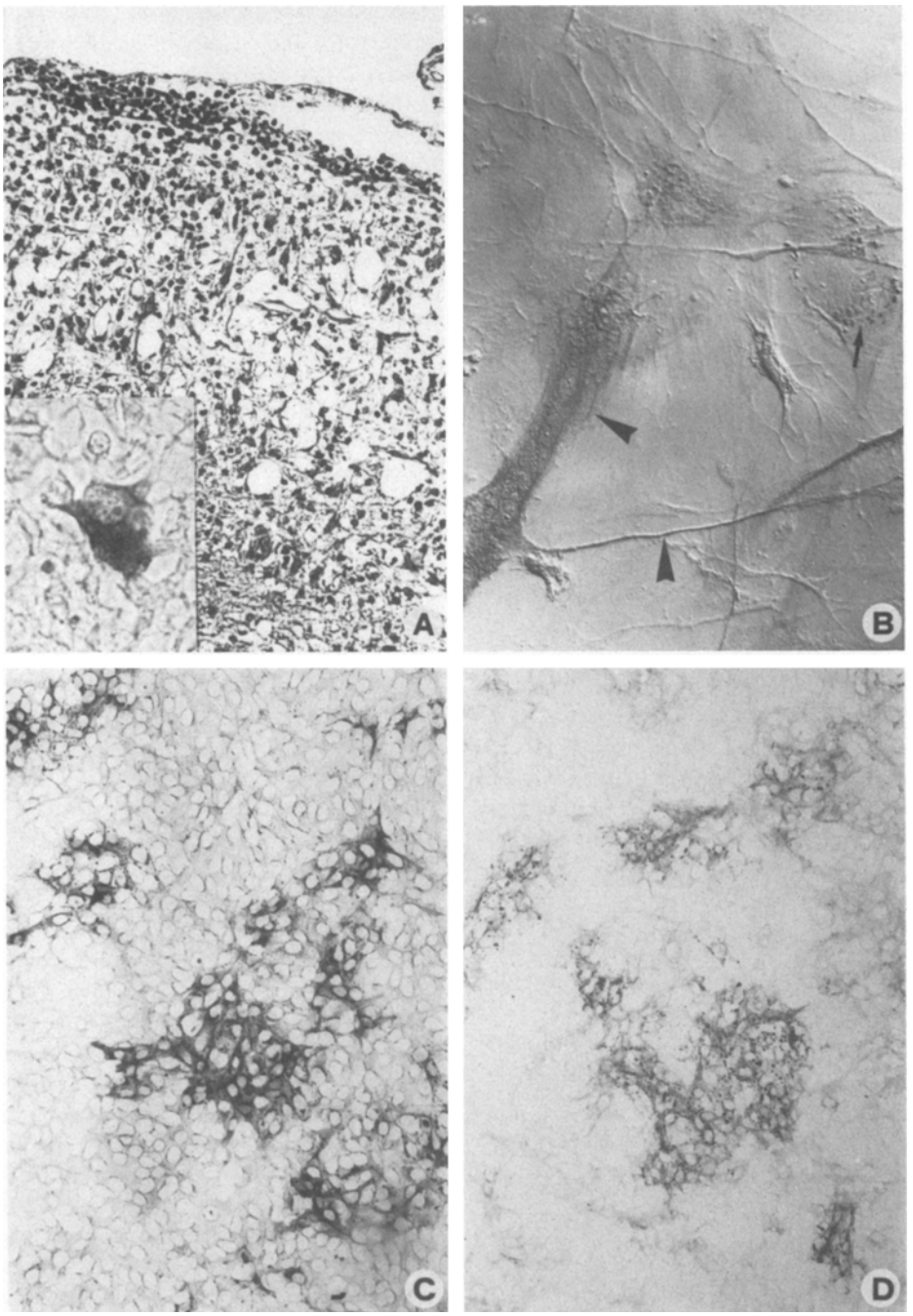


Figure 2. *A* Necrotizing white matter lesion with tissue vacuolation and infiltration with inflammatory cells in a dog after infection with virulent A75/17-CDV, ×250, HE. Inset: CDV antigen in a single glial cell is demonstrated with mAB D110-PAP, ×750. *B* Dog glial cell culture infected with avirulent OP-CDV and stained 5 days p.i. with mAB L1 – PAP. Infected glial cell with process (arrow head) and inclusion bodies (arrow) are positive, ×400. *C, D* Vero cells infected with A75/17-CDV at passage 13 and stained with rabbit anti-CDV (*C*) and mAB L1 (*D*). Many small cell clusters contain CDV antigen. Note that there is no apparent difference in the number of virus-containing cells, ×400.

p.i. On post mortem examination, both dogs had a marked interstitial pneumonia, thymic atrophy and numerous inclusion bodies in the intestinal mucosa and urinary bladder. One dog had mild demyelinating lesions and focal glial nodules in the brain; the other dog had a severe multifocal demyelinating encephalitis (fig. 2A). CDV-antigen was demonstrated immunocytochemically in the affected tissues. The two dogs that had received passaged A75/17-CDV developed a mild transient lymphopenia one week p.i. and remained otherwise healthy during the whole observation period. At post mortem examination, no significant lesions were found. Viral antigen was not found in sections of representative tissues.

*Discussion*

We describe here a mAB, named L1, that distinguishes between virulent and attenuated CDV. We further show that the epitope recognized by this mAB is acquired after repeated passaging of virulent CDV through Vero cell cultures, and that the acquisition of this epitope coincides with loss of virulence.

Loss of virulence is a frequently-observed phenomenon after virulent viruses have been adapted to cell lines, but its mechanism is poorly understood. It appears that certain sites in the CDV genome, responsible for the loss of or the regaining of virulence are preferred sites of mutation, requiring only a very limited number of passages in an appropriate system to change in either direction [3, 15]. It is possible that such rapid changes occur on the basis of selection pressure by the host system favouring certain mutations. Considering the high mutation rate of RNA viruses [16], the alternative explanation would be the continuous presence of a number of mutants within a given virus population, with the host system favouring certain variants; loss of or return to virulence would then be a reflection of fluctuations in the numerical distribution of the different variants in the population.

It has also been proposed that adaptation can, for example, be induced by integration of host-specific cell membrane elements in the envelope of viruses replicating in a non-permissive system [3].

Virulence has been related to structural features of the viral envelope proteins [17]. Changes in the surface glycoproteins of CDV, namely of the H or F proteins, which are responsible for adhesion and fusion [18], could presumably lead to altered virulence. The reverse process, the selection of escape mutants with neutralizing mABs, to obtain attenuated virus, has been successfully performed in various viral systems [19 - 21]. However, mAB L1 binds to the NP protein of CDV. Therefore, it is possible that the observed change in the NP of CDV, coinciding with adaptation, is an epiphenomenon which is not directly responsible for the changes in pathogenicity. On the other hand, we cannot exclude the possibility that an epitope in the NP protein could influence virulence. For example, a change could lead to an altered assembly of the nucleocapsid protein, or variation in the attachment of the nucleocapsid via the matrix protein to the future envelope prior to budding of the virus from the cell membrane. Cloning and sequencing of the epitope recognized by mAB L1 will be performed in the future, and may lead to further explanation of the observed phenomena.

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## The broken axis approach – a new way to analyze bi-directional circular data

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*Abstract.* A new technique is demonstrated that allows detection of bi-directional asymmetric modes in circular data. The method makes it possible to search for and find the best possible description of data sets that are distributed on a circle. It was primarily developed for analysis of avian orientation data, but it is equally well suited for circular data in general. Critical levels of statistically significant deviations from uniformity, according to analyses by the new technique, are provided.

*Key words.* Circular distributions; bi-directional data; asymmetrical modes; orientation; bird migration.