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ORIGINAL ARTICLE

DNA vaccines encoding proteins from wild-type and attenuated canine distemper virus protect equally well against wild-type virus challenge

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Abstract Immunity induced by DNA vaccines containing the hemagglutinin (H) and nucleoprotein (N) genes of wild-type and attenuated canine distemper virus (CDV) was investigated in mink (*Mustela vison*), a highly susceptible natural host of CDV. All DNA-immunized mink seroconverted, and significant levels of virus-neutralizing (VN) antibodies were present on the day of challenge with wild-type CDV. The DNA vaccines also primed the cell-mediated memory responses, as indicated by an early increase in the number of interferon-gamma (IFN-γ)-producing lymphocytes after challenge. Importantly, the wild-type and attenuated CDV DNA vaccines had a long-term protective effect against wild-type CDV challenge. The vaccine-induced immunity induced by the H and N genes from wild-type CDV and those from attenuated CDV was comparable. Because these two DNA vaccines were shown to protect equally well against wild-type virus challenge, it is suggested that the genetic/antigenic heterogeneity between vaccine strains and contemporary wild-type strains are unlikely to cause vaccine failure.

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Introduction

Canine distemper virus (CDV) is a member of the genus *Morbillivirus*, family *Paramyxoviridae*, that is closely related to measles virus (MeV), which infects humans [1]. These highly virulent contagious viruses cause severe diseases in their respective hosts worldwide, illustrating the need for the development of more efficacious and safer vaccines.

The lymphotropic CDV can cause a systemic, potentially fatal disease associated with severe immunosuppression in a broad range of domestic and wild carnivores including dog, mink and ferrets [2, 3]. Despite the use of attenuated live vaccines, outbreaks of distemper in both vaccinated and unvaccinated individuals have been reported [4–14].

The hemagglutinin (H) protein is the key determinant in viral entry, as it mediates the binding of the virus to the signalling lymphocyte activation molecule receptor (SLAM/CD150) at the surface of susceptible cells and thereby initiates virus infection [15, 16]. A possible reason for vaccine failure may be variation between the H proteins of the CDV strains used in vaccines and those of the currently circulating wild-type strains [9, 10, 17–19].

CDV strains worldwide can be divided into distinct geographically separated subtypes based on the H gene, namely, America-1, America-2, European, Arctic, Asia-1 and Asia-2 [17, 19–23]. The current and widely used CDV vaccines (such as Onderstepoort and Snyder Hill) are based on different attenuated strains isolated before 1960 and belong to the America-1 group [15]. It is uncertain whether the America-1 CDV strains still are circulating in the field, since they have not been seen in the last five decades [24]. The greatest diversity both genetically and antigenically is seen between field CDV isolates and the traditional vaccine

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strains [10, 25–27]. The marked genetic and antigenic variation between wild-type CDV and vaccine CDV strains has been suggested to play a role in vaccine failures in animals immunized with attenuated live vaccine against circulating wild-type CDV [10, 19, 25, 27, 28]. Therefore, it is of high relevance to study the protective effects of DNA vaccines against wild-type CDV.

DNA vaccines have several advantages over attenuated live vaccines: (1) they contain no infectious components, while serious or even fatal infections can occur subsequent to vaccination with live CDV vaccines in some wildlife species [29–32], (2) they stimulate both long-lasting cellular and humoral immune response without any risk of reversion to virulence [33], and (3) they have the ability to induce immunity against morbilliviruses in offspring in the presence of maternal immunity [34–37]. A disadvantage of plasmid-DNA-based vaccines is the inefficient uptake of plasmid by cells because of inefficient delivery. This inefficient uptake results in less antigen production and the humoral immune response is thus limited. The first study of DNA vaccination in a natural host of CDV showed that dogs immunized with plasmids encoding the H, nucleoprotein (N) and fusion (F) genes were protected against severe clinical disease, while only a limited humoral response was induced [38]. The humoral response induces mainly virus-neutralizing (VN) antibodies directed against the H protein. A minor fraction of the VN antibodies are directed against the F protein, and these probably prevent fusion between the viral envelope and the host-cell membrane [39–43].

In earlier studies, it was demonstrated that vaccination with DNA vaccines consisting of the H and N genes from the attenuated CDV Onderstepoort strain conferred solid cross-protection against infection of mink with virulent wild-type CDV strains [44–46]. The studies showed that the vaccine-specific VN antibody and cell-mediated responses that were induced provided robust protection against disease development in both adult and young mink [44–46].

In the present study, a new DNA vaccine, containing plasmids encoding the H, N and F genes from wild-type CDV derived from a distemper outbreak in partly vaccinated dogs in Denmark in 1991 (DK91) [5], was developed. A possible explanation for vaccine failure found during the Danish distemper outbreak could be interference by maternally-derived antibodies [5]. Another explanation could be inappropriate vaccination due to immunization immediately before or after viral exposure.

In this study, the immunogenicity of a novel wild-type DNA vaccine and a DNA vaccine encoding the H and N genes from the attenuated Onderstepoort strain was investigated by challenge inoculation with the same wild-type CDV used to create the wild-type DNA vaccine. The DK91 wild-type CDV strain induces massive lymphocyte-associated viremia, lymphopenia and multisystemic infection in mink [46, 47]. Viremia and lymphopenia are characteristic for virulent CDV infections in its highly susceptible natural hosts [44, 47–49]. Long-term protective immunity was investigated here, as the challenge inoculation was performed 28 weeks after the last immunization with the DNA vaccines.

Materials and methods

Experimental animals

A total of twenty CDV-seronegative female wild-type mink (*Mustela vison)*, 10-12 months of age, were purchased from Østergård Farm (Roskilde, Denmark). The mink had no records of Aleutian mink disease virus or mink enteritis virus. Experimental procedures were in accordance with the requirements of the Danish Animal Care and Ethics Committee.

Viruses

The wild-type CDV strain DK91B (in short, DK91) was derived from a dog with acute fatal distemper during an epidemic in Denmark in 1991 [5]. This wild-type isolate was propagated by passage of organ homogenates three times in mink before use in the present study as challenge inoculum. Spleen homogenates of 10 % or 20 % (w/v) were prepared in RPMI1640, cleared by centrifugation at 1500 × *g* for 10 minutes and frozen in aliquots at −80 °C. The Danish wild-type isolates DK91 and DK91C (from another acute fatal clinical case from the 1991 epidemic in Denmark) were used to produce the wild-type DNA vaccine [5, 50]. In a similar way, CDV-negative spleen homogenates were prepared from uninfected animals. The Onderstepoort strain of CDV was used for virus neutralization (VN) test.

Preparation of DNA vaccines

cDNAs encoding CDV DK91 H, N, and F proteins were subcloned into the eukaryotic expression plasmid vector pVR1012 (Vical, San Diego, CA, USA), which contains the cytomegalovirus (CMV) promoter [51]. To facilitate making these constructs, site-directed mutagenesis was used to introduce SalI sites at the 5' ends and BglII at the 3' ends of the H and F cDNAs, and SalI sites at the 5' ends and XbaI sites at the 3' ends of N cDNA. The resulting plasmids were named pVRCDV-H (1890 bp), pVRCDV-N (1615 bp) and pVRCDV-F (2204 bp). The inserts were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and analyzed in a Prism 310 Genetic Analyzer (Applied Biosystems). For confirmation of expression, pVRCDV-H, pVRCDV-N and pVRCDV-F

were introduced by transfection into Vero cells (SuperFect Transfection Reagent, QIAGEN). Expression of the corresponding CDV proteins was confirmed by using an immunofluorescence assay as described previously [25].

The plasmids pCDV-H and pCDV-N consisted of the expression plasmid vector pVIJ (p) [52] containing the insert of the H gene (1815 bp) or the N gene (1573 bp) of the CDV Onderstepoort strain [44, 46, 53]. The expression from pCDV-H and pCDV-N has been described and confirmed previously [44, 53]. The plasmids were purified using an EndoFree Plasmid Giga Kit according to the supplier's protocol (QIAGEN) with a few modifications [44, 53]. Purified DNA plasmids were dissolved in phosphate-buffered saline to a final concentration of 1 μg/μl.

Administration of DNA vaccines

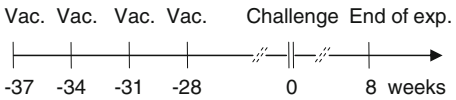
Immunizations were carried out under anaesthesia with ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (1 mg/kg) (Ketaminol Vet. and Narcoxyl Vet., Intervet, Denmark), which were administered intramuscularly. The vaccine dose was 800 μg of each plasmid. One third of the dose was administered intradermally, and the rest, intramuscularly as described previously [44]. Four mink received the pCDV-H and pCDV-N plasmids (Fig. 1). Four received a mixture of pVRCDV-H and pVRCDV-N plasmids, and four received a mixture of pVRCDV-H, pVRCDV-N and pVRCDV-F plasmids. Two were given the empty plasmid vector pVR1012 (pVR), and two did not receive any plasmid (non-vaccinated). The mink were vaccinated four times at three-week intervals (Fig. 1).

Experimental infection

Challenge inoculation was performed 28 weeks after the last immunization. Fourteen mink were inoculated with 1.6×10^5 $TCID_{50}$ per ml of the DK91 wild-type CDV strain homogenate, and three control animals were inoculated with non-infectious spleen homogenate (Fig. 1). Under anaesthesia, 1 ml of the homogenate suspension 10 % (w/v) was dripped into each conjunctiva and nostril and 4 ml of the 20 % (w/v) homogenate was injected intraperitoneally. Two days later, the animals were given a second dose of 1 ml of the 10 % spleen homogenate in the conjunctiva and nostrils. They were then examined daily for clinical signs of disease throughout the challenge period, and finally, eight weeks after challenge, they were anesthetized and then euthanized with barbiturate.

Mink specimens

Blood samples for serum collection and flow cytometric analysis were drawn by puncture of the vena cephalica



Vaccine	Challenge	No. of mink
pCDV-H/-N	+	*n* = 4(3)*
pVRCDV-H/-N	+	*n* = 4
pVRCDV-H/-N/-F	+	*n* = 4(3)*
pVR/non-vaccinated	+	*n* = 4
Control	-	*n* = 3

Fig. 1 Four mink were vaccinated with a mixture of plasmid vectors pVIJ (p) expressing the hemagglutinin (H) and nucleoprotein (N) proteins of the Onderstepoort strain of CDV (pCDV-H/-N). Eight were vaccinated with plasmid vectors pVR1012 (pVR) expressing H, N proteins and with or without fusion (F) protein of the wild-type CDV strain DK91 (pVRCDV-H/-N/-F or pVRCDV-H/-N). *One mink in the pVRCDV-H/ -N/ -F group and one in the pCDV-H/ -N group died unexplainably just before the last vaccination and around five months later, respectively, which reduced the number of animals to three in each of those groups. Two animals received empty pVR, and two were left unvaccinated (pVR/non-vaccinated). The animals were vaccinated four times at three-week intervals and challenged (day 0 and 2) with the wild-type strain DK91 28 weeks after the last vaccination. Three mink were included as uninfected controls

accessoria [54]. Tissues samples were transported on dry ice and stored at −80 °C until two-step RT-PCR were performed.

Virus neutralization (VN) test

VN antibody determinations were performed in Vero cell cultures using the $TCID_{50}$ format microtiter assay and the Vero-cell-adapted Onderstepoort strain as described previously [46]. The titers were calculated by the Reed and Munch method [43].

Two-step RT-PCR assay

Total RNA preparations were isolated using RNA Now (Ozyme, Biogentex, St Quentin Yvelines, Frances) from 80-100 mg homogenised tissues (brain tissue was taken from the cranial and caudal part of cerebrum and cerebellum, mesenteric lymph node, spleen and lung). Two-step RT-PCR reactions were performed as described previously [46]. Briefly, cDNA was synthesised from 1 μg of total RNA using reverse transcriptase and random priming with hexamers (Advantage RT-for-PCR Kit, Clontech, Paulo Alto, CA, USA). PCR was performed with the "universal" primer pair against the phosphoprotein gene first described by Barrett et al. [55]. As a control for the RNA extraction, primers for cellular glyceraldehyde-3-phosphate

dehydrogenase were used. The limit of detection of this assay was 0.03 $TCID_{50}$ in 1 μg of total RNA [46].

Flow cytometric analysis of peripheral blood leukocytes

Flow cytometric analysis was carried out on a FACS Calibur flow cytometer (Becton Dickinson, CA, USA) as described previously [46]. In a forward-scatter-versus-side-scatter diagram, populations of lymphocytes, monocytes and granulocytes were gated and quantified (2×10^3 peripheral blood leukocytes were collected). The absolute leukocyte counts were performed using TruCount quantification beads (Becton Dickinson) [56]. Within the lymphocyte/monocyte gates, 2×10^4 cells were collected for single-cell cytokine production. An additional 1×10^4 cells were collected for detection of intracellular CDV-N antigen. The percentages of cytokine-positive cells were calculated after subtraction of positive signals from isotype-matched immunoglobulin control preparations. A cross-reacting monoclonal antibody to bovine interferon gamma (IFN-γ) (catalogue no. MCA 1783, Serotec, Oxford, United Kingdom) was used [57]. A monoclonal antibody against the CDV-N protein was used for viral antigen detection [43]. A rabbit antibody to mouse IgG $F(ab')_2$ fragments conjugated to fluorescein isothiocyanate (code no. F0313, DakoCytomation) was used as secondary antibody. For statistical evaluations, Student's *t*-test was used. All *P*-values were two-tailed and were considered statistically significant when the associated probability was less than 0.05.

Excluded animals

One control mink exhibited extraordinarily low lymphocyte counts on the day of inoculation with non-infectious organ homogenate. The reason for the low lymphocyte count remained unknown, and the animal was excluded from the trial. Another mink that had received empty plasmid (pVR) had a high lymphocyte count on the day of challenge inoculation and was also excluded.

Results

Virus-neutralizing (VN) antibody responses after DNA immunization

Mink were immunized four times with plasmids encoding the CDV genes of the Onderstepoort strain (pCDV-H/ -N) or with the plasmids encoding CDV genes of the wild-type DK91 strain (pVRCDV-H/ -N/ -F) as summarized in Fig. 1. Already after two immunizations, all animals vaccinated with the pCDV-H and -N had medium levels of serum VN antibodies (average titer: 1.9 $\log_{10}$; Fig. 2). In all DNA-immunized animals, VN antibodies were measured six weeks after the last immunization, corresponding to 22 weeks before challenge (-22w in Fig. 2). The VN antibody titers remained constant without any significant variations until challenge with the wild-type CDV strain DK91. The control animals did not develop any detectable serum VN antibodies during the experimental period (Fig. 2).

Long-term protective effects of DNA immunization in mink

Mink were challenged intraperitoneally and on the conjunctival and nasal mucosa 28 weeks after the last immunization on days 0 and 2 (Fig. 1). The wild-type CDV strain DK91 caused a mild virulent infection. The animals in the pVR/non-vaccinated group became lymphopenic (defined in the legend to Table 1) and viremic (defined here as positive for cell-associated CDV-N antigen) during the first weeks after challenge (Figs. 3, 4; Table 1). Subsequent multisystemic infection developed with the presence of CDV RNA in the lymphoid system (lymph node and spleen), lung, cerebrum and cerebellum (Table 1). Thus, DNA immunization of mink had a long-term protective effect against lymphopenia, viremia and multisystemic infection.

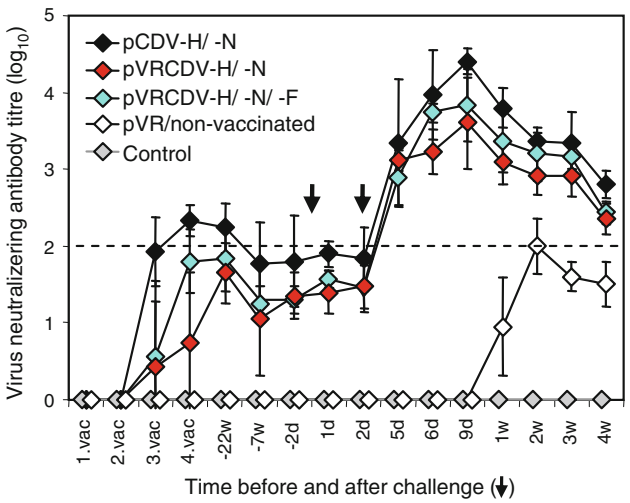


Fig. 2 CDV-specific virus-neutralizing (VN) antibody response induced by DNA vaccination and after challenge (arrows) with the DK91 wild-type strain. Blood samples from vaccinated mink (pCDV-H/ -N: $n = 3$; pVRCDV-H/ -N: $n = 4$; pVRCDV-H/ -N/ -F; $n = 3$), pVR/non-vaccinated mink ($n = 4$) and uninfected controls ($n = 3$) were collected on the indicated days (d) and weeks (w). The horizontal dotted line indicate $\log_{10} = 2$, which equals a neutralizing titer of 100. The values shown are geometric means ± standard deviation. The x-axis is not drawn to scale

Humoral and cellular immune responses after challenge with wild-type CDV

To evaluate the humoral and cellular immune responses after challenge, we measured the titer of VN antibodies and the percentages of IFN-γ-producing lymphocytes in the peripheral blood. The results showed that all of the DNA-vaccinated animals exhibited a memory VN antibody response that was already present five days after challenge (between 1.8- and 2.0-fold increase; Fig. 2). The VN antibody titers remained above 100 until the end of the experiment (Fig. 2). Titers above 100 are considered indicative of protection [49, 58]. Serum VN antibodies in pVR/non-vaccinated mink were not detectable until two weeks after challenge, and the average serum VN antibody titers remained below or at 100 (Fig. 2).

The DNA vaccines were also found to prime the cell-mediated memory responses, as an early increase in the number of IFN-γ-producing lymphocytes was measured by flow cytometry (Fig. 5). Interestingly, the early response of IFN-γ-producing lymphocytes found in DNA-vaccinated animals (Fig. 5a, b; Table 1) was statistically significant when compared to the pVR/non-vaccinated group (Fig. 5c and Table 1). In all DNA-vaccinated animals, we detected an average of 10 % or more IFN-γ-producing lymphocytes on days 6 and 9 after challenge, while less than 3 % IFN-γ producing lymphocytes were found in the pVR/non-vaccinated group (Fig. 5 and Table 1). In the pVR-/non-vaccinated animals, levels of IFN-γ producing lymphocytes comparable to the ones found in the immunized animals appeared much later, on days 21 and 28 after challenge (Fig. 5c and Table 1). Less than 1 % IFN-γ-producing lymphocytes were found in the pVR/non-vaccinated mink on day 6 after challenge, at the time when the animals were lymphopenic and viremic. Uninfected control animals were included to estimate the background level of IFN-γ-producing lymphocytes. The average background level was 4.0 % to 9.5 % IFN-γ-producing lymphocytes throughout the experimental period (Fig. 5d and Table 1).

