JOURNAL OF VIROLOGY, Dec. 2003, p. 12579–12591 Vol. 77, No. 23
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.23.12579–12591.2003

A Ferret Model of Canine Distemper Virus Virulence and Immunosuppression

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Received 15 May 2003/Accepted 22 August 2003

Canine distemper virus **(CDV) infects many carnivores, including ferrets and dogs, and is the member of the *Morbillivirus* genus most easily amenable to experimentation in a homologous small-animal system. To gain insights into the determinants of CDV pathogenesis, we isolated a strain highly virulent for ferrets by repeated passaging in these animals. Sequence comparison of the genome of this strain with that of its highly attenuated precursor revealed 19 mutations distributed almost evenly in the six genes. We then recovered a virus from a cDNA copy of the virulent CDV strain's consensus sequence by using a modified reverse genetics system based on B cells. We infected ferrets with this virus and showed that it fully retained virulence as measured by the timing of rash appearance, disease onset, and death. Body temperature, leukocyte number, lymphocyte proliferation activity, and cell-associated viremia also had similar kinetics. We then addressed the question of the relative importance of the envelope and other viral constituents for virulence. Viruses in which the envelope genes (matrix, fusion, and hemagglutinin) of the virulent strain were combined with the other genes of the attenuated strain caused severe rash and fever even if the disease onset was delayed. Viruses in which the nucleocapsid, polymerase, and phosphoprotein genes (coding also for the V and C proteins) of the virulent strain were combined with the envelope genes of the attenuated strain caused milder signs of disease. Thus, virulence-inducing mutations have accumulated throughout the genome.**

Measles virus (MV) is endemic in many developing countries (3, 32), and epidemics occur in every human population as soon as the herd immunity drops (1, 20, 39). Annually, MV infects approximately 40 million people and results in nearly 1 million deaths (19, 37). Most often, these deaths are the consequence of a general immunosuppression caused by the MV infection, of which the loss of delayed type hypersensitivity (DTH) response and inhibition of lymphocyte proliferation are diagnostic clinical manifestations (29, 47, 56). This immunosuppression results in increased susceptibility to secondary infections that in turn can have a detrimental effect on the survival of the infected individual (9).

Due to the lack of a small-animal system in which MV pathogenesis is fully recapitulated, the mechanisms of MV-induced immunosuppression are still poorly understood. MV is highly specific for humans, and even closely related nonhuman primates like chimpanzees or macaques develop only a mild rash and transient lymphopenia instead of the full spectrum of disease, including respiratory and gastrointestinal signs (49, 50, 59). There are rodent models available, based on either transgenic mice expressing CD46, one of the MV receptors, with human tissue-like specificity (31, 36) or cotton rats (34), which allow MV replication to a certain degree, but they reproduce only some aspects of MV pathogenesis and virulence.

MV belongs to the *Morbillivirus* genus, which also includes important animal pathogens like rinderpest (RPV) and canine distemper (CDV) viruses. In contrast to RPV, which has to be studied in cattle and is a reportable disease, CDV infects a broad range of terrestic and aquatic carnivores, including ferrets and dogs, and both species have been used to study virus-host interactions in a homologous small-animal model (2, 16, 26), putting CDV in an ideal position to address the mechanisms of *Morbillivirus* pathogenesis and virulence.

All morbilliviruses are highly contagious and are transmitted by aerosol. The initial infection occurs in epithelial cells and lymphoid tissue in the nasopharynx, and primary replication takes place in the lymphatic tissue of the respiratory tract. A transient fever as well as the onset of lymphopenia can be observed between 3 and 6 days after infection, which coincides with the first viremia that results in the infection of all lymphatic tissue (21, 25, 44, 59). The second viremia with high fever follows several days later, leading to infection of epithelial cells throughout the body (10, 35). It is accompanied by the onset of rash and marks the beginning of the symptomatic phase, characterized by serous nasal discharge, conjunctivitis, and anorexia. Gastrointestinal and respiratory signs may follow and are often complicated by secondary bacterial infections. In the case of CDV infections, an acute encephalomyelitis may occur in association with or immediately after the systemic disease depending on the individual strain (8, 60), and hyperceratosis of footpads and epithelium of the nasal plane are often observed.

The ability to recover recombinant morbilliviruses from cDNA has greatly increased the understanding of their biology (5, 17, 41, 55), but translation of in vitro findings into in vivo models has been difficult, since most recombinant systems are based on highly attenuated vaccine strains that cause no signs of disease in their respective hosts. Thus, these viruses are unsuited for the characterization of pathogenesis mechanisms.

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The recovery of a recombinant wild-type MV with retained virulence in cynomolgous monkeys (50) constitutes a first step in this direction, but the difficulties associated with primate experiments make it a challenging model to work with.

This study is based on a pair of CDV strains: the parental strain 5804Han89 (5804) and its derivative, 5804P, which we obtained by passaging the parental strain three times in ferrets and is obligatorily lethal for these animals. The parental strain was adapted to growth in Vero cells and is attenuated. We show that these strains differ in 19 residues, resulting in 12 amino acid exchanges distributed evenly throughout the genome. After generating infectious cDNA clones of both strains we recovered recombinant viruses by using a modified system based on a B-cell line to maintain virulence. Indeed, we proved that virulence of the recombinant viruses in ferrets reproduced that of the respective parental strain. We then tested whether the envelope complex (matrix [M], fusion [F], and hemagglutinin [H] proteins) or the replication complex (nucleocapsid [N], polymerase [L], and phosphoprotein [P] with the nonstructural proteins V and C) were more important for virulence and immunosuppression.

MATERIALS AND METHODS

Viruses. The attenuated wild-type isolate CDV5804 (55) and the virulent CDV strain Snyder Hill (ATCC VR-526) were used in this study. Virus recovery and propagation were performed in the marmoset B-cell-derived cell line B95a (24).

Selection of a CDV strain highly virulent in ferrets. To obtain a ferret-adapted CDV strain, three animals were inoculated intranasally with 10^4 50% tissue culture infectious doses ($TCID_{50}$) of the Vero cell-adapted wild-type strain CDV5804. Only one animal developed signs of disease and was sacrificed when it became moribund. Its liver, spleen, and kidney were homogenated in 10 ml of OptiMem (Invitrogen), and 100 μl of this organ homogenate was used to infect two animals. This process was repeated one more time, pooling the organs of both animals, and the virus isolated by cocultivation of canine peripheral blood mononuclear cells (PBMC) with an organ homogenate of the last-infected group was designated CDV5804P.

Generation of stable cell lines. Vero cells (ATCC CCL-81) constitutively expressing the canine SLAM molecule (VerodogSLAMtag) were generated by selecting Zeocin-resistant clones after transfection with pCGdogSLAMtagZeo, an expression plasmid coding for the canine SLAM molecule and the Zeocin resistance gene connected with an IRES element. To facilitate identification of canine SLAM-expressing clones, an hemagglutinin tag was attached to the N terminus of the protein as described by Tatsuo et al. (51). A high-expressing clone isolated after two steps of limited dilution cloning was used for all experiments. All cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal calf serum (Invitrogen), and VerodogSLAMtag cells were propagated in the presence of 1 mg of Zeocin/ml (Invitrogen).

Generation of full-length cDNAs of the attenuated and virulent CDV strain. Cloning of overlapping fragments covering the entire viral genome was performed as described previously (55). The RNA of the attenuated wild-type strain CDV5804Han89 (CDV5804) was isolated from infected Vero cells and that of its virulent progeny CDV5804P was isolated from infected canine PBMC by using the RNeasy RNA isolation kit (Qiagen). The RNA was reverse transcribed by using Superscript (Invitrogen), and the overlapping fragments were PCR amplified (Expand High Fidelity PCR system; Roche Biochemicals) and cloned into Topo cloning vectors (Invitrogen). At least three clones of each fragment were sequenced (ABI PRISM 377 DNA Sequencer; Perkin-Elmer Applied Biosystems) to define a consensus sequence. Only clones that corresponded exactly to the consensus sequence were used to assemble the full-length cDNA clones.

A new system for recovery of wild-type viruses. The recombinant viruses were recovered by using an MVA-T7-based system (28). B95a cells were infected with MVA-T7 with a multiplicity of infection of 0.8 and were seeded in 6-well plates with a density of 10^6 cells per well. The transfection was carried out by using Lipofectamine 2000 (Invitrogen). Four micrograms of the respective antigenomic plasmid and a set of three plasmids (1 μg of N, 1 μg of P, and 0.5 μg of L protein-expressing plasmids in 10 mM Tris-HCl, pH 8.5), from which the proteins of the CDV polymerase complex are expressed, were diluted in 200 μl of OptiMEM. After 5 min of incubation at room temperature, 200 μl of OptiMEM mixed with 8 μl of Lipofectamine 2000 was added to the solution, followed by vortexing. After incubation for 30 min at room temperature the mixture was added to the cells. The supernatant was removed the next day, and the cells were maintained in DMEM with 10% fetal calf serum. Three days after the transfection, cells of each well were transferred to a 75-cm^2 dish in which VerodogSLAMtag cells were seeded at 60 to 80% confluency. Within 24 to 48 h syncytia were detected, and four were picked for each virus and were transferred onto fresh B95a cells seeded at 30% confluency in 24-well plates. These infected cells were expanded first into 6-well plates and then into 75-cm^2 dishes. When the cytopathic effect was pronounced the cells were scraped into the medium and subjected once to freezing and thawing. The cleared supernatants were used for all further experiments. Virus titers were determined by limited dilution and are expressed as $TCID_{50}$ in VerodogSLAMtag cells.

Animal infection and DTH tests. Unvaccinated male ferrets (Mustela putorius furo) 12 weeks and older (Marshall Farms) were used for all experiments. Prior to infection animals were vaccinated three times in weekly intervals against tetanus with 0.5 of tetanus toxoid (Fort Dodge) intramuscularly and were tested for the presence of antibodies against CDV by neutralization assay and enzyme-linked immunosorbent assay (54). Groups of four animals were infected with 10^4 $TCID_{50}$ of the respective virus intranasally under general anesthesia (10 mg of Telazol/kg of body weight; Fort Dodge). Animals were monitored daily for signs of disease, and the body temperature was recorded. At the time of infection and weekly thereafter, a DTH test was performed by injecting 0.05 ml of tetanus toxoid intradermally in the abdominal region, and 2 ml of blood was collected from the jugular vein and was divided evenly between an EDTA- and a heparin-coated tube (Vacutainer; Becton Dickinson). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

Proliferation activity of PBMC. A small amount of blood was used directly for a white blood cell count (Unopette; Becton Dickinson), and plasma as well as PBMC were isolated from 1 ml of heparinized blood by using Ficoll (Amersham Biosciences) gradient centrifugation. These PBMC were used to determine in vitro proliferation activity with a nonradioactive cell proliferation ELISA (Roche Biochemicals). Cells of each animal were split in two duplicates and were either stimulated with 15 μg of phytohemagglutinin (PHA; Sigma)/ml or left untreated and incubated for 24 h. Then 5-bromo-2′-deoxyuridine (BrdU) was added to a final concentration of 10 μM, and cells were incubated for another 24 h before they were transferred into black 96-well plates, washed, and fixed at 65°C for 1 h. The BrdU that incorporated into the DNA of dividing cells was detected by using a peroxidase-linked anti-BrdU antibody and was revealed with a chemiluminescent substrate. The signal was detected by using a microplate luminescence counter (TopCount NXT; Packard). The proliferation activity was expressed as the ratio between stimulated and nonstimulated cells, allowing for comparison of samples that differ in absolute cell numbers due to the virus-induced leukopenia.

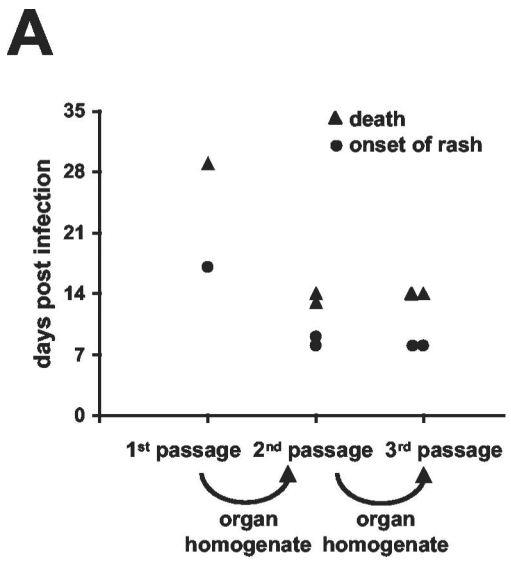
The erythrocytes in the EDTA-treated blood were lysed in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.01 mM EDTA [pH 7.2 to 7.4]). The remaining PBMC were split in two aliquots, one of which was transferred onto VerodogSLAMtag cells and cultivated in the presence of 3 μg of Concanavalin A (Sigma)/ml to detect cell-associated viremia. RNA was isolated from the remaining cells and was stored at −70°C.

Grading of clinical signs. The protocol extended for 5 weeks after the infection. A grading system was established to evaluate the severity of clinical signs and to determine time points for the removal of animals from the study. Animals that failed to eat for more than 48 h experienced weight loss of 15 to 20%, became severely dehydrated, developed central nervous system signs (head pressing, circling behavior, paralysis, seizure), displayed any other important reduction in functional status (severe pneumonia and/or diarrhea), or became moribund before the end of the protocol were euthanized for humane purposes with an overdose of pentobarbital (Nembutal; Abbott Laboratories).

Nucleotide sequence accession numbers. The sequences of CDV5804 and CDV5804P have been deposited in GenBank under accession numbers AY386315 and AY386316, respectively.

RESULTS

Isolation of a CDV strain highly virulent for ferrets. The wild-type strain CDV5804 originates from a 1989 distemper outbreak in an animal shelter in northern Germany that killed most of the dogs in the shelter, and thus was considered highly



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	NT	AA	5804 → 5804P
N gene	2	2	I174T G519R
P gene	1	2	I123T (P and V orf) S119P (C orf)
PM UTR	1		
M gene	2	2	G13D T312S
F gene	4	2	A202S } 50% K208R } T641I
H gene	2	2	R106Q Y549H
L gene	7	2	P636T E651G
total	19	12	

FIG. 1. Generation of a CDV strain virulent for ferrets and sequence comparison with its precursor. (A) Selection of a virulent CDV strain. Onset of rash is indicated by dots, and time of death is indicated by triangles. Each pair of symbols represents one animal. (B) Nucleotide (NT) and amino acid (AA) differences between CDV5804 (5804) and CDV5804P (5804P). The amino acid exchanges are indicated with the one-letter code. The numbers refer to the residue position in the corresponding protein. The 50% label indicates that half of the clones had the A202S mutation while the other half carried the K208R mutation.

pathogenic. The virus was isolated from the PBMC of a dog and subsequently adapted to grow in Vero cells. Since the original isolate and earlier passages were no longer available, the eighth passage in Vero cells was used to characterize its virulence in ferrets. Two of the three animals infected intranasally with 10^4 $TCID_{50}$ developed a transient fever but no further signs of disease and mounted a strong neutralizing antibody response. The third animal maintained an elevated temperature and developed a chin rash 17 days postinfection (d.p.i.). Signs of disease worsened progressively over the next 10 days, and the animal was euthanized 29 d.p.i (first passage in ferrets is shown in Fig. 1A, left side).

An organ homogenate of this animal was used to infect two animals (second passage in ferrets) that both developed signs of disease around 7 d.p.i. and were euthanized 5 to 6 days later (Fig. 1A, center). Two additional animals (third passage in ferrets) were infected with an organ homogenate of those two animals, and the course of disease followed the same pattern (Fig. 1A, right). The virus isolated by cocultivation of canine PBMC with an organ homogenate of the last infected group was designated CDV5804P.

CDV5804 and CDV5804P differ in 19 residues distributed evenly throughout the genome. To characterize the genetic differences that accounted for the increase in virulence between the two strains we sequenced their entire genomes. A total of 19 mutations were identified: 11 resulted in amino acid exchanges, 7 were silent, and 1 was located in the 5′ untranslated region of the M gene (Fig. 1B). The mutation in the P gene resulted in two amino acid exchanges: one in the P protein and one in the C protein, a nonstructural protein that is translated from an overlapping open reading frame. In 5 out of 10 clones covering the F_2 subunit sequenced the mutation A202S was observed, generating an additional N-linked glycosylation consensus sequence NXS, whereas all other five clones had the nearby mutation K208R. We found that the newly generated site was indeed used, increasing the number of N-linked glycans attached to the F_2 subunit to four. Since viruses with either sequence caused similar clinical signs (data not shown), we performed all experiments with a K208R carrying virus to maintain the three glycan arrangement of the F_2 subunit conserved among morbilliviruses (53).

New B-cell-based system for CDV recovery. Our experience with the CDV5804 strain suggested that even a few passages on Vero cells may result in attenuation. To prevent attenuation the CDV recovery system had to be based on a cell line that allows propagation of virulent CDV. B95a cells, a marmoset B-cell line that expresses high levels of the common morbillivirus receptor SLAM, was selected because of the ability of these cells to support propagation of virulent RPV and MV without attenuation (50, 58). This cell line is transformed with Epstein-Barr virus, which has a narrow host range and infects only humans and certain nonhuman primates (23) and is thus not expected to replicate in ferrets. B95a cells were infected with the highly virulent challenge strain Snyder Hill (CDV_{SH}) at a multiplicity of infection of 0.01, and the virus was propagated over three passages. Three animals each were inoculated intranasally with 10^4 $TCID_{50}$ of the original CDV_{SH} or the virus passaged three times in B95a cells. Animals in both groups developed first signs of a rash around 7 d.p.i. followed by seizures due to encephalitis around 15 d.p.i. (data not shown), indicating that CDV grown in B95a cells maintains full virulence. Since B95a cells are also efficiently transfected, they were used for the initial transfection as well as the propagation of the newly recovered viruses.

A second issue that had to be addressed was the early identification of infected foci, since wild-type CDV strains do not form large syncytia in B95a cells. Towards this we generated a Vero cell line constitutively expressing canine SLAM, the only

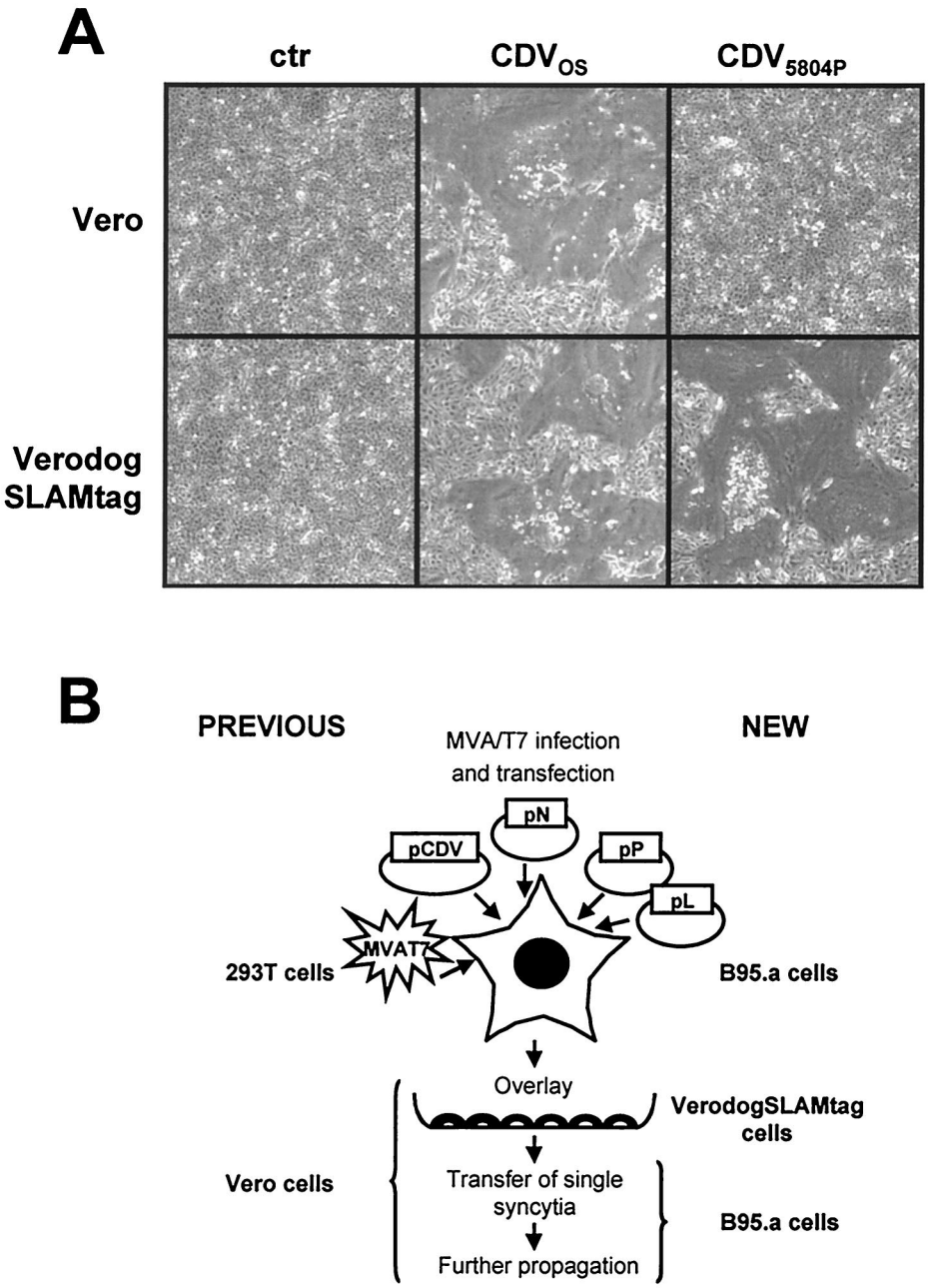

FIG. 2. New recovery system for wild-type CDV. (A) Comparison of cytopathic effects observed after infection of Vero (top row) and VerodogSLAMtag cells (bottom row). Cell lines were infected with the attenuated CDV vaccine strain Onderstepoort as control (CDV_{OS}, center column) or the virulent wild-type strain CDV5804P (CDV_{5804P}, right column) at a multiplicity of infection of 0.01, and phase-contrast pictures were taken 48 h after infection. ctr, control. (B) New system allowing recovery of wild-type viruses. The expression plasmids for the N, P, and L proteins and the full-length CDV plasmid are shown with the MVA-T7 replication-deficient vaccinia virus providing the T7 polymerase. The conventional recovery process for the generation of vaccine strains is based on the transfection of 293T cells followed by the identification of infected foci and the subsequent virus propagation in Vero cells (indicated on the left). Wild-type CDV is recovered and propagated in B95a cells; foci are identified after overlaying the transfected B95a cells onto VerodogSLAMtag cells (indicated on the right).

CDV receptor (VerodogSLAMtag) (51) identified so far. Indeed, we observed that infection of these cells with either vaccine or wild-type CDV strain leads to extensive syncytia formation (Fig. 2A, bottom row). Thus, we established a recovery system based on the transfection of B95a cells previously infected with MVA-T7, followed by the overlay of VerodogSLAMtag cells. Syncytia are isolated and transferred onto fresh B95a cells, and a virus stock is produced by expanding those B95a cells (Fig. 2B). All viruses generated in the study were recovered by using the B95a/VerodogSLAMtag system.

Pathogenesis of the recombinant viruses recapitulates that of the parental strain. To assess the virulence of the recovered

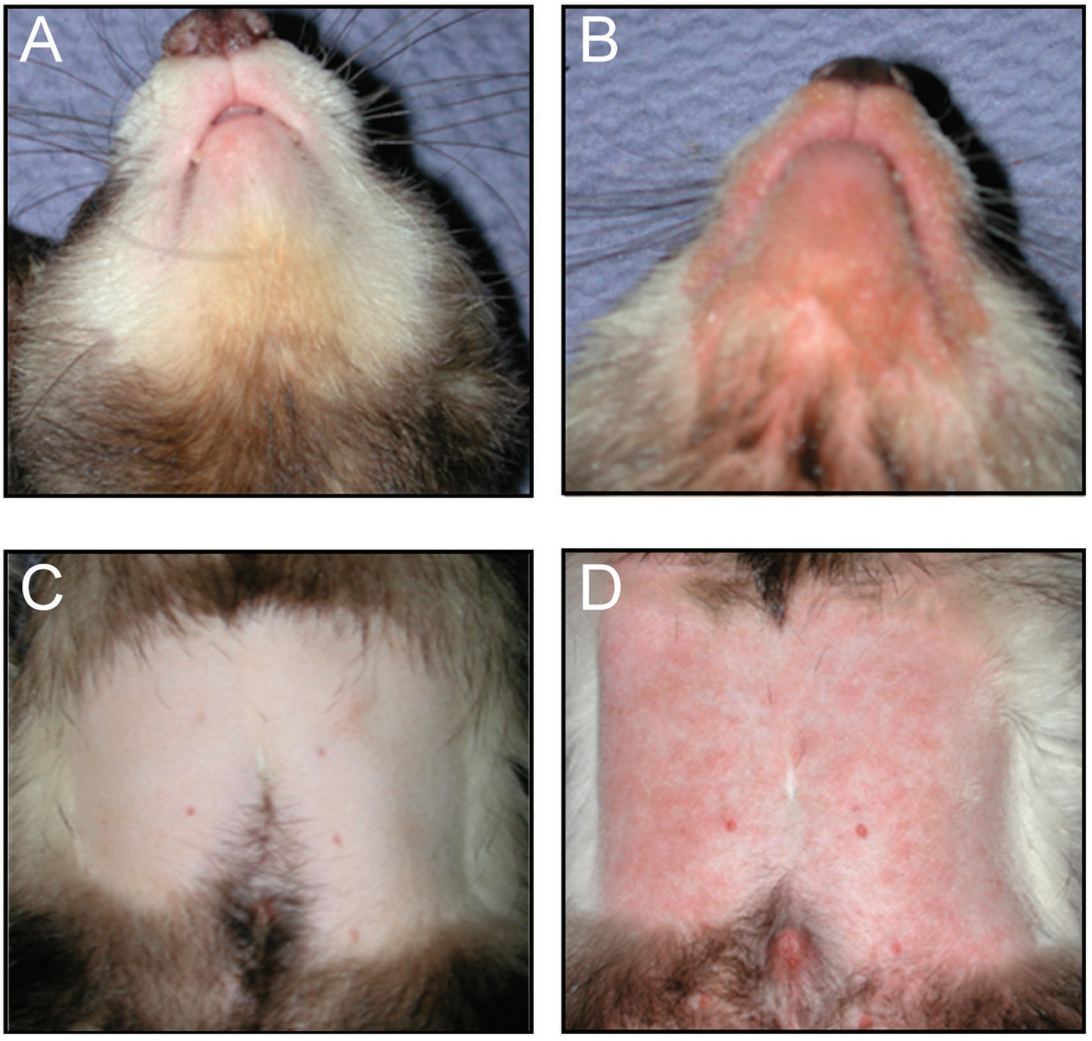

FIG. 3. Photograph of an animal infected with the recombinant attenuated (A and C) or virulent (B and D) strain. Chin and mouth (B) and abdominal rash (D) of a ferret at 12 d.p.i.

viruses, we inoculated groups of four animals intranasally with 10^4 $TCID_{50}$ of either the attenuated (r5804) or the virulent (r5804P) recombinant strain. The animals infected with the recombinant virulent strain r5804P developed first signs of rash 6 to 8 d.p.i. that rapidly progressed to a severe full-body rash (Fig. 3), corresponding closely to the course of animals infected with the parental strain (Fig. 4A, circles). All animals infected with virulent strains had to be euthanized between 12 and 16 d.p.i. because they were moribund. Signs of disease included severe dehydration caused by diarrhea, which was sometimes accompanied by pneumonia as well as purulent conjunctivitis (Fig. 4A, triangles). In contrast, none of the animals infected with the recombinant attenuated strain developed any signs of disease.

The body temperature of animals infected with virulent strains started to rise when the first rash was observed and increased steadily up to 39.5°C. Sometimes a drop in temperature was observed by the time animals became moribund. We found that the two animals infected with the parental attenuated strain experienced a phase of increased temperature starting around 5 to 7 d.p.i. and lasting about a week; however, animals infected with r5804 experienced only minimal changes in body temperature (Fig. 4B).

A characteristic of infection consistent for all strains was the severe white blood cell number drop 1 week after the infection. However, while leukocyte numbers of animals that received the virulent strains remained low, those of animals that were infected with attenuated strains progressively recovered, reaching preinfection or near preinfection values at 35 d.p.i. when the experiment was terminated (Fig. 4C). Finally, all animals infected with attenuated strains mounted a strong neutralizing immune response reaching protective titers of 100 and above within 14 d.p.i., whereas groups that received virulent strains never developed a sufficient response, with maximum titers around 40 at the time of euthanasia (Fig. 4D).

Cell-associated viremia was detected by cocultivation of isolated PBMC with VerodogSLAMtag cells. All groups were viremic at 7 d.p.i., and while virus was present in the PBMC of animals infected with virulent strains until the time of euthanasia (Fig. 5B and D, black columns), groups infected with the

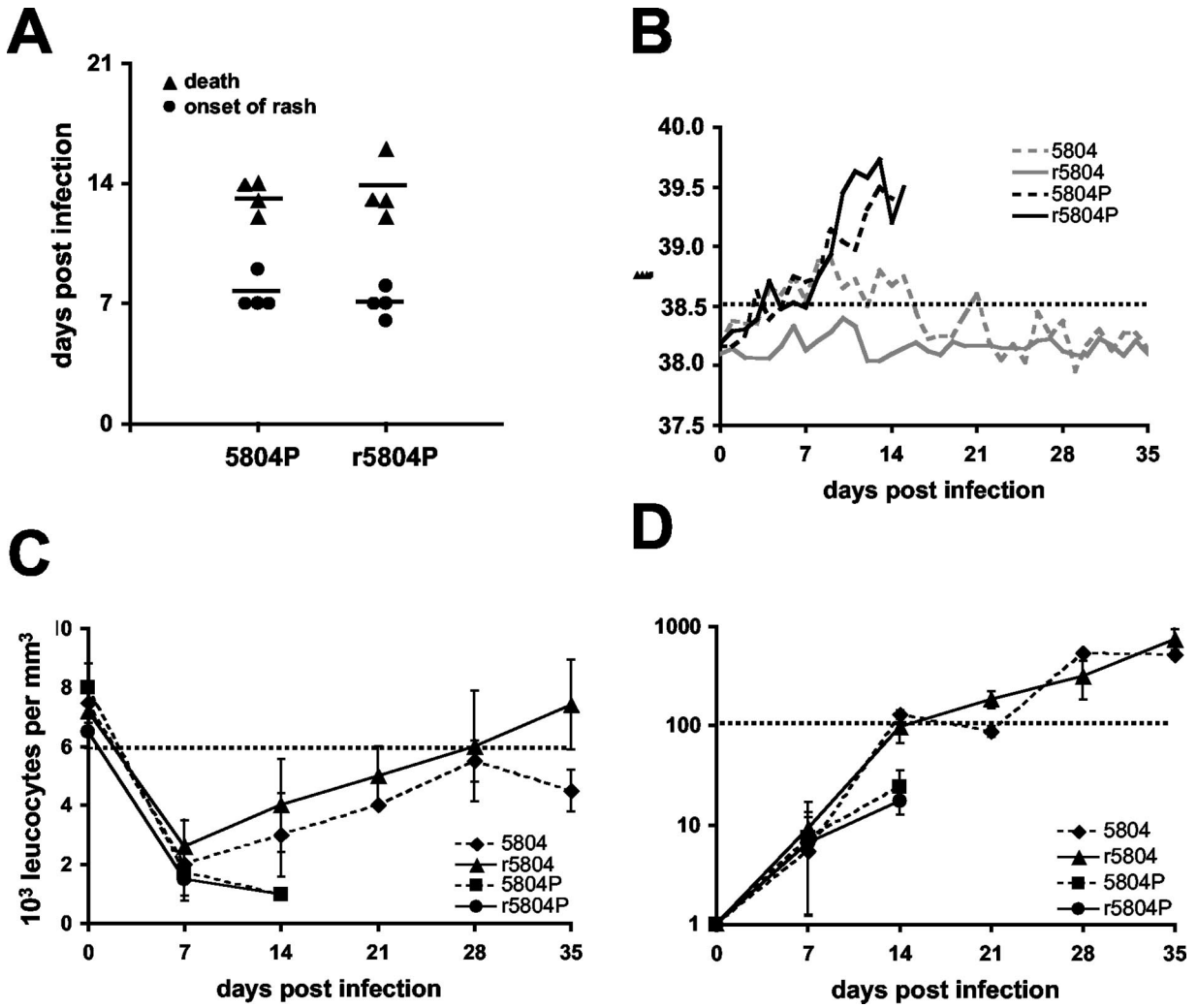

FIG. 4. Course of disease in animals infected with parental or recombinant, attenuated or virulent viruses. (A) Comparison of disease progression in animals infected with the original (5804P) or recombinant (r5804P) virulent strains. Onset of rash is indicated by dots, and time of death is indicated by triangles. Each pair of symbols represents one animal. (B) Rectal temperatures of animals infected with the different attenuated or virulent strains (5804, $n = 2$; r5804, $n = 4$; 5804P, $n = 4$; r5804P, $n = 4$). Temperatures were recorded daily. The temperature (38.5°C) above which animals were considered to be pyrexic is indicated by a dotted line. Gray lines are used for attenuated viruses, and black lines are used for the pathogenic strains. Interrupted lines are used for the parental viruses, and continuous lines are used for strains recovered from infectious cDNA. Mean values are indicated. Leukocyte count (C) and neutralizing antibody titer (D) of animals infected with the different viruses (same animals as described in panel B) are shown. Blood samples were taken at the indicated time points. Lines are drawn with the same conventions as those used for panel B. Error bars are included for each data point. The leukocyte concentration (6,000 cells per mm^3) below which animals are considered leukopenic and the protective neutralizing antibody titer (≥100) are indicated by a dotted line in panels C and D, respectively.

attenuated strains cleared the virus 14 to 21 d.p.i. (Fig. 5A and C). Again, a small difference was observed between the parental and recombinant attenuated strain, suggesting that the parental strain might be slightly more virulent. In summary, the course of disease caused by parental and recombinant viruses is similar.

Severity of immunosuppression correlates with virulence. The loss of DTH response to antigens that were efficiently recognized prior to infection has long been recognized as a sign of morbillivirus infection (56). To measure immunosuppression, all animals were immunized against tetanus prior to the infection, and the DTH response to tetanus toxoid was used to assess this aspect of immunosuppression in CDV infection. While all animals developed an induration of at least 10 mm in diameter two days after intradermal injection of tetanus toxoid at the initial time of infection, no response was detected at day 7. Of the four animals infected with the attenuated strain (Fig. 5E), the two that showed a DTH response at 14 d.p.i. had also cleared the viremia at that time (Fig. 5C), suggesting that these two parameters were linked. Three out of four animals infected with the virulent strain did not survive long enough to perform and evaluate the DTH test at day 14, and the remaining animal did not show any response but experienced viremia (Fig. 5D and F).

To compare the proliferation activity of lymphocytes from animals infected with either r5804 or r5804P we determined

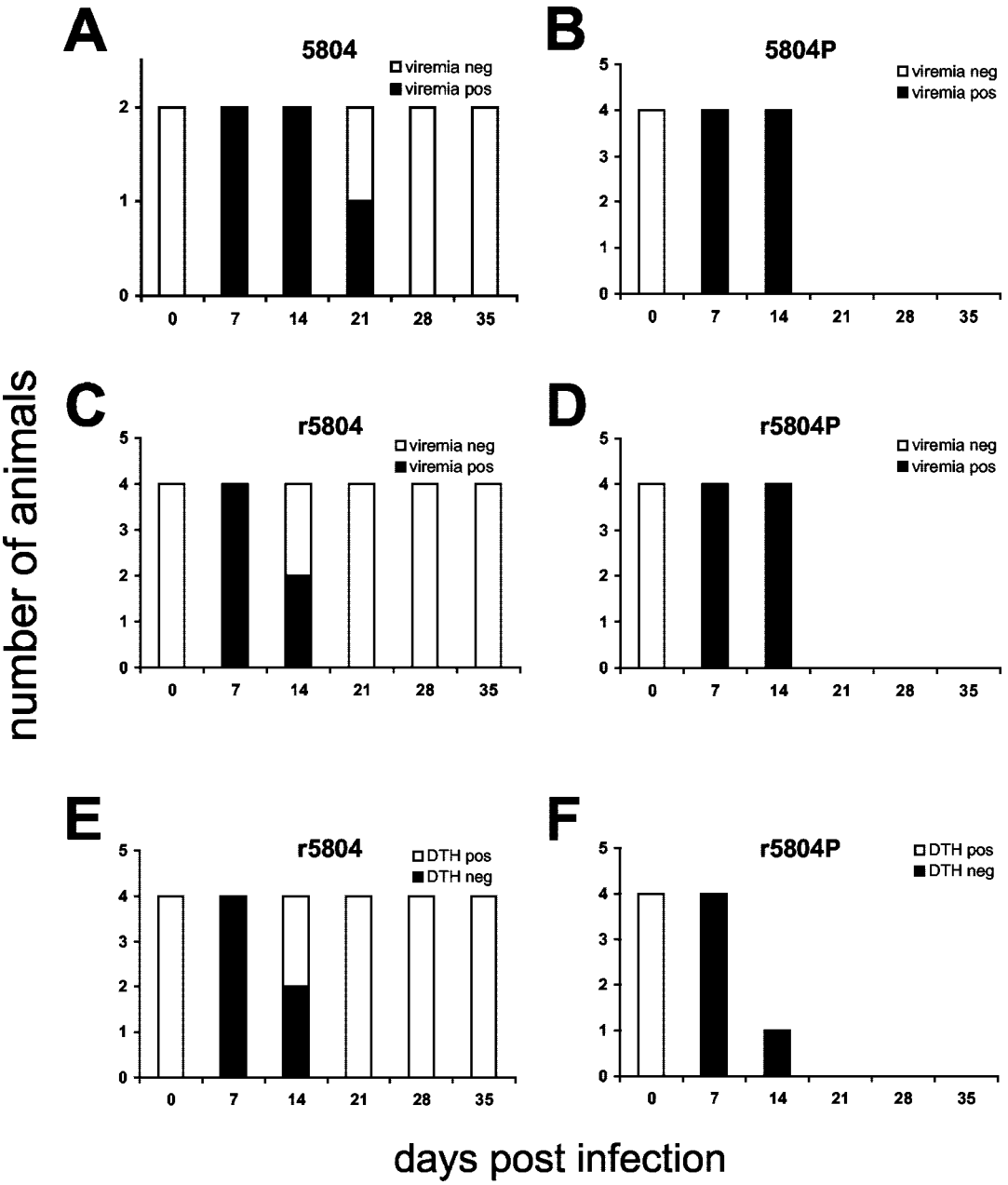

FIG. 5. Cell-associated viremia and DTH responses of animals infected with attenuated and virulent strains. (A to D) Cell-associated viremia in the groups infected with the original viruses 5804 (A) and 5804P (B) and the recombinant viruses r5804 (C) and r5804P (D). PBMCs were isolated weekly from 0.5 ml of blood and were cocultivated with VerodogSLAMcells. Cell-associated viremia was detected by syncytium formation occurring between 1 and 3 days after the PBMC isolation. The white portions of the columns represent virus-negative animals, the black portions represent virus-positive animals. (E and F) DTH responses of animals infected with the recombinant attenuated (r5804) (E) and virulent (r5804P) (F) viruses. Animals were immunized against tetanus prior to the experiment, and a positive DTH response (diameter of induration, $\geq$10 mm) was detected at the time of infection. The white portions of the columns represent animals with a positive DTH response; the black portions represent DTH-negative animals.

the ratio of BrdU incorporation of PHA-stimulated versus nonstimulated PBMC from each animal at different times after the infection. PHA was chosen because of its low toxicity for ferret lymphocytes in pilot experiments. For the animals infected with the virulent strain, proliferation activity was severely reduced within 7 d.p.i. and remained low until the time of euthanasia. In contrast, the group that received the attenuated strain showed only a transient reduction of proliferation activity, compared to that of noninfected control animals, and recovered by the end of the protocol (Fig. 6).

Envelope exchange results in intermediate disease phenotypes. To determine whether most virulence determinants are associated with the viral envelope genes or the other genes, we generated two reassortant viruses: r5804envP, in which the N,

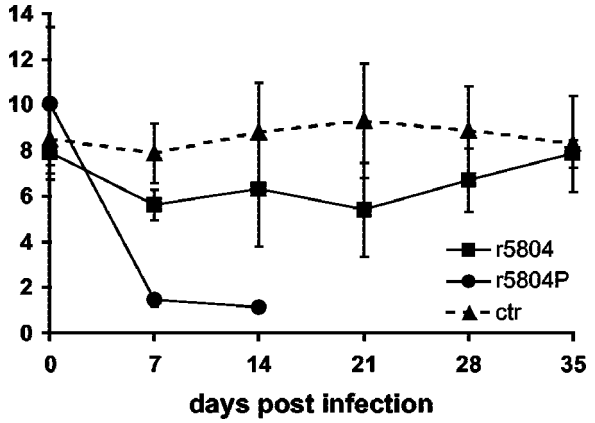

FIG. 6. In vitro lymphocyte proliferation activity of ferrets infected with the recombinant attenuated or virulent viruses. PBMC of each animal were isolated by Ficoll gradient centrifugation and split in four equal aliquots, stimulated with PHA or left untreated and incubated with BrdU. BrdU incorporation was detected by using a peroxidase-linked anti-BrdU antibody and was revealed with a chemiluminescent substrate. The proliferation activity was expressed as the ratio between stimulated and nonstimulated cells. The mean values of animals infected with r5804 ($n = 4$) are represented by squares connected by a continuous line, those of animals infected with r5804P ($n = 4$) are represented by dots connected by a continuous line, and those of three noninfected control animals are indicated by solid triangles connected by an interrupted line. Error bars are shown.

P, and L genes of the attenuated strain were combined with the envelope M, F, and H genes of the virulent strain, and r5804repP with the complementary gene arrangement (Fig. 7A). Both viruses were recovered by using the modified system, and groups of six (r5804envP) and four (r5804repP) animals were inoculated intranasally with 10^4 $TCID_{50}$ of the respective virus.

Animals infected with r5804envP displayed a delayed disease onset, with first signs of rash between 8 and 12 d.p.i. (Fig. 7B, center column), which successively developed into a severe whole-body rash similar to that observed in animals infected with r5804P in intensity in four out of the six animals infected. In two animals the rash was mostly concentrated in the chin and neck regions with additional spots all over the body. Mild diarrhea and inflammation in the anal region were observed in all animals, indicating involvement of the gastrointestinal tract. The rash peaked about 7 days after onset, followed by a rapid, complete recovery, and it was completely healed between 17 and 22 d.p.i.

In contrast, animals infected with r5804repP developed first signs of rash between 6 and 8 d.p.i. (Fig. 7B, right column). The rash progressed over the following 3 days to cover the whole body qualitatively as in animals infected with r5804P or r5084envP, but it never reached more than intermediate severity (data not shown). The infection progressed less severely than in animals infected with the pathogenic strain; no further signs of disease were observed, and the rash was completely healed around 13 d.p.i.

A temperature peak between 3 and 5 d.p.i. was observed for all animals infected with the original virulent strain r5804P as well as the chimeric viruses r5804envP and r5804repP (Fig. 7C). This peak is thought to be associated with the first viremia, during which the virus spreads from its initial center of infection into all lymphoid tissues. In animals infected with r5804envP (Fig. 7C, green line), this peak was followed by 2 to 3 days of normal temperature, after which a phase of pyrexia was observed that coincided with the development of rash and other signs of disease and subsided around the time those signs disappeared. Animals infected with the complementary virus r5804repP were pyrexic for a brief period after the initial peak also corresponding to the height of their symptomatic phase (Fig. 7C, yellow line).

Similar to the groups infected with the unaltered viruses, all animals infected with the chimeric viruses experienced a severe drop in white blood cell counts at 7 d.p.i. (Fig. 7D, green rhombuses and yellow triangles). In contrast to animals infected with the original virulent virus, in the other three groups lymphocyte counts continuously recovered over the course of the experiment, reaching preinfection levels around 28 d.p.i. Similarly, animals receiving the chimeric viruses developed protective antibody levels around 14 d.p.i. that continued to rise until the end of the protocol (Fig. 7E, green rhombuses and yellow triangles).

Cell-associated viremia and DTH response appeared and disappeared in concert in animals infected with r5804envP and r5804repP (Fig. 8A to D). Compared to the attenuated strain r5804, viremia was extended by 2 to 3 weeks, and during this time DTH responses were suppressed, with one animal (Fig. 8A, asterisk) in the r5804envP group being viremic until 8 weeks after the infection. In this animal the antibody titers remained between 30 and 60 throughout this time and only rose above 100 once the viremia was cleared. For animals infected with r5804envP the delayed disease onset observed was reflected in the time course of lymphocyte proliferation activity (Fig. 8E, green rhombuses). While the proliferation activity in animals infected with r5804P was severely inhibited at 7 d.p.i., that of animals infected with r5804envP reached similarly low levels at 14 d.p.i., after which a rapid recovery was observed. In contrast, animals infected with r5804repP displayed a course of proliferation inhibition similar to that observed for the attenuated strain r5804 (Fig. 8E, yellow triangles). These findings indicate that virulence-associated mutations are distributed both in the envelope as well as in the polymerase complex genes.

DISCUSSION

The *Paramyxoviridae* family includes causative agents for several classical diseases in humans, such as measles, mumps, and parainfluenza, and viruses afflicting livestock, such as RPV and bovine respiratory syncytial virus, to name only a few. Despite the availability of reverse genetics systems for most of these viruses, the systematic characterization of virulence factors on the molecular level has been slow, at least in part due to the lack of suitable and practical animal models in combination with closely related pairs of attenuated and virulent strains that can be used for reverse genetics. In this study we established a homologous small-animal system giving insights in the mechanisms of CDV immunosuppression and virulence. These insights may be relevant for morbillivirus infections in general.

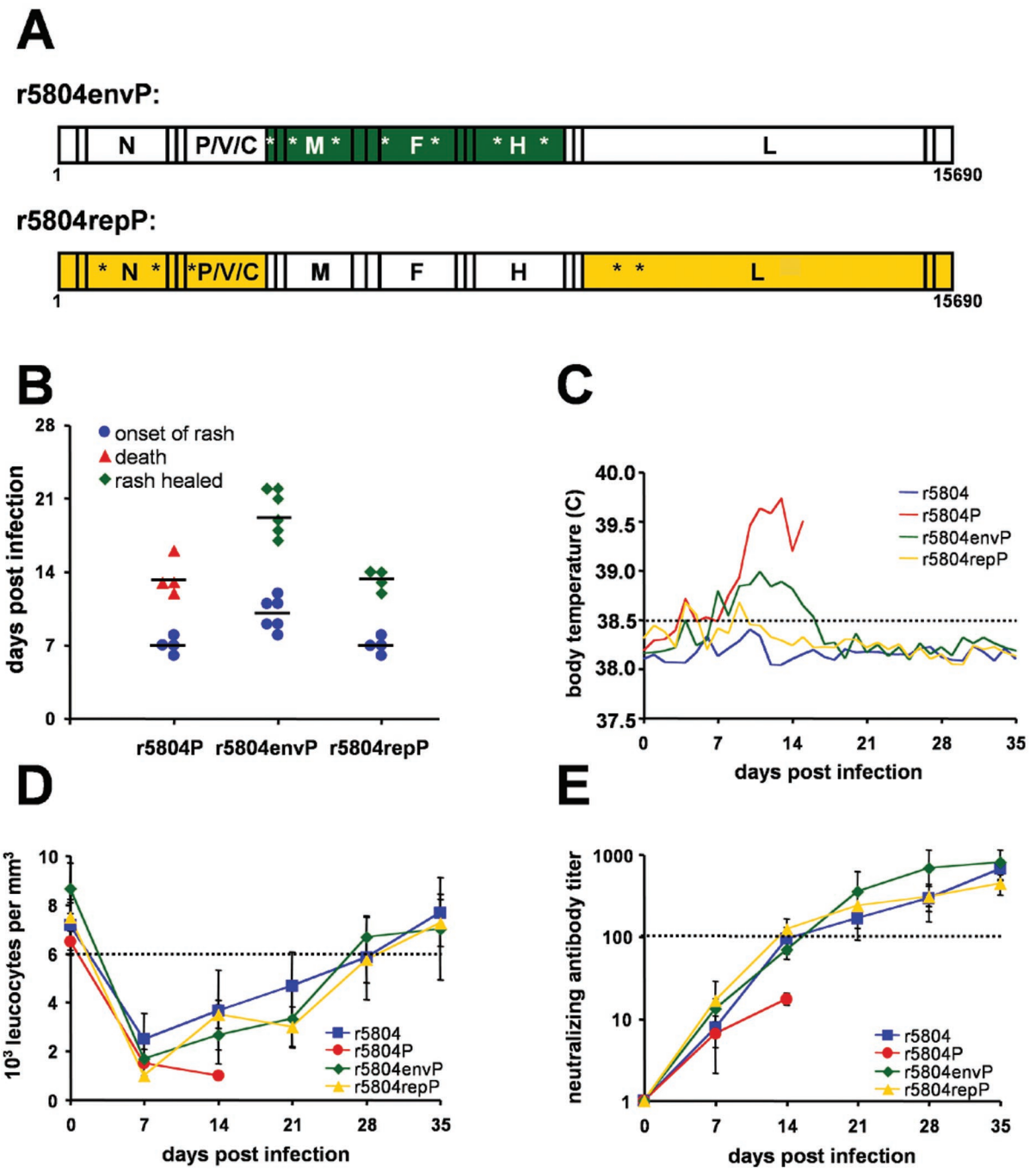

FIG. 7. Scheme of the genome of viruses with reassorted genes and course of disease they elicited. (A) Scheme of the r5804envP genome consisting of the virulent envelope genes (M, F, and H) in combination with the attenuated replication complex genes (N, P, and L) and of the r5804repP genome with the inverse gene combination. The genes originating from strain r5804P are indicated in green for r5804envP or yellow for r5804repP. Locations of mutations are indicated by stars. (B) Disease progression in animals infected with the virulent strain r5804P and the chimeric viruses r5804envP and r5804repP. Onset of rash is indicated by dots, time of death by triangles, and healing of rash by rhombuses. Each pair of symbols represents one animal. (C) Rectal temperatures were recorded daily (r5804envP, $n = 6$; r5804repP, $n = 4$). The temperature (38.5°C) above which animals were considered to be pyrexic is indicated by a dotted line. The mean values of all animals infected with 5804 are represented by a blue line, and those of all animals infected with r5804P are represented by a red line. Values of animals infected with r5804envP are represented by a green line, and those of animals infected with r5804repP are represented by a yellow line. Leukocyte count (D) and neutralizing antibody titer (E) of animals infected with the different viruses are shown. Blood samples were taken at the indicated time points. The color coding is as described for panel C. Error bars are included for each data point. Total leukocyte counts and neutralizing antibody titers were determined as described in Materials and Methods. The leukocyte concentration (6,000 cells per mm^3) below which animals are considered leukopenic and a protective neutralizing antibody titer (≥100) are indicated by a dotted line in panels D and E, respectively.

