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Canine Distemper Virus (CDV) Infection of Ferrets as a Model for Testing *Morbillivirus* Vaccine Strategies: NYVAC- and ALVAC-Based CDV Recombinants Protect against Symptomatic Infection

CHARLES B. STEPHENSEN,[1,2]* JANET WELTER,[2] SUBHASHCHANDRA R. THAKER,[1]† JILL TAYLOR,[3] JAMES TARTAGLIA,[3] AND ENZO PAOLETTI[3]

Department of International Health, School of Public Health,[1] and Department of Comparative Medicine, School of Medicine,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294-0019, and Virogenetics Corporation, Rensselaer Technology Park, Troy, New York 12180[3]

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Canine distemper virus (CDV) infection of ferrets causes an acute systemic disease involving multiple organ systems, including the respiratory tract, lymphoid system, and central nervous system (CNS). We have tested candidate CDV vaccines incorporating the fusion (F) and hemagglutinin (HA) proteins in the highly attenuated NYVAC strain of vaccinia virus and in the ALVAC strain of canarypox virus, which does not productively replicate in mammalian hosts. Juvenile ferrets were vaccinated twice with these constructs, or with an attenuated live-virus vaccine, while controls received saline or the NYVAC and ALVAC vectors expressing rabies virus glycoprotein. Control animals did not develop neutralizing antibody and succumbed to distemper after developing fever, weight loss, leukocytopenia, decreased activity, conjunctivitis, an erythematous rash typical of distemper, CNS signs, and viremia in peripheral blood mononuclear cells (as measured by reverse transcription-PCR). All three CDV vaccines elicited neutralizing titers of at least 1:96. All vaccinated ferrets survived, and none developed viremia. Both recombinant vaccines also protected against the development of symptomatic distemper. However, ferrets receiving the live-virus vaccine lost weight, became lymphocytopenic, and developed the erythematous rash typical of CDV. These data show that ferrets are an excellent model for evaluating the ability of CDV vaccines to protect against symptomatic infection. Because the pathogenesis and clinical course of CDV infection of ferrets is quite similar to that of other *Morbillivirus* infections, including measles, this model will be useful in testing new candidate *Morbillivirus* vaccines.

Measles virus (MV) and canine distemper virus (CDV) are closely related members of the genus *Morbillivirus* in the family *Paramyxoviridae* (37). While morbilliviruses are closely related to one another, they do not cross-infect unnatural hosts with any facility (21), perhaps because of the specific interaction of the viral hemagglutinin (HA) protein with a species-specific cellular receptor for each virus (23). MV infects only humans, although nonhuman primates can become infected from their caretakers when held in captivity (37). Thus no suitable small-animal model exists for directly studying MV pathogenesis or for vaccine development. However, in their natural hosts different morbilliviruses cause quite similar diseases (19, 32, 34). The mechanisms of protective immunity are closely related as well (11, 14, 33, 44). We have therefore chosen to evaluate the utility of CDV infection of ferrets as a model of MV infection, particularly for the evaluation of new measles vaccine strategies. The ability of CDV vaccine constructs to prevent symptomatic distemper in ferrets should help predict the ability of similar vaccines to protect children against measles. In addition, measles vaccines can be directly evaluated in ferrets for their ability to protect against CDV challenge, since heterologous vaccination of ferrets and other species with MV can protect against subsequent challenge with CDV (3, 17). Work toward the development of a new measles vaccine is necessary because the current live-virus vaccines have significant limitations. Most notably, the current vaccine cannot be safely given to infants below 9 months of age in a dose sufficiently high to be immunogenic in the presence of maternally derived serum antibody directed against MV (2, 27). Development of a safe and effective vaccine which could be administered to infants below 6 months of age would greatly enhance measles prevention programs in the developing world.

There is a substantial amount of literature showing that *Morbillivirus* subunit vaccines which include the HA and F proteins provide solid protection against challenge infections. Many of these studies, however, have employed unnatural hosts and unnatural routes of challenge, including intracerebral challenge with MV or CDV in rodents and with rinderpest virus (RPV) in rabbits (8, 10, 13, 22, 24, 52, 54, 55). Four studies have examined protection in the natural host: two have demonstrated protection of cattle against subcutaneous (s.c.) RPV challenge (41, 57), and two studies have used both a natural host (dog) and natural route of challenge (intranasal [i.n.]) with CDV (22, 38). Four studies have examined heterologous protection: two employed MV recombinant vaccines to protect against CDV challenge in dogs (48, 49), one utilized an RPV recombinant to protect against peste des petits ruminants virus infection in goats (31), and one utilized a CDV immune-stimulating complex preparation to vaccinate against phocine distemper virus infection in seals (53).

The previous studies with CDV have utilized purified HA

* Corresponding author. Mailing address: Department of International Health, School of Public Health, 308 Tidwell Hall, 720 S. 20th St., University of Alabama at Birmingham, Birmingham, AL 35294-0008. Phone: (205) 934-1732. Fax: (205) 975-3329. E-mail: stephenc@crl.soph.uab.edu.

† Present address: 232 North Rd., Bedford, MA 01730-1013.

and F proteins to immunize small numbers of dogs against CDV challenge (22, 38). Such studies have not been done in ferrets, which suffer a much more severe course of infection than do dogs, with a case fatality rate of essentially 100% (34). In fact, attenuated strains of CDV suitable for vaccination of dogs and other species can cause symptomatic and sometimes fatal infections in ferrets (16, 32). Subunit vaccines are thus an attractive alternative for immunizing ferrets. For this reason, and to evaluate the utility of the CDV-ferret model for testing *Morbillivirus* vaccines, we have tested two subunit vaccines incorporating both the HA and F proteins of CDV. One vaccine utilized the highly attenuated NYVAC strain of vaccinia virus (46), and the other was constructed in the ALVAC strain of canarypox virus, which does not productively replicate in mammalian hosts (50). Ferrets were also vaccinated with an attenuated, live-virus CDV vaccine.

MATERIALS AND METHODS

Ferrets. European ferrets (*Mustela putorius furo*) were purchased from Marshall Farms (North Rose, N.Y.) and were housed in small groups.

Generation of NYVAC- and ALVAC-based recombinant CDV vaccines. The Onderstepoort strain of CDV was obtained from M. Appel (James A. Baker Institute for Animal Health, Cornell University, Ithaca, N.Y.). Vero cell monolayers were inoculated with CDV and harvested when early cytopathic effect was evident. Total RNA was extracted as described by Chirgwin et al. (18), using the guanidinium isothiocyanate-cesium chloride method. First-strand cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) with CDV F- and HA-specific primers and the RNA from CDV-infected cells as template. The HA- and F-specific open reading frames were then amplified by PCR (26) using custom synthesized oligonucleotides (Applied Biosystems 380B, Foster City, Calif.). The derived HA and F genes were inserted into NYVAC and ALVAC insertion vectors by standard methods previously described (46, 50). In each vector both HA and F genes are inserted in a 5′ to 5′ orientation with both genes under the transcriptional control of the early/late vaccinia virus H6 promoter which has been described previously (39). Appropriate expression of the CDV F and HA genes was confirmed by immunoprecipitation analysis performed essentially as described by Tartaglia et al. (46) by using a polyclonal CDV-immune serum derived from dog. Generation of the NYVAC-RG and ALVAC-RG recombinant vaccines has been described by Tartaglia et al. (46) and Taylor et al. (50), respectively.

Vaccinations. Ferrets were vaccinated intramuscularly with 10^8 PFU of the NYVAC and ALVAC constructs, with 0.2 ml per dose. One group of ferrets received an attenuated, live-virus vaccine (Distem-RTC, Schering Corp., Union, N.J.) prepared in chicken tissue culture for use in minks. This vaccine has been extensively tested in ferrets (5). Ferrets were immunized at 14 and 18 weeks of age.

Challenge infection. Ferrets were challenged (i.n.) at 22 weeks of age with 10^3 50% tissue culture infective dose ($TCID_{50}$) units of the Snyder Hill strain of CDV in 0.2 ml while under anesthesia to prevent sneezing (5 to 10 mg each of tiletamine and zolazepam per kg). The virus was dripped into each nostril with a micropipette while the ferret's nose was pointed upward to allow the inoculum to drain into the nasal passages and trachea. Preliminary work indicated that 0.5 $TCID_{50}$ units did not cause symptomatic infection within 4 weeks of challenge but that 5 $TCID_{50}$ units produced typical distemper within 18 days.

Monitoring clinical course of distemper. After challenge, animals were monitored at least daily. Rectal temperature, body weight, and activity level (normal or diminished), were recorded, as was the presence or absence of any of the following five clinical signs of distemper: conjunctivitis, central nervous system (CNS) signs (seizures and circling behavior), a chin rash typical of distemper, generalized erythema, and dyspnea (an indicator of pneumonia). Since ferrets do not recover from CNS involvement (which eventually causes protracted seizures), animals with CNS signs were immediately euthanized. Animals which become moribund without showing CNS signs (e.g., due to pneumonia) