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Development of a Challenge-Protective Vaccine Concept by Modification of the Viral RNA-Dependent RNA Polymerase of Canine Distemper Virus▽

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We demonstrate that insertion of the open reading frame of enhanced green fluorescent protein (EGFP) into the coding sequence for the second hinge region of the viral L (large) protein (RNA-dependent RNA polymerase) attenuates a wild-type canine distemper virus. Moreover, we show that single intranasal immunization with this recombinant virus provides significant protection against challenge with the virulent parental virus. Protection against wild-type challenge was gained either after recovery of cellular immunity postimmunization or after development of neutralizing antibodies. Insertion of EGFP seems to result in overattenuation of the virus, while our previous experiments demonstrated that the insertion of an epitope tag into a similar position did not affect L protein function. Thus, a desirable level of attenuation could be reached by manipulating the length of the insert (in the second hinge region of the L protein), providing additional tools for optimization of controlled attenuation. This strategy for controlled attenuation may be useful for a "quick response" in vaccine development against well-known and "new" viral infections and could be combined efficiently with other strategies of vaccine development and delivery systems.

Canine distemper virus (CDV) is an enveloped negative-strand RNA virus which, along with measles virus (MV) and rinderpest virus (RPV), belongs to the *Morbillivirus* genus, a serologically closely related genus of the *Paramyxoviridae* family of the order *Mononegavirales*. Morbilliviruses have a significant global impact on both human and animal health, and although CDV primarily infects canines, infections of other terrestrial and aquatic carnivores have been reported (13, 33). Mortality rates due to CDV infection vary among susceptible species (1) and range from 0% for domestic cats (22) to 100% for ferrets (54, 56). For domestic dogs, lethality rates are approximately 50% (18), although some outbreaks in African wild dogs led to mortality rates of up to 95% (52).

In natural hosts, such as ferrets or dogs, CDV infection can represent a very useful model for the study of general morbillivirus pathogenesis and immunogenesis. These are therefore generally considered to be better models than modified/adapted MV in rodent model systems, in which systemic infection is severely restricted (27). Because ferrets are exquisitely susceptible to CDV, they also provide a useful means to assess protection after vaccination by subsequent challenge with a wild-type pathogenic virus. Ferrets are popular domestic pets, but current CDV vaccines, which were developed specifically for dogs, are often insufficiently attenuated for ferrets and can cause symptomatic and sometimes fatal infections after administration (56). This could be due to the specific way in which viruses have been attenuated for vaccine preparation. For example, for the common CDV vaccine strain Onderstepoort (CDV^{OP}), attenuation involved 57 passages of wild-type virus in ferrets, 208 passages in chicken embryos, 62 to 66 passages on chicken embryo cell cultures, 13 to 14 passages on ferret kidney cell cultures, and finally, more than 100 passages on Vero cells (6, 31). It is probable that during passage, multiple mutations in different genes occurred, which might make the vaccine inefficacious or even dangerous for different species, particularly some exotic animals (35), although the precise molecular determinants which are responsible for the underattenuation in ferrets are unknown (2). It is generally considered that the development of species-specific CDV vaccines is cost-prohibitive (11). Therefore, an alternative strategy of controlled attenuation theoretically suitable for several species is desirable.

The CDV genome is 15,690 nucleotides (nt) in length and consists of a short, 52-nt leader region followed by six genes, encoding the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins, which are separated by 3-nt intergenic regions and concluded by a short 38-nt trailer region (44). As in the other morbilliviruses, nonstructural V and C proteins are encoded within the P gene. The C protein is expressed from an overlapping reading frame, while V is expressed by cotranscriptional RNA editing (3, 8). In many members of the subfamily *Paramyxovirinae*, hexamers of 6 nt are likely to be associated with the N protein (29). The comprehensive structure of these ribonucleoprotein (RNP) complexes is still not known, but it has been suggested that the N protein of vesicular stomatitis virus binds to the sugar-phosphate backbone of the viral RNA, exposing the bases to the outside. Thus, the RNAs of the members of the order *Mononegavirales* could be transcribed without dissociation from the nucleoproteins (23). The P and L proteins form the RNA-dependent RNA polymerase (RdRp) complex, which is incorporated in all progeny virions. Neither the genomic nor antigenomic RNA of a paramyxovirus can be translated as mRNA.

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Therefore, the proteins involved in transcription and replication have to be provided in *trans* to generate recombinant viruses by reverse genetics (19, 42).

Conceptually, the simplest and easiest means to attenuate a virus such as CDV would be to introduce mutations into the receptor-binding, cleavage, and/or fusion-related sites in the H and F glycoprotein genes. However, mutations in these sites could potentially affect vaccine efficacy, as they may encode highly protective B-cell epitopes. Furthermore, the outcomes of changes in receptor usage are unpredictable. Currently, the tendency is to develop more rational approaches to attenuation or to use "vectored" vaccines. For example, canarypox virus was developed as a safe carrier for mammals and has been used to elicit protective immune responses by efficient delivery of F and H glycoproteins of MV (51) and CDV (38, 57) to the mucosal immune system. Furthermore, a single immunization with a live vaccinia virus-based vaccine carrying the F and H glycoprotein genes of RPV was partially (60%) protective against wild-type CDV challenge (25). Nevertheless, live attenuated viruses still provide the best and most versatile immune protection against a virulent virus, and hence, the controlled attenuation of viruses is a desirable approach for the development of novel vaccines.

Sequence analysis of morbillivirus L proteins revealed two regions with high sequence variability, termed hinges, between three more-conserved regions, termed domains (34, 41). Previously, it was possible to introduce the open reading frame (ORF) of enhanced green fluorescent protein (EGFP) within the second hinge (H2) region of the L genes of both MV and RPV. The modified RdRp was significantly less efficient in replicating and transcribing viral RNA (40), and in cattle infections, wild-type RPV was shown to be attenuated (5, 14). Delays in virus spread within the host due to inefficiencies in the replication machinery may allow time for the immune system to develop a response, and this in turn may contribute to attenuation under conditions in which the major antigens are not altered, providing protection against the wild-type virus. We demonstrate here that insertion of the EGFP ORF into the H2 region of the CDV L protein gene also attenuates a wild-type virus, showing the general applicability of this approach. Moreover, we show that immunization with this recombinant virus also provides partial protection from challenge with the virulent parental virus.

Thus, the objective of this work is to be a proof-of-concept study regarding the development of a challenge-protective vaccine by controlled attenuation of virus via modification of the L protein.

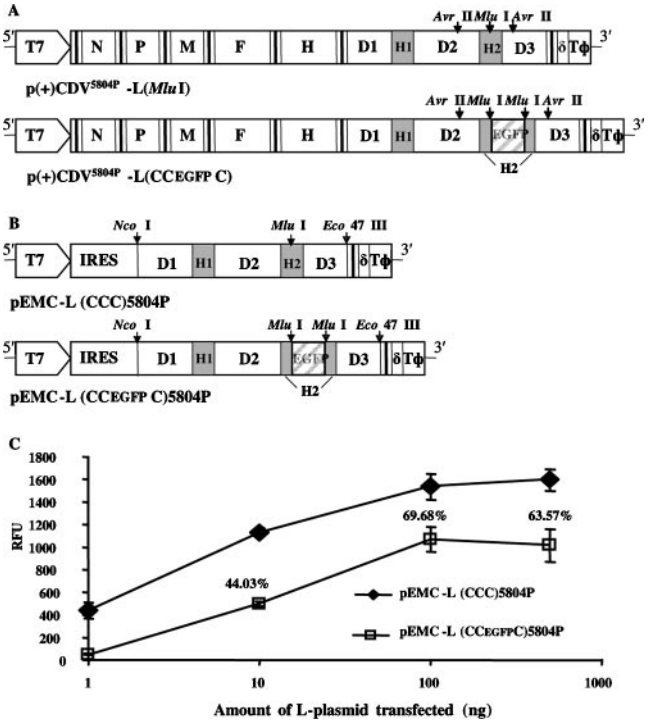

FIG. 1. Construction of recombinant viruses. The positions of the T7 promoter (T7), the internal ribosome entry site (IRES), three domains of the RdRp (D1 to D3), the hinge regions of the RdRp (H1 and H2), the hepatitis delta ribozyme (δ), and the T7 terminator (Tφ) are indicated (not to scale). Bold lines indicate the positions of the intergenic trinucleotide spacers. (A) Schematic representation of rCDV^{5804P} and rCDV^{5804P}-L(CC$_{EGFP}$C) full-length genome plasmids. Three conservative domains and two hinge regions of the L protein are shown. The EGFP ORF was cloned into the MluI site. (B) Schematic representation of plasmids expressing rCDV^{5804P} and rCDV^{5804P}-L(CC$_{EGFP}$C) L proteins. (C) Relative activities of unmodified and EGFP-modified CDV^{5804P} polymerases. Various amounts of pEMC-L(CCC)5804P (diamonds) and pEMC-L(CC$_{EGFP}$C)5804P (squares) were used in minigenome transfection assays. The amount of DsRed2 produced was determined by flow cytometry and expressed in relative fluorescence units (RFU). Percentages represent the relative activities of the modified RdRp compared to the unmodified form of the protein.

MATERIALS AND METHODS

Cells and viruses. Vero cells stably expressing the CDV receptor dog signaling lymphocyte activation molecule (SLAM) (54) (VeroDogSLAM cells) were grown in minimum essential medium (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen) under selective pressure of 0.1 mg/ml Zeocin (Invitrogen). Primary ferret peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with Histopaque 1077 (Sigma) and were grown in RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 50 μM β-mercaptoethanol. The full-length plasmid encoding the recombinant CDV^{5804P} antigenome was kindly provided by V. von Messling (INRS, Institut Armand-Frappier, Montreal, Canada). This plasmid was modified by introducing an MluI restriction site into the second hinge region of the gene encoding the L protein, followed by insertion of the EGFP ORF (see below). Recombinant wild-type CDV^{5804P} (rCDV^{5804P}) and EGFP-modified rCDV^{5804P}-L(CC$_{EGFP}$C) were rescued by standard reverse genetic techniques (19, 20). Viruses were passaged three times on VeroDogSLAM cells. Vaccinia virus Ankara (MVA-T7), used in the recovery of the recombinant CDVs, was grown in chicken embryo fibroblasts as described previously (15).

Plasmid construction. A 926-bp fragment (bp 13467 to 14392) spanning the second hinge region of the L protein gene between two AvrII restriction sites (Fig. 1A) was amplified by a two-step overlapping PCR, with incorporation of an MluI restriction site into the second hinge region (bp 14151 to 14156). The fragment was ligated into a full-length CDV^{5804P} antigenomic plasmid encoding the 5804P ferret-adapted virulent strain of CDV (54) after digestion with AvrII to generate clone p(+)CDV^{5804P}-L(MluI). The 720-bp nucleotide sequence encoding EGFP, without its stop codon, was amplified using primers containing MluI restriction sites (lowercase) (priEGFPMluIF [5′-AAAacgcgtATGGTGAG CAAGGGCGAGGAG-3′] and priEGFPMluIR [5′-AAAacgcgtcgcCTTGTAC AGCTCGTCCAT-3′]) and was cloned into the MluI site in the second hinge region of the L gene of p(+)CDV^{5804P}-L(MluI) (Fig. 1A). Eukaryotic expression plasmids pEMC-L(CCC)5804P and pEMC-L(CC$_{EGFP}$C)5804P were generated by cloning of PCR-amplified sequences encoding L(CCC)5804P and L(CC$_{EGFP}$CC)5804P from appropriate full-length plasmids into pEMCassette (14), using NcoI and Eco47III (Fig. 1B). All plasmids were sequenced using a Perkin-Elmer ABI

Prism 310 genetic analyzer, and their integrity was confirmed by PCR and restriction enzyme digestion.

Assessment of polymerase function. The ability of the pEMC-L(CCC)5804P and pEMC-L(CCEGFPC)5804P plasmids to express functional L proteins was determined in a standard MV minigenome replication/transcription assay (42), using a negative-sense minigenome plasmid expressing the red fluorescent protein DsRed2 [p(−)MVDIDsRed2], kindly provided by L. Rennick (The Queen's University of Belfast, Northern Ireland, United Kingdom). The minigenome plasmid, along with supporting plasmids expressing the N protein (pEMC-N) and the P protein (pEMC-P) of CDV (19) and either the unmodified [pEMC-L(CCC)5804P] or modified [pEMC-L(CCEGFPC)5804P] L protein to be tested, was transfected into MVA-T7-infected VeroDogSLAM cells in a manner similar to that described previously (46). At 3 days posttransfection, cells expressing DsRed2 were analyzed by flow cytometry using a CyAn ADP flow cytometer (Dako). Cells transfected without the minigenome or L-encoding plasmids were analyzed using identical settings to establish background fluorescence and to gate the appropriate region in consequent runs of DsRed2-expressing cells.

Growth analysis. VeroDogSLAM cells, cultured to 80% confluence in six-well plates, were infected at a multiplicity of infection (MOI) of 0.01 for 1 h. The inoculum was removed at 1 h postinfection, and samples were taken from the supernatant or from cells collected by scraping the monolayer for cell-associated virus. Further samples were taken at 12, 24, 36, 48, 60, and 72 h postinfection, and supernatants and cells were stored at −70°C. Virus titers were determined by infection of VeroDogSLAM cells in quadruplicate with appropriate dilutions of the virus preparations and were expressed as 50% tissue culture infective doses ($TCID_{50}$)/ml, using the Reed-Muench algorithm.

SDS-PAGE and immunoblotting. VeroDogSLAM cells were infected at an MOI of 0.01 with $rCDV^{5804P}$-L(CCEGFPC) and lysed at 3 days postinfection (dpi). Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions, and immunoblots were developed with anti-EGFP polyclonal antibodies (1/100 dilution; BD) according to standard protocols.

RNA preparation and RT-PCR. Total RNAs were prepared from virus-infected VeroDogSLAM cells and PBMCs by using TRIzol reagent (Invitrogen) and were used as templates for reverse transcription-PCR (RT-PCR) (one-step RT-PCR kit; Qiagen) with specific primers spanning the H2 region of the L gene (priCDV5804PAvrIIF [5′-GAGCCTAGGTTATTTACTGTG-3′] and priCDV 5804PAvrIIR [5′-TCTCCTAGGAATAAACCGTGT-3′]). Controls in which the RT step was omitted were used to confirm that amplicons were not produced from contaminating plasmid DNA, and β-actin housekeeping gene primers were used to verify the integrity of the total RNA.

Confocal scanning laser microscopy. VeroDogSLAM cells or primary ferret PBMCs were grown on 13-mm glass coverslips in 35-mm petri dishes to 80 to 90% confluence. Cells were infected with $rCDV^{5804P}$-L(CCEGFPC) at an MOI of 0.01. The nuclei were counterstained with propidium iodide (100 ng/ml), and the coverslips were mounted on slides with Citifluor (Amersham). Cells were fixed in 4% (wt/vol) paraformaldehyde, and EGFP autofluorescence was examined by microscopy as described previously (16).

Assessment of virulence of $rCDV^{5804P}$ and $rCDV^{5804P}$-L(CCEGFPC). Fifteen 4- to 5-month-old male European unvaccinated ferrets (*Mustela putoris furo*) were used in animal experimentation under an approved Home Office license. Animals were obtained from Harlan United Kingdom, Ltd., and were fed commercial pelleted food concentrates. Food and water were supplied ad libitum. Before use, all animals were checked for the absence of anti-CDV antibodies by enzyme-linked immunosorbent assay (ELISA) and a neutralization assay to ensure the similarity of their immune statuses. Animals were infected intranasally with 10^4 $TCID_{50}$ of $rCDV^{5804P}$-L(CCEGFPC), a recombinant Onderstepoort large-plaque variant of CDV ($rCDV^{OP}$), or $rCDV^{5804P}$. Rectal temperatures were recorded daily, and body weights were measured weekly. Animals were examined daily for clinical symptoms of CDV infection. Animals were bled on the day of infection and weekly thereafter. Nasal, conjunctival, anal, and preputial swabs were collected, frozen, and then thawed, and doubly diluted samples were added to VeroDogSLAM cells for virus detection. A challenge experiment with the $rCDV^{5804P}$ ferret-adapted wild-type strain was carried out 6 weeks after the primary immunization infection. The systemic humoral immune response after infection was evaluated by ELISA and by neutralization assays, while the cellular immunity status was evaluated by a PBMC proliferation assay and delayed-type hypersensitivity (DTH) reactions with keyhole limpet hemocyanin (KLH). At the end point, animals were euthanized and gross and histopathology investigations were performed.

Blood sample analysis. Blood was collected in heparinized collection tubes (BD Vacutainer). Blood (10 μl) was added to ammonium chloride-kalium (ACK) lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.001 mM EDTA; 190 μl), and the total white blood cell (WBC) count was determined using a hemocytometer. Blood smears were prepared and stained with Giemsa stain (Sigma), and differential lymphocyte counts were made. Due to the wide normal range, lymphopenia was evaluated as a sustained decrease of lymphocyte counts rather than a low absolute count value. Blood samples (0.5 ml) were centrifuged, and sera were collected and stored at −20°C for further analysis. The remainder of the blood was layered onto Histopaque 1077, and gradients were centrifuged to separate the PBMCs. Half of the PBMCs were utilized for proliferation assays with a cell proliferation ELISA bromodeoxyuridine (colorimetric) kit (Roche) according to the manufacturer's instructions. The ratio of levels of bromodeoxyuridine incorporation between phytohemagglutinin (PHA)-stimulated and nonstimulated PBMCs was evaluated as a marker of potential cellular proliferation responses. The use of this relative colorimetric index compensates for differences in the numbers of cells in samples. One-quarter of the PBMCs were used for determination of cell-associated viremia by an infectious center assay, applying serially diluted PBMCs onto VeroDogSLAM cells. Syncytium formation revealed the presence of virus in the samples. The remainder of the PBMCs were stored at −70°C.

Virus neutralization assay and ELISA. For serum anti-CDV antibody assessments, double dilutions of sera were preincubated with 10^3 $TCID_{50}$ of $rCDV^{5804P}$ for 1 hour and then added to 10^5 VeroDogSLAM cells in 96-well plates. The reciprocal value of the maximal dilution of serum that protected cells from infection was taken as the neutralizing antibody titer. For ELISA, 96-well plates (Nunc) were coated with $rCDV^{5804P}$-containing supernatant (10^3 $TCID_{50}$/well) in 50 mM carbonate buffer for 1 hour and then blocked in 5% (wt/vol) skim milk (0.3 ml). Serum samples were diluted 1:50, added to wells in triplicate, incubated for 1 hour, and washed. A horseradish peroxidase-labeled goat anti-ferret immunoglobulin G (IgG) serum (1/2,000 dilution; Bethyl) was added to detect anti-CDV IgG in the ferret sera. After extensive washing and addition of 3,3′,5,5′-tetramethylbenzidine (TMB) One Solution substrate (Promega), the reaction was read at 650 nm, with reference at 450 nm, in a Labsystems Multiscan 352 ELISA reader according to the manufacturer's instructions, and the level of serum anti-CDV IgG was recorded in relative arbitrary units based on the absorbance of processed substrate in the wells.

DTH assay. KLH (Sigma) and incomplete Freund's adjuvant (Sigma) were used to develop a DTH assay. Animals were immunized intramuscularly twice with KLH (0.25 ml) mixed with incomplete Freund's adjuvant (0.25 ml) at fortnightly intervals. Two weeks after the second injection, KLH (0.05 ml) was injected intradermally into the skin of the abdomen to determine basic levels of DTH. The diameter of infiltrate at the site of injection was measured and recorded 48 h after injection. Assays were performed weekly starting on the day of virus infection.

RESULTS

EGFP-modified L protein has affected functionality in vitro. The CDV^{5804P} L protein is a 2,184-amino-acid, multifunctional polypeptide. Two L protein-expressing plasmids (Fig. 1B) were constructed on the basis of the earlier reported pEMCassette (14), into which an Eco47III site was introduced, which did not lead to amino acid changes. However, pEMCassette was designed for the expression of the MV polymerase, and hence the last 15 nucleotides of the L ORF were derived from the MV L protein such that the CDV L protein expressed from these constructs is 2,183 amino acids long and has two amino acid differences. This minor modification was earlier shown to have no significant effect on RPV RdRp activity (5). The relative functionality of the modified wild-type pEMC-L(CCC)5804P and EGFP-containing pEMC-L(CCEGFPC)5804P polymerase-encoding plasmids (Fig. 1B) was examined by assessing DsRed2 reporter gene expression in an MV minigenome assay. The MV-based minigenome assay was used because the corresponding CDV plasmids have demonstrated unacceptably high levels of intrinsic activity, probably due to the presence of cryptic promoters (4). Omission of L-encoding plasmids provided a negative control, which defined the background levels of DsRed2 signal in the fluorescence-activated cell sorter anal-

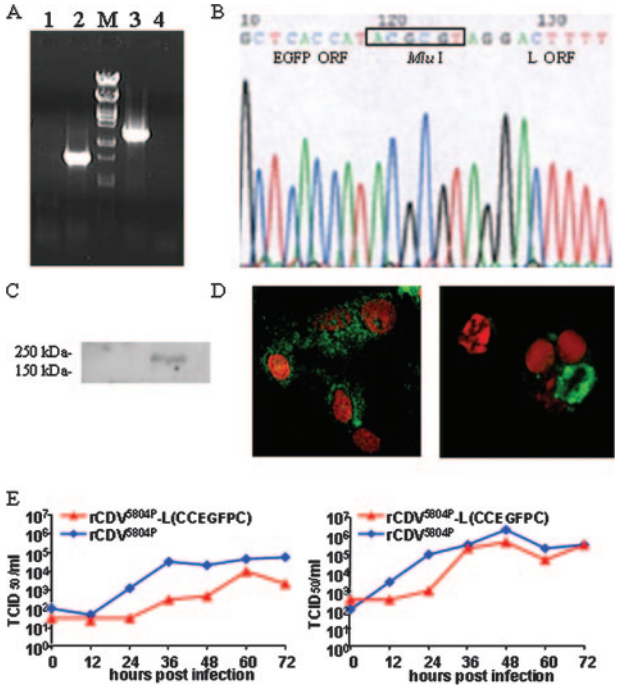

FIG. 2. Rescue of recombinant viruses. (A) RT-PCR products generated with primers spanning the MluI site in the L protein gene. Lanes: 1, no-RT control with RNA extracted from CDV^{5804P}-infected cells; 2, RNA extracted from CDV^{5804P}-infected cells (926-bp product); M, DNA marker (λ DNA digested with PstI); 3, RNA extracted from rCDV^{5804P}-L(CCEGFPC)-infected cells (1,646-bp product); 4, no-RT control with RNA extracted from rCDV^{5804P}-L(CCEGFPC)-infected cells. (B) Sequence analysis of RT-PCR product from lane 4 in panel A indicating that the EGFP ORF is inserted in frame and in the correct orientation. (C) Western blot developed with polyclonal anti-GFP antibodies. SDS-PAGE was carried out under reducing conditions. Left lane, VeroDogSLAM cell lysate; right lane, lysate of VeroDogSLAM cells infected with rCDV^{5804P}-L(CCEGFPC). (D) Autofluorescence of rCDV^{5804P}-L(CCEGFPC)-infected VeroDogSLAM cells (left) and ferret lymphocytes (right). The nuclei were stained with propidium iodide, and the signal in the green channel was produced by autofluorescence of the L protein. (E) Growth curves of viruses on VeroDogSLAM cells. Cells were infected at an MOI of 0.01. Viral titers in the supernatant (left panel) and cell-associated viral titers (right panel) are shown.

ysis. Both L-encoding plasmids were added in various amounts (1 to 500 ng), and minigenome rescue experiments were performed in triplicate. The modified wild-type pEMC-L(CCC) 5804P- and EGFP-containing pEMC-L(CCEGFPC)5804P-encoded polymerase proteins were found to possess dose-dependent specific polymerase function. The latter protein had decreased activity levels of between 60 and 30% dependent on the respective concentrations of the plasmids (Fig. 1C).

Characterization of recombinant viruses. Reverse genetics using standard protocols was used to generate live recombinant viruses (20). The identities of rCDV^{5804P} and rCDV^{5804P}-L(CCEGFPC) were confirmed by RT-PCR (Fig. 2A) and sequencing (Fig. 2B). Western blot analysis with anti-GFP antibodies confirmed the presence of EGFP epitopes in a high-molecular-weight protein (Fig. 2C). The functionality of the EGFP was revealed by examination of rCDV^{5804P}-L(CCEGFPC)-infected VeroDogSLAM cells and primary ferret lymphocytes by confocal microscopy (Fig. 2D). Growth curves of cell-associated and supernatant viruses demonstrated that both rCDV^{5804P} and rCDV5804P-L(CCEGFPC) were produced in similar amounts in vitro (Fig. 2E). However, EGFP-modified virus was delayed in growth by 12 h for cell-associated virus and by at least 24 h for supernatant virus release. rCDV^{5804P}-L(CCEGFPC) could grow in primary ferret lymphocytes as well as in VeroDogSLAM cells. This demonstrated its potential to multiply in lymphocytes that are relevant to in vivo infection.

Clinical and pathological assessment of primary infection in ferrets. All animals infected with rCDV^{5804P} developed the full range of clinical signs, with rash onset at 9 to 11 dpi. Animals were euthanized at terminal stages of disease at 15 to 18 dpi (Fig. 3E). No clinical signs beyond moderate transient lymphopenia (Fig. 4C and E) were observed in all animals infected with rCDV^{5804P}-L(CCEGFPC) and in the control group infected with rCDV^{OP}. Body temperatures were within normal physiological limits for the groups of ferrets infected with rCDV^{5804P}-L(CCEGFPC) and rCDV^{OP} (data not shown). All rCDV^{5804P}-infected animals (no. 12, 19, 20, and 21) developed hyperthermia, and a drop of temperature was observed in two cases (animals 12 and 20) at the terminal stage of the disease (Fig. 3F). Two other moribund ferrets (animals 19 and 21) were euthanized before the temperature drop for ethical reasons. All animals of this group had distemper symptoms, i.e., anorexia, hyperthermia, rash (Fig. 3C), lymphopenia (Fig. 4B), rhinitis, and conjunctivitis (Fig. 3D). Mild diarrhea was also observed in two animals (no. 12 and 20). All animals showed a decrease in body weight by 13 dpi (Fig. 3A), while the animals in the rCDV^{5804P}-L(CCEGFPC)-infected group still gained weight (Fig. 3B). Autopsy revealed bronchopneumonia (Fig. 3I and J), cystitis, liver degeneration of both infiltration and decomposition origins, blood hypovolemia, and neutrophilia, which caused an increase in WBC count in spite of the general lymphopenia (Fig. 4A). Moribund animals had an altered structure of lymphoid tissue in the spleen, with clear degeneration of white pulp (Fig. 3K).

Clinical and pathological assessment of a virulent CDV challenge. The virulent rCDV^{5804P} infection developed differently in the group vaccinated with rCDV^{5804P}-L(CCEGFPC) (animals 10, 11, 13, 15, 16, and 24) from that in the group with primary infection with rCDV^{5804P}. After challenge with rCDV^{5804P}, only one ferret (no. 13) of six developed the full complex of distemper symptoms, and this animal was euthanized at 22 days postchallenge (Fig. 3G). However, the onset of disease was delayed (Fig. 3G and H), clinical signs were less severe (rash and ocular discharge were less prominent), and the survival time was longer than that for animals infected with rCDV^{5804P}. Four more animals had transient rash, two animals had transient lymphopenia (Fig. 4D) but not lymphoid depletion in the spleen (Fig. 3L), and one had no clinical signs at all. Individual body temperature curves for animals challenged with rCDV^{5804P} after primary infection with rCDV^{5804P}-L(CCEGFPC) showed hyperthermia with a terminal drop of body temperature for ferret 13 (Fig. 3H). The control group vaccinated with rCDV^{OP} developed no clinical signs of infection after challenge with rCDV^{5804P}, apart from a lymphopenia (lymphocyte count drop more than twice) in ferrets 6 and 18 (Fig. 4F).

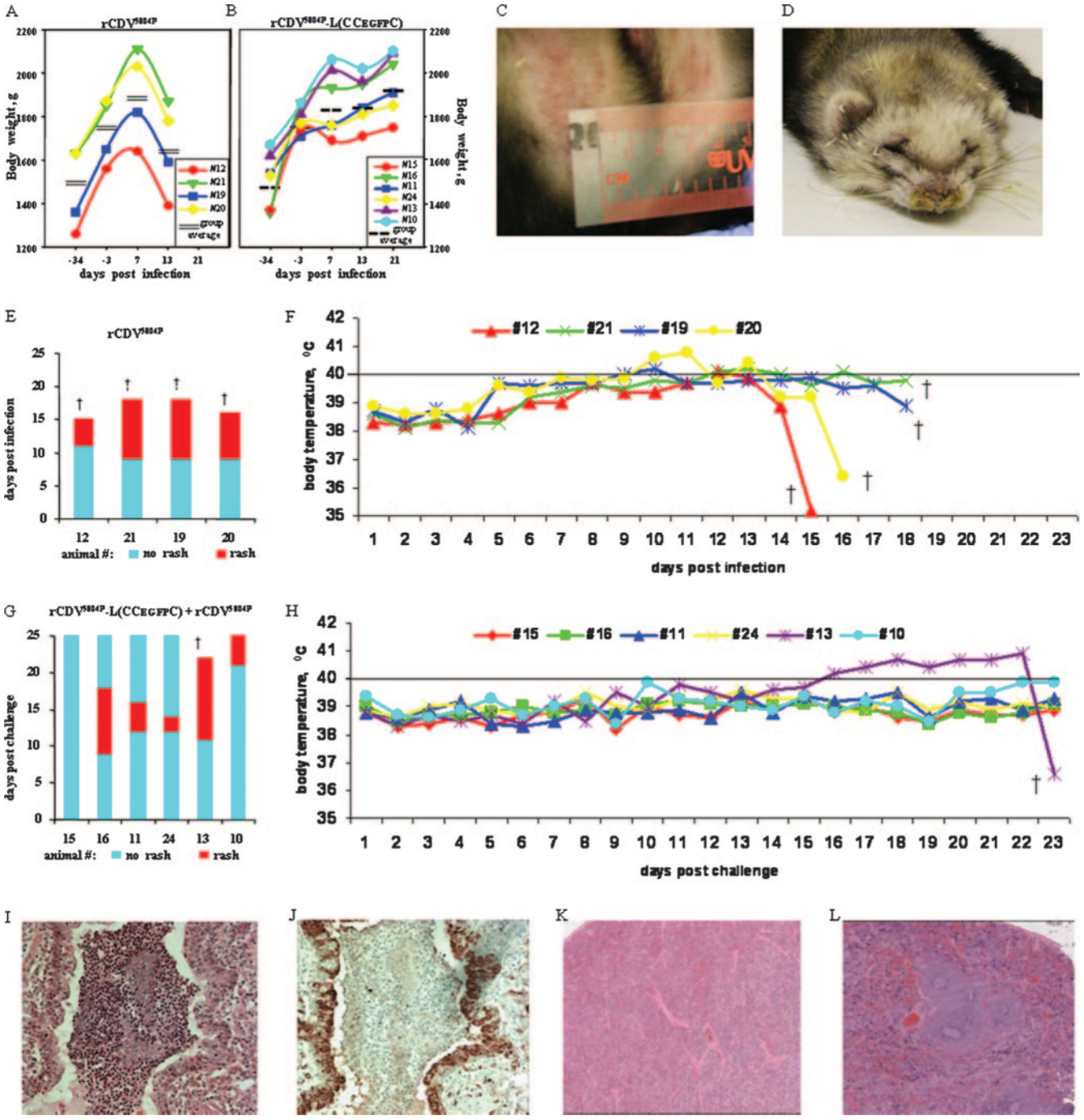

FIG. 3. Disease development in animals infected with rCDV^{5804P} only (A, C to F, and I to K) or infected with rCDV^{5804P}-L(CCEGFPC) (B) and then challenged with rCDV^{5804P} (G and H). (A and B) Ferret body weight dynamics after primary infection. (C) Abdominal skin rash. (D) Mucopurulent nasal and ocular discharge. (E and G) Individual data on rash onset, recovery, and terminal stage of disease. (F and H) Individual body temperature curves postinfection (F) and postchallenge (H). †, ferrets were severely moribund and were euthanized. (I and J) Histopathology of lung tissue showing acute purulent bronchopneumonia. Hematoxylin-eosin staining (I) and immunohistochemical staining with monoclonal antibodies (VMRD, Inc.) against the CDV N protein (J) were performed. (K and L) Histology structure of spleens from animals infected with rCDV^{5804P} (K) or infected with rCDV^{5804P}-L(CCEGFPC) and then challenged with rCDV^{5804P} (L). Note the lymphoid depletion and absence of lymphoid follicles in the spleen of the moribund animal.

Immunological assessment of vaccinated ferrets. DTH assays demonstrated that nonspecific cellular immunity was suppressed transiently after infection with rCDV^{5804P}-L(CCEGFPC), while suppression was sooner, affected all animals, and lasted until death after primary infection with rCDV^{5804P} (Fig. 5A and B). Infection with the latter also led to suppression of the ability of PBMCs to proliferate after PHA stimulation. In groups infected with rCDV^{OP} and rCDV^{5804P}-L(CCEGFPC),

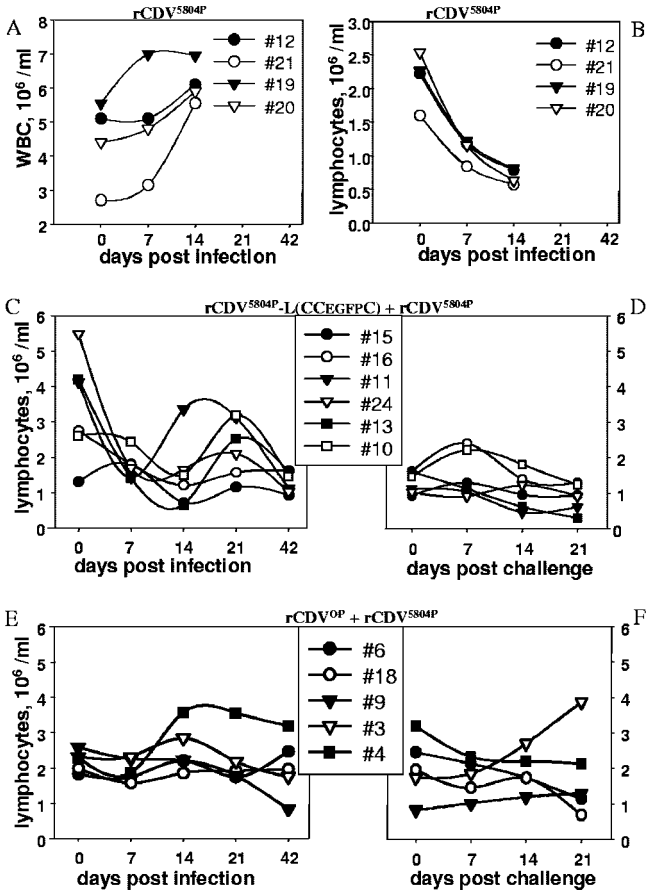

FIG. 4. Hematological changes during infection. (A) WBC counts in peripheral blood. The curves show individual data for the group primarily infected with rCDV^{5804P}. (B to F) Differential counts of lymphocytes. The curves show individual data for the group primarily infected with rCDV^{5804P} (B) and the groups infected with rCDV^{5804P}-L(CCEGFPC) (C) and rCDV^{OP} (E) and then challenged with rCDV^{5804P} (D and F, respectively). Legends display animal numbers.

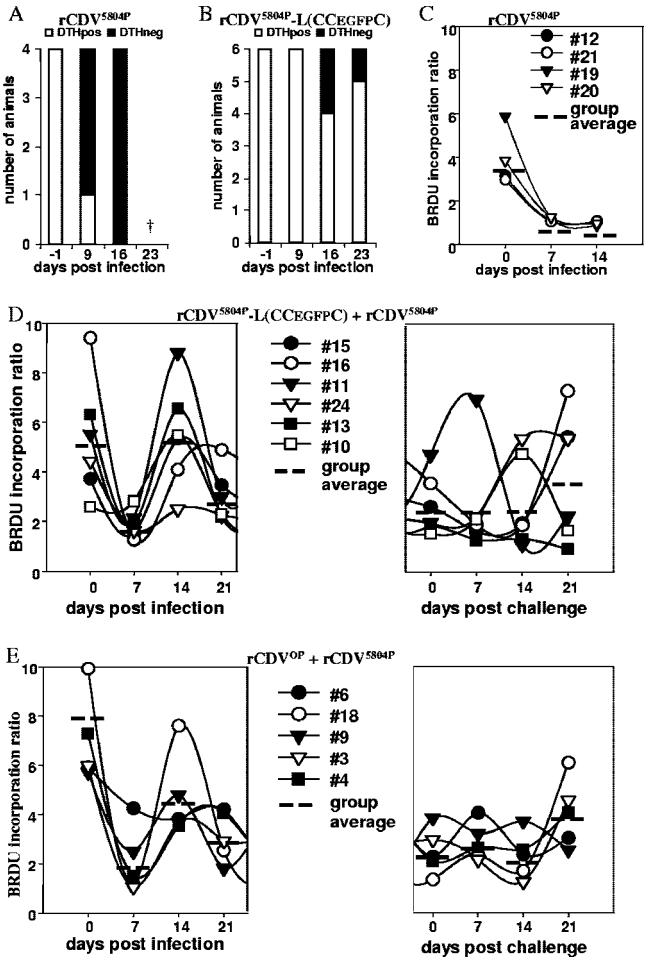

FIG. 5. Nonspecific cellular immunity assessment. DTH reaction profiles of animals infected with rCDV^{5804P} (A) or rCDV^{5804P}-L(CCEGFPC) (B) are shown. Proliferation potencies of PHA-stimulated PBMCs from individual animals in the group primarily infected with rCDV^{5804P} (C) and in the groups infected with rCDV^{5804P}-L(CCEGFPC) (D) and rCDV^{OP} (E) and then challenged with rCDV^{5804P} are also shown. Legends display animal numbers. Group averages are shown by bold horizontal lines. BRDU, bromodeoxyuridine.

most animals recovered cellular immunity, as measured by proliferation capacity (Fig. 5D and E) or DTH reactivity (all animals of the above groups but animal no. 13 developed a DTH reaction, although weak, probably due to a desensitization effect [data not shown]) on the day of challenge. Some animals in these groups developed a specific humoral immune response to CDV, as detected by ELISA and virus neutralization assay (Fig. 6A and B).

All of the animals in the control group infected with rCDV^{OP}, except for ferret 9, had measurable levels of neutralizing antibodies on the day of challenge (Fig. 6B). Ferret 9 demonstrated significant potency for a cellular immune response in the DTH assay and the proliferative response assay (Fig. 5E), and none of the animals demonstrated clinical signs of disease after rCDV^{5804P} challenge. Ferrets 11, 15, and 16 in the group previously infected with rCDV^{5804P}-L(CCEGFPC) showed a cellular immune response by DTH and proliferation assays on the day of challenge. While this potential to proliferate was suppressed on the day of challenge in animals 24, 13, and 10 (Fig. 5D), ferret 24 had a high (>400) titer of neutralizing antibody (Fig. 6A) and animals 24 and 10 showed positive DTH reactions on the day of challenge and recovered cellular immunity before the day of rash onset (day 14) (Fig. 5D). Only one animal (no. 13) had suppressed cellular immunity from the day of challenge until the day of rash onset (Fig. 5D) and did not developed a specific humoral immune response (Fig. 6A).

Correlation between immune status of animals and challenge outcomes. Two groups of ferrets previously immunized with attenuated CDV strains [rCDV^{5804P}-L(CCEGFPC) and rCDV^{OP}] were challenged with fully virulent rCDV^{5804P}, and similar infection conditions (batch of virus, dose, route, and technique of administration) to those used for ferrets infected with rCDV^{5804P} were applied. In the group immunized with rCDV^{OP}, only two ferrets (animals 3 and 4) seemed to be protected from challenge by both humoral and cellular immunity (Table 1). They had developed neutralizing antibodies, had restored cellular immunity at the time of challenge, and did not develop clinical or pathological signs of disease. Interestingly, only these two animals from both immunized

FIG. 6. Development of specific anti-CDV neutralizing antibodies. Plaque neutralization titers for individual animals infected with rCDV^{5804P}-L(CCEGFPC) (A) and rCDV^{OP} (B) followed by challenge with rCDV^{5804P} are shown as reciprocal dilutions.

groups registered PBMC-associated viruses at day 7 postimmunization. Ferret 6 acquired protection, albeit with a significantly lower titer of neutralizing antibodies, and developed minor lung lesions, which were partially recovered at the end of the experiment. Despite the loss of PBMC proliferation activity, ferret 18 developed neutralizing antibodies, and signs of recovered lung lesions were registered during its autopsy. However, ferret 9 did not develop a humoral immune response to CDV, but its PBMC proliferation activity was maximal at the day of challenge, and no clinical or pathological signs of distemper were detected in this animal.

In the group immunized with rCDV^{5804P}-L(CCEGFPC), one ferret (animal 13) developed the full clinicopathological complex of distemper, although with a clear delay of 4 to 6 days in comparison to nonimmunized animals (Table 1), while the other five ferrets in the same group had only transient hyperthermia (1 of 5 animals), rash (4 of 5 animals), and lymphopenia (2 of 5 animals) (Table 1). Ferret 13 did not develop neutralizing antibodies before clinical signs were manifested, its PBMC proliferation activity was very low on the day of challenge, and it was DTH negative. Ferret 10 developed a weak and blurred symptom complex of CDV infection. It had not developed humoral protection at the time of challenge. However, cellular immunity was active, as it demonstrated a

TABLE 1. Clinical and pathological outcomes for ferrets in the challenge experiment

Primary infection and ferret no.	Challenge virus	Severity of clinical sign (after primary infection [first group] or after challenge)[a]				Animal classified as moribund on day:	Severity of pathology findings (at postmortem [first group] or at the end of the exp't)[a]			Immune status indicator (at 2 weeks postinfection [first group] or on the day of challenge)[a]		
		Hyperthermia	Rash/ erythema	Purulent discharge	Lymphopenia		Lung lesions	Intoxication	Lymphoid depletion	DTH	PBMC proliferation	Neutralizing antibodies
rCDV^{5804P}												
12	None	+++	+++	+++	+++	15	+++	+++	+++	−	−	−
21	None	+++	+++	+++	+++	18	+++	+++	+++	−	−	−
19	None	+++	+++	+++	+++	18	+++	+++	+++	−	−	−
20	None	+++	+++	+++	+++	16	+++	+++	+++	−	−	−
rCDV^{5804P}-L-CC$_{EGFP}$C												
15	rCDV^{5804P}	−	−	−	−	−	−	−	−	+	+	−
16	rCDV^{5804P}	−	+	−	±	−	−	−	−	+	+	−
11	rCDV^{5804P}	−	+	−	+	−	+	−	±	+	+	−
24	rCDV^{5804P}	−	+	−	−	−	−	−	±	−	+	+
13	rCDV^{5804P}	+++	++	++	+++	22	+++	++	+++	−	−	−
10	rCDV^{5804P}	±	+	−	+	−	+	−	+	+	−	±
rCDV^{OP}												
6	rCDV^{5804P}	−	−	−	++	−	+	−	±	+	+	±
18	rCDV^{5804P}	−	−	−	++	−	+	−	±	+	−	+
9	rCDV^{5804P}	−	−	−	±	−	−	−	−	+	+	−
3	rCDV^{5804P}	−	−	−	−	−	−	−	−	+	+	+
4	rCDV^{5804P}	−	−	−	−	−	−	−	−	+	+	+

[a] +++, severe; ++, moderate; +, mild; ±, unclear; −, absent.

clear positive DTH response to KLH on the day of challenge. Two other animals of this group, ferrets 15 and 16, developed a mild symptomatic picture of disease, limited to transient rash and lymphopenia. They did not develop a humoral response to CDV, but the capacity of their cellular immunity was high, as assessed by proliferation and DTH assays. In contrast, ferret 24 had compromised cellular immunity but developed a high titer of neutralizing antibodies, which allowed it to survive challenge with a very mild and transient rash and lymphopenia. Ferret 11 was the only animal with no clinical symptoms and with PBMC-associated viremia on days 7 and 14 postchallenge. It had some rash on days 12 to 16 postchallenge, deep lymphopenia and neutralizing antibodies developed at 14 to 21 days postchallenge, and it survived with minimal lung lesions.

In summary, on the day of challenge, all animals from the group primarily infected with $rCDV^{OP}$ either developed specific humoral immunity against CDV or had measurable potency to cellular immune responses, while for the group primarily infected with $rCDV^{5804P}$-L(CCEGFPC) this was observed in all animals apart from ferret 13, which neither developed antibodies nor recovered cellular immunity. Unsurprisingly, ferret 13 showed clinical signs of disease after CDV^{5804P} challenge and succumbed to the infection (Fig. 3G).

DISCUSSION

It has been shown previously that the second hinge region of the MV and RPV L proteins can tolerate the insertion of EGFP (5, 14). Here we demonstrate that the CDV L protein can also tolerate this insertion and that the conformation of the chimeric protein does not impact the autofluorescent properties of EGFP. Moreover, the RdRp retains both replicase and transcriptase activities when assayed in a minigenome analysis, albeit at reduced levels. The overall activity level of pEMC-L(CCC)5804P and the effect of the insertion of the EGFP ORF into the ORF for the CDV L protein are comparable to the results of minigenome assays with MV or RPV helper plasmids (14). It was possible to rescue a recombinant virus which expresses this chimeric L protein. Moreover, autofluorescence of the L protein in infected cells allowed determination of the dynamics of viral infection in tissue culture as well as the intracellular localization of L protein. The reduced L protein activity correlated with slower virus growth, similar to that observed for analogous MV and RPV mutants (5, 14). Nevertheless, $rCDV^{5804P}$ and $rCDV^{5804P}$-L(CC$_{EGFP}$C) attained similar total titers on VeroDogSLAM cells, albeit at a lower rate. These results illustrate that probably all morbillivirus L proteins can tolerate the insertion of EGFP into the second hinge region. The generation of $rCDV^{5804P}$-L(CC$_{EGFP}$C) provides an opportunity to perform challenge experiments in the ferret as a tractable small-animal model of morbillivirus pathogenesis and also to assess its potential as a live attenuated vaccine.

Modified live attenuated virus strains demonstrate different degrees of safety in susceptible species. High mortality rates have been reported for mink and black-footed ferrets after distemper vaccination (7, 17, 21, 48). Moreover, spontaneous reversion has been demonstrated in "back passages" of vaccine CDV in inoculated ferrets. For example, pulmonary congestion was observed in one of four ferrets immunized with the SOLO-JEC-7 vaccine, which had been "back passed" eight times in ferrets, while ferrets infected with virus from the ninth back passage showed congested spleens with evidence of splenomegaly, suggesting that the vaccine virus became more virulent for ferrets (59). The development of the CDV^{OP} vaccine strain included 57 sequential passages of a wild-type CDV isolate in ferrets and 13 or 14 passages in ferret kidney cells (6), and this might explain the levels of virulence observed when ferrets are immunized using this strain.

Major neutralizing immunological determinants in the H and F glycoproteins of wild-type strains of CDV may be altered in standard approaches to the generation of live attenuated vaccines, as the virus may acquire the ability to utilize other host receptors (50). Controlled attenuation by manipulating the L gene could provide a significant advantage, as the antigenicity of the wild-type H, F, and other viral proteins is fully preserved. Presenting the full repertoire of neutralizing epitopes is very important for viral vaccines, as neutralizing antibodies can have a major effect on disease outcome. Moreover, the spontaneous reverse mutation required to precisely excise the EGFP ORF for reversion to virulence is unlikely to occur.

In many viral infections, the rate of virus replication in the host correlates with virulence (12, 28, 32). Morbilliviruses are no exception (10, 49, 55), and the decreased growth rate of the recombinant virus demonstrated in vitro may affect its ability to spread quickly and may give the host the opportunity to limit and overcome infection by production of a specific adaptive cellular and/or humoral immune response. In analyzing the challenge experiments, it is important to remember that ferrets are outbred, and hence the intrinsic immunological properties of individual ferrets may explain observed variations. The immune response induced even after single primary intranasal infection with rCDV5804P-L(CC$_{EGFP}$C) secured 84% protection against CDV^{5804P} challenge, despite the absence of viremia at 7 dpi. The group of ferrets infected only with virulent $rCDV^{5804P}$ developed disease with the full range of clinical, pathological, and immunosuppressive components. Specifically, all four animals from the group infected intranasally with 10^4 $TCID_{50}$ of $rCDV^{5804P}$ developed hyperthermia, abdominal skin rash, mucopurulent conjunctivitis and rhinitis, anorexia and body weight loss, movement and mood suppression, and bronchopneumonia in all cases with CDV infection of bronchial epithelium, and diarrhea was observed in two cases. Animals of this group had blood hypovolemia, neutrophilia, and increased triglyceride levels in the serum (data not shown). As revealed by histopathology, these animals had fat degeneration of hepatocytes, not only due to lipid infiltration caused by starvation and fat depot mobilization but also due to toxic decomposition (data not shown). In contrast, none of the animals infected with $rCDV^{5804P}$-L(CC$_{EGFP}$C) and $rCDV^{OP}$ developed any of the above symptoms. Reductions in WBC counts after CDV infection of ferrets have been reported earlier (53, 55). However, we did not observe this in our experiments. Data for individual ferrets suggest increases rather than decreases in WBC counts, which could not be explained by dehydration only. Due to the extremely wide range of WBC counts in groups and the high variability in WBC counts in individual ferrets during the acclimatization period, it was not possible to demonstrate statistically significant differences in

WBC counts between groups during infection. Leukocytosis has been documented for CDV-infected dogs (24, 36). However, in common with other morbillivirus infections (26, 30, 37, 39, 43, 47), differential counts of lymphocytes demonstrated lymphopenia after infection with rCDV^{5804P}. Therefore, we used differential lymphocyte counts to evaluate the progression of disease.

In conclusion, the data here indicate that protection against wild-type challenge could be gained either after the reestablishment of cellular immunity postimmunization or after the development of neutralizing antibodies. Sufficient time for recovery after vaccination and boosting of infections with an attenuated virus could significantly improve protection against wild-type virus. The 240-amino-acid insertion of EGFP does seem to overattenuate the virus. Earlier, we showed that a six-amino-acid insertion of the c-Myc epitope tag in the same position in MV did not affect L protein function (14). Hence, a desirable level of attenuation could be reached by manipulating the length of the insert in the second hinge region of the L protein, thus providing additional tools for optimization of controlled attenuation. This strategy for controlled attenuation may be useful for vaccine development against well-known and "new" emerging viral infections (e.g., dolphin morbillivirus and porpoise morbillivirus in cetaceans and phocine distemper virus in pinnipeds [45]). Furthermore, it may be possible to use this approach to attenuate other emerging paramyxoviruses if alignments of L proteins are examined in greater detail and combined with other systems of controlled attenuation (9), such as engineered mutations in genes encoding innate immunity evasion proteins (e.g., NS, V, and C proteins) (55, 58). However, decreasing polymerase activity is potentially a more universal approach, as it relates to intrinsic viral features affecting replication speed rather than to interactions between virus and host. Furthermore, the level of attenuation could theoretically be modulated in our system through controlling the length and type of insert in the L protein.

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