Discussion

The aim of the present study was to investigate the immunogenic and protective effect of DNA vaccines based on different CDV strains against canine distemper in a natural host. CDV strains isolated from vaccinated dogs with CDV-related disease have been found to be distinguishable from

Table 1 Long-term protective capacity of the DNA vaccines against CDV

Group	Lymphopenia[a] after challenge						Viremia[b] after challenge					CDV RNA in[c]					IFN-γ[d] after challenge					
	6d	9d	2w	3w	4w	8w	6d	9d	2w	3w	4w	Lymph node	Spleen	Lung	Cerebrum	Cerebellum	0d	6d	9d	2w	3w	4w
pCDV-H/ -N	□	□	□	□	□	□	□□□	□□□	□□□	□□□	□□□	0/3	0/3	0/3	0/3	0/3	■	■	■	■	■	■
pVRCDV-H/ -N	□	□	□	□	□	□	□□□□	□□□□	□□□□	□□□□	□□□□	0/4	0/4	0/4	0/4	0/4	■	■	■	■	■	■
pVRCDV-H/ -N/ -F	□*	□	□	□	□	□	- - -	- - -	- - -	- - -	- - -	0/3	0/3	0/3	0/3	0/3	-	-	-	-	-	-
pVR/non-vaccinated	■	■	□	□	□	□	■■■	■■■	▨▨▨	□□□	□□□	4/4	4/4	3/4	2/4	1/4	■	□	■	■	■	■
Control	□	□	□	□	□	□	□□□	□□□	□□□	□□□	□□□	0/3	0/3	0/3	0/2	0/2	■	■	■	■	■	■

Data are expressed as number of animals testing positive/number tested. Box symbols are explained below. "–", not tested

The mink were challenge 28 weeks after the last immunization and euthanized 8 weeks after challenge with the wild-type DK91 strain. Overt clinical signs were not observed

[a] Lymphocytes were quantitated by flow cytometry at days 0, 6 and 9 and weeks 2, 3, 4 and 8 after CDV challenge. Each box represents the average counts for the group of mink. Grey boxes indicate a >2.2-fold decrease in lymphocyte count when compared to the pre-inoculation counts (day 0). White boxes represent a ≤2.2-fold decrease. *One mink vaccinated with pVRCDV-H, -N, -F had transient lymphopenia similar to that observed in the pVR/non-vaccinated group

[b] The presence of viral N-antigen in the peripheral blood lymphocytes was analyzed by flow cytometry at days 0, 6 and 9 and weeks 2, 3 and 4 after challenge. Black boxes indicate ≥20 % positive lymphocytes, grey boxes indicate >5 % positive lymphocytes, striped boxes indicate ≤5 % positive lymphocytes, and white boxes indicate that viremia was not detected (defined as less than 3 % positive lymphocytes). Each box represents individual animals

[c] The presence of viral RNA was tested in various tissues post-mortem by fragment amplification of CDV phosphoprotein RNA in a two-step RT-PCR

[d] Percentage of IFN-γ-producing lymphocytes at days 0, 6 and 9 and weeks 2, 3 and 4 after challenge. Black boxes indicate ≥10 % IFN-γ-positive lymphocytes, grey boxes indicate <10 % IFN-γ-positive lymphocytes, and white boxes indicate <1 % IFN-γ-positive lymphocytes. Each box represents the average percentage for each group of animals

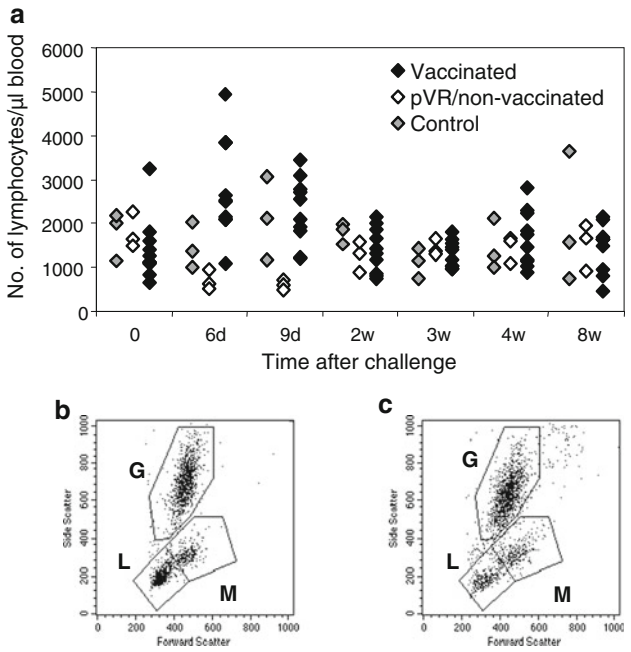


Fig. 3 (**a**) Lymphocyte levels in vaccinated mink (pCDV-H/ -N; $n = 3$: pVRCDV-H/ -N/ -F; $n = 3$: pVRCDV-H/ -N; $n = 4$), pVR/non-vaccinated mink ($n = 3$) and uninfected controls ($n = 3$) were measured by flow cytometry on the indicated days (d) and weeks (w). Since the lymphocyte counts varied, the lymphocyte counts from the individual animals were compared to their pre-inoculated counts on day 0 as illustrated in Table 1. (**b**) Forward-scatter-versus-side-scatter dot plot of the lymphocyte (L), monocyte (M) and granulocyte (G) populations from a mink with a normal lymphocyte count at day 0 and (**c**) with a transient drop in the lymphocyte count on day 6 after challenge

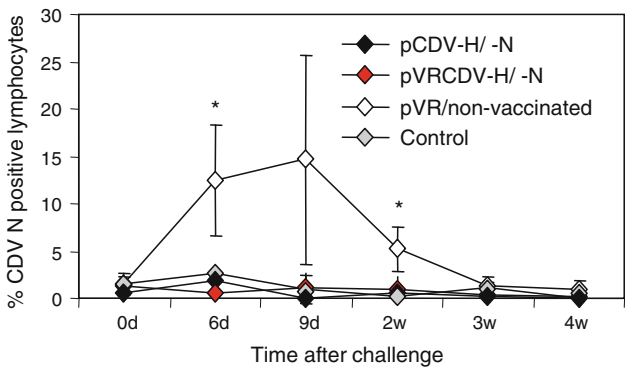


Fig. 4 CDV-N protein detection by flow cytometry in peripheral blood lymphocytes of vaccinated (pCDV-H/ -N; $n = 3$: pVRCDV-H/ -N; $n = 4$) or pVR/non-vaccinated mink ($n = 4$) on the indicated days (d) and weeks (w) after challenge with the DK91 strain. Uninfected controls were included ($n = 3$). Viral antigen quantification was performed on gated lymphocyte populations in forward-scatter-versus-side-scatter dot plots (as illustrated in Fig. 3). * indicates a significant difference between the percentage of CDV-N-positive lymphocytes from pCDV-H/-N- and pVRCDV-H/-N-vaccinated mink and those receiving empty plasmids (pVR) or unvaccinated mink

the currently used vaccine strains [4–6, 8, 11, 12, 14, 24]. The heterogeneity found among CDV vaccine strains and wild-type CDV strains have been speculated to be responsible for the incomplete protection of the vaccinated animals [5, 19, 59].

To assess whether a wild-type DNA vaccine (pVRCDV-H/ -N/ -F) is more efficacious against wild-type CDV than a DNA vaccine based on an attenuated strain (pCDV-H/ -N), the humoral and cellular responses were evaluated in mink. We found that both DNA vaccines were able to prime immunological memory responses. Even though the animals vaccinated with the wild-type plasmids (pVRCDV-H/-N/-F) did not produce an antibody response above 100 prior to challenge, all of the immunized animals, with a single exception, were solidly protected against lymphocyte-associated viremia, multisystemic infection and lymphopenia. One mink that was vaccinated with pVRCDV-H/ -N/ -F developed transient lymphopenia. Importantly, the vaccine regimens tested in the present study induced a comparable protective immunity measured as VN antibodies and IFN-producing lymphocytes against wild-type CDV. It can still not be ruled out that the sequence variations between the H genes of vaccine and circulating wild-type strains can contribute to inadequate immunity in some vaccinated animals.

Our study indicates that both VN antibodies and cell-mediated memory responses contribute to protection against CDV. The relative contribution of VN antibodies and cell-mediated memory responses to protection remains unclear. However, to achieve solid disease protection against CDV and MeV, both cell-mediated and VN antibody responses must be induced by the vaccines [35, 36, 38, 53]. An advantage of the DNA vaccines is that there is no risk of vaccine-induced disease from residual virulence, which must be considered in the case of attenuated live vaccines, as illustrated by the formerly used vaccine based on the Rockborn strain [31]. The Rockborn vaccine was used globally from 1962 to the mid-1990s, when it was withdrawn as a consequence of several reported suspected cases of severe vaccine-induced disease (e.g., post-vaccinal encephalitis) in dogs who had received the less-attenuated vaccine [31].

In the current study, 10- to 12-month-old mink were immunized four times with different combinations of plasmids encoding the H, N and F genes from CDV. The DNA vaccines were administered by the intradermal and intramuscular routes, as this combination had previously been found to induce higher levels of serum antibodies than the intramuscular route alone [44, 46]. By combined intradermal and intramuscular injections, both Langerhans cells and myocytes can be primed. Furthermore, this allows a sufficient volume of plasmids to be injected, since only a relatively small volume of DNA vaccine can be administered