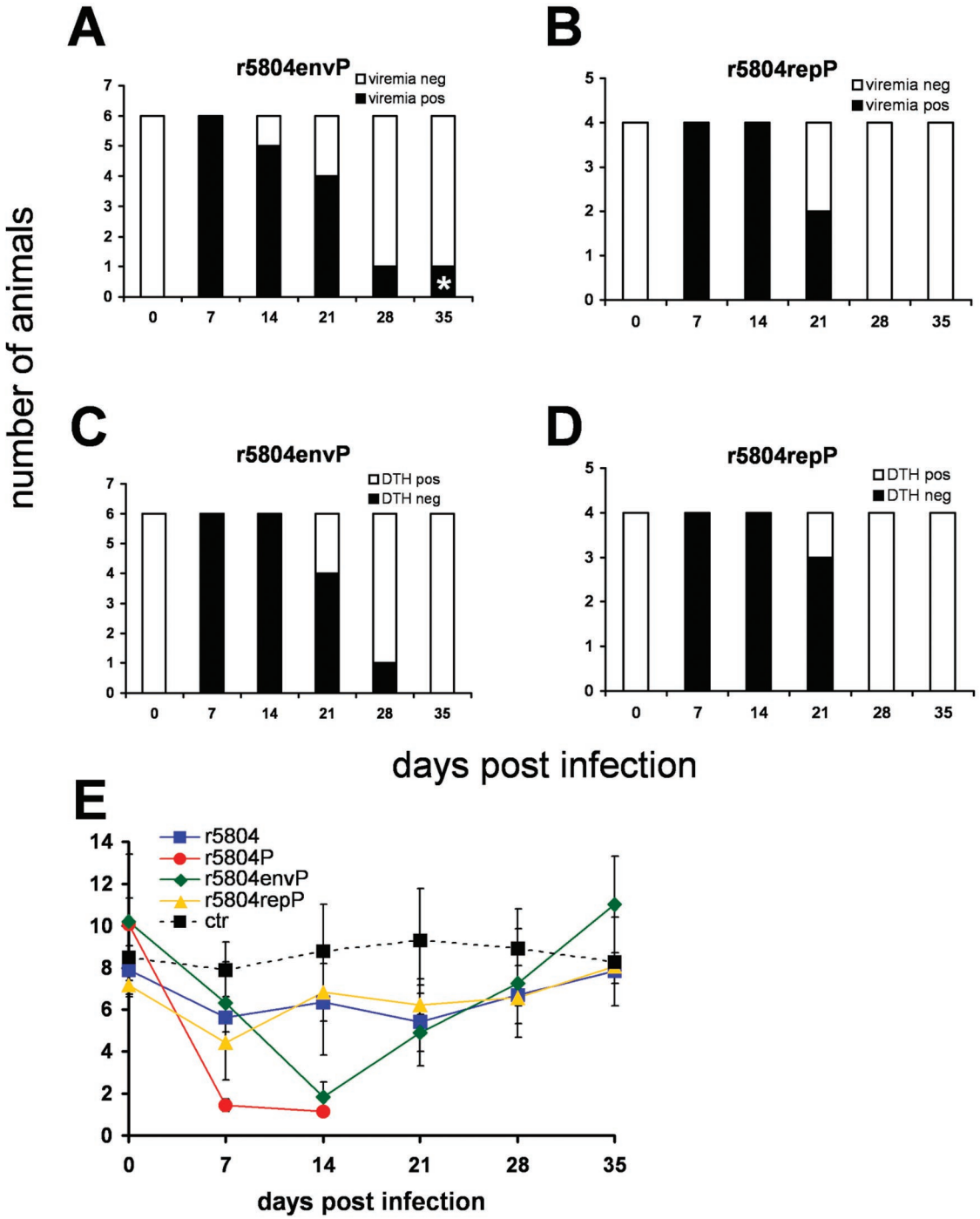

FIG. 8. Cell-associated viremia, DTH responses, and in vitro lymphocyte proliferation activity of viruses with reassorted genes. (A and B) Cell-associated viremia detected after infection with r5804envP (A) and r5804repP (B). PBMCs were isolated weekly from 0.5 ml of blood and were cocultivated with VerodogSLAMcells. Cell-associated viremia was monitored by detection of syncytium formation between 1 and 3 days after the PBMC isolation. The white portions of the bars represent virus-negative animals, and the black portions represent virus-positive animals. (C and D) DTH responses of animals infected r5804envP (C) and r5804envP (D). Animals were immunized against tetanus prior to the experiment, and a positive DTH response was detected at the time of infection. The white portion of the columns represent animals with a positive DTH response, the black portions represent DTH-negative animals. (E) Comparison of in vitro proliferation activity of PBMC from animals infected the unaltered viruses r5804 ($n = 4$) and r5804P ($n = 4$) or the chimeric viruses r5804envP ($n = 6$) and r5804repP ($n = 4$). Methods were as described in the legend to Fig. 6. The color coding is as described for Fig. 7D. The proliferation activity of three noninfected control animals at each time point is indicated by black squares connected by an interrupted black line. Error bars are shown.

Recombinant virulent wild-type CDV from cDNA. To examine whether a recombinant virus derived from cDNA retained the pathogenic characteristics of its parent, we infected groups of ferrets with equal amounts of infectious units of the two virus preparations. Animals infected with both viruses had similar timings of rash onset (6 to 8 d.p.i.), clinical signs, and death (12 to 16 d.p.i.), indicating that r5804P retained all the pathogenic characteristics of 5804P. In comparing the two attenuated viruses we found that the recombinant strain r5804 was slightly more attenuated than its natural parent 5804.

RNA virus genomes are heterogeneous (14, 15, 48), and it was proposed that this heterogeneity contributes to the efficient infection of multiple tissues (4, 27). Indeed, we monitored heterogeneity in multiple cDNAs obtained from infected cells and devised an approach assuring reconstitution of the consensus sequence. Nevertheless, the RNA heterogeneity of the original inoculum is likely to be higher than that of the inoculum derived from a cDNA clone and amplified only once. Thus, it was not possible to predict whether virus recovered from our consensus sequence-based CDV genomic cDNAs would completely reproduce the pathogenic characteristics of the parental inocula. The fact that virulence of r5804P corresponded closely to that of the parental strain 5804P indicates that in this case the consensus sequence derived from a homogenate of different organs maintained the competence to rapidly reconstitute a fully virulent genomic quasispecies.

In contrast, the recombinant virus derived from strain 5804 was slightly more attenuated than its parent. Thus, in this case the corresponding consensus sequence may not have allowed reconstitution of full genomic diversity, or a quasispecies memory effect may have occurred (43). In any case, the closely related pair of an attenuated and a virulent virus described here constitutes an ideal basis for the genetic analysis of virulence factors.

Infection of ferrets with r5804P recapitulates many aspects of morbillivirus pathogenesis. One reason to develop the CDV-ferret model is its potential use as homologous small-animal model to study morbillivirus virulence and pathogenesis. We observed a dramatic course of disease in ferrets infected with the virulent strains, involving severe leukopenia and gastrointestinal and respiratory signs and leading to death of the animals within 2 to 3 weeks. This course of disease is very similar to that described for cattle infected with virulent RPV (7, 57); severe disease is also observed during sporadic morbillivirus epidemics in aquatic mammals (22) as well as when CDV jumps the species barrier (42). In contrast, MV infections in humans and nonhuman primates, CDV infections in dogs, and peste des petits ruminant infections in goats and sheep are less dramatic. And while leukopenia, rash, and gastrointestinal and respiratory involvement are still typical signs of disease, the majority of infected individuals survives (2, 13, 20, 59). Nevertheless, the tropism and tissue distribution of many, if not all, morbillivirus infections is similar. Importantly, the severity of the disease in the CDV-ferret model allows a sensitive readout of attenuation.

Importance of the envelope proteins and other factors in virulence and immunosuppression. A relevant question regarding morbillivirus pathogenesis is the relative importance of the envelope and replication protein complexes as well as the nonstructural proteins V and C for virulence and immunosuppression (50, 58). Our sequence analysis of the 5804 and 5804P genomes indicated that the 19 mutations differentiating these two strains were almost evenly distributed over the genome. Therefore, to address the above question, we produced two recombinant CDVs with different combinations of the polymerase (including V and C) and envelope complexes of the virulent and attenuated strain.

Viruses in which the virulent envelope complex was combined with the other proteins from the attenuated strain elicited a delayed onset of rash and fever but caused severe signs of disease, while disease onset of the complementary viruses corresponded to the original wild-type strain but a milder course was observed. In addition, we found that the extent of immunosuppression as measured in loss of DTH response and inhibition of lymphocyte proliferation correlated generally with disease severity, and in the majority of cases DTH tests were negative while animals were viremic.

In this context it is important to note that the P genes code not only for the P protein, a polymerase cofactor, but also for the two nonstructural proteins V and C. The function of these proteins in controlling the quality of the immune response and interfering with the interferon system was established in other members of the *Paramyxoviridae* subfamily (18). For RPV and MV the nonstructural proteins have only ancillary functions for replication in cultured cells (6, 40, 45), but their absence reduces the extent of MV spread in rodent models (30, 52). Moreover, recently the MV V protein was shown to suppress cytokine signal transduction (38). The relevance of the CDV V and C proteins for pathogenesis is presently under investigation.

These results allow speculations about possible pathogenicity mechanisms associated with the envelope and the replication complexes: disease onset correlates with the source of the replication complex, suggesting that entry does not limit viral spread through the highly susceptible lymphoid cells. In contrast, the severity of rash and fever correlates with the source of the envelope, suggesting that cell entry may be a limiting factor for viral spread to epithelial cells at later infection stages, when most of the signs of disease occur. In this context it is interesting that in related viruses virulence has been associated with mutations in the envelope protein complex: in Newcastle disease and influenza viruses pathogenicity and tropism are associated with mutations in the cleavage-activation site of the fusion-hemagglutinin protein (11, 12, 46).

In summary, gain of virulence in our system involves multiple genes and is not associated with a single function. In line with our results with CDV, in MV attenuating mutations have been identified in different genes (50). The relatively low costs of experimentation in ferrets and the availability of these animals, together with the characterization of several parameters of virulence and attenuation presented here, will clear the way for a comprehensive study of the determinants of morbillivirus virulence, including not only the envelope and replication proteins but also the accessory C and V proteins that counteract host defenses (18, 33).

ACKNOWLEDGMENTS

We thank Sompong Vongpunsawad for excellent technical support.

This work was supported by grants of the Mayo and Siebens Foundations and by an Emmy Noether award of the German Research Foundation (DFG) to V.V.M.

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