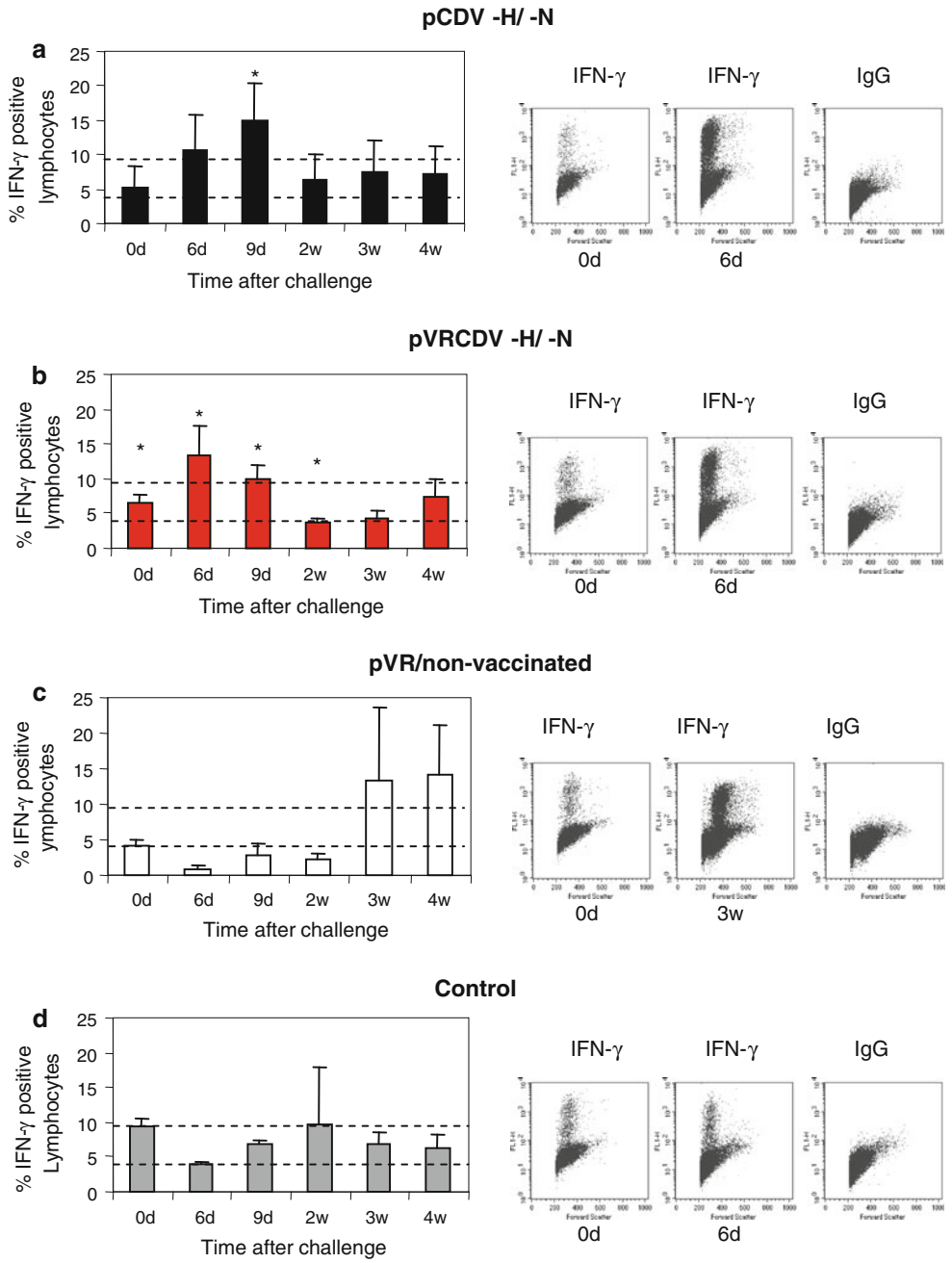


Fig. 5 IFN-γ-producing lymphocytes from vaccinated mink with pCDV-H/ -N ($n = 3$) (**a**) or with pVRCDV-H/ -N ($n = 4$) (**b**), from mink receiving empty plasmids (pVR) or non-vaccinated mink ($n = 4$) (**c**), and from uninfected control mink ($n = 3$) (**d**) on the indicated days (d) and weeks (w) after challenge. Corresponding IFN-γ and IgG antibody staining profiles of cells within a combined lymphocyte/monocyte gate of an individual mink are included. The lymphocyte/monocyte gates were identical to the one shown in Fig. 3. The horizontal dotted lines indicate the average background level of IFN-γ-producing lymphocytes estimated from the control mink. * indicates a significant difference between the percentage of IFN-γ-producing lymphocytes from pCDV-H/-N- and pVRCDV-H/-N-vaccinated mink and those receiving empty plasmids (pVR) or unvaccinated mink

intradermally. This study showed a strong antibody-based memory response in the DNA-vaccinated mink. Already after two immunizations, all of the animals vaccinated with the pCDV-H and pCDV-N plasmids had medium levels of serum VN antibodies. In contrast, animals receiving the wild-type DNA vaccines had only a limited level of serum VN antibodies. The nucleotide sequences of the open reading frames (ORF) of the DK91 H, N and F genes are 92.1 %, 93.9 % and 91.3 % identical, respectively, to those of the corresponding ORFs of the Onderstepoort vaccine strain. These sequence differences may affect the neutralization titer obtained, as the Vero-cell-adapted Onderstepoort

vaccine virus was used in the VN antibody assay [28, 60]. Therefore, in further studies, it would be preferable to test serum samples for neutralizing activity against the homologous wild-type virus from which the relevant vaccine is generated to clarify whether the low antibody levels might be due to an antigenic difference between the CDV strains. However, in the present study we were able to shown that both DNA vaccines induced a solid neutralizing antibody response in mink after challenge. Moreover, a previous *in vitro* study has shown that the sequence difference between wild-type and vaccine strains did not have any significant influence on the cross-reactivity of CDV-positive sera in neutralizing assays [9].

The wild-type DNA plasmids (pVRCDV-H/-N/ -F) were designed to contain the H, N or F genes (starting 12, 12 and 86 nucleotides upstream of the start codon and ending 55, 31 and 135 nucleotides downstream of the stop codon, respectively), while only the ORFs of Onderstepoort H and N were inserted into the plasmids (pCDV-H/ -N). Both expression plasmids contained the cytomegalovirus (CMV) promoter. The extra Kozak nucleotides in the pVRCDV-H/-N/ -F may have an influence on the expression of the proteins. For DNA vaccines, it is essential to prime antigen-presenting cells to present the antigens on both MHC I and MHC II to T cells. In addition to the ability of the plasmids to express the antigen *in vivo*, other factors such as post-translational modification can have an influence on the presentation of the antigen on the antigen-presenting cells [61].

The F gene was included in the DNA vaccine with the wild-type strain to assess if it induced a stronger overall immune response. In the present study, the VN antibody levels of the two groups of mink receiving pVRCDV-H/ -N or pVRCDV-H/-N/-F were found to be comparable, without any significant differences. In future studies, it will be interesting to evaluate the contribution of F plasmids to the cell-mediated memory response. In this study, immunization with plasmids encoding the H and N genes primed a cell-mediated memory response, as shown by an early increase in the percentage of IFN-γ-producing lymphocytes.

We observed no overt clinical signs of distemper, which is in contrast to a previous experiment in 6-month-younger mink infected with the wild-type CDV strain DK91 [46]. The reason for this lack of clinical symptoms is most likely the different doses of challenge material used (a tenfold lower challenge dose was used in the present study), although the lack of clinical symptoms could also be attributed to the age of the animals, as young animals are more susceptible to severe disease, than older ones [58, 62–65]. However, the extent of lymphopenia and lymphocyte-associated CDV N antigen found in the pVR/non-vaccinated group corresponds to data obtained in our previous studies [46, 47]. Our finding of a protective immune response in DNA-vaccinated mink against challenge with a virulent strain of CDV encourages further studies. It is important to test the DNA vaccines further against a challenge that induces overt clinical signs in unvaccinated animals.

In summary, these results demonstrated induction of VN antibody and cell-mediated (IFN-γ) immune responses in DNA-vaccinated mink compared to pVR/non-vaccinated mink after challenge with wild-type CDV. An immunological memory response was observed in all of the DNA-immunized animals, and the vaccine-induced immunity provided long-term protection against CDV challenge. Our results showed cross-protection between DNA vaccines based on the vaccine strain against wild-type CDV challenge. The protective capacity was comparable with that induced by the wild-type vaccine. However, the heterogeneity between the currently circulating wild-type and vaccine strains must still be considered in future vaccine strategies against CDV to ensure cross-protection. One approach to minimize potential vaccine failures due to genetic and antigenic differences between attenuated strains used to generate the DNA-vaccines and circulating virulent strains of CDV is to immunize with DNA vaccines containing both the H and more-conserved internal antigens. Immunization with the conserved internal antigens N or matrix protein seems to ensure a broader cross-protection against influenza viruses, which undergo antigenic shift and drift [66].

In perspective, the continuous outbreaks of distemper and the ongoing measles outbreaks in Europe and Africa illustrate the need for the development of new, improved vaccines for prophylaxis, and in the case of measles, eventual eradication [67]. The relatively large amount of plasmid DNA and multiple injections used in this study to induce immunity compared to the existing licensed modified live-virus vaccines must be minimized before the DNA vaccines can be used commercially. However, we believe that our finding of a long-term protective effect against wild-type challenge encourages further studies on DNA immunization to reduce the dose of DNA and the number of vaccinations required. One approach to improve the immune response could include immunostimulatory factors like cytokines. Co-administration of a DNA vaccine encoding the MeV H, F and N proteins with an IL-2 molecular adjuvant has been shown to increase the antibody response and the cell-mediated immune response and enhance the level of protection against viremia in newborn macaques [36].

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Conflict of interest The authors declare that they have no conflict of interest.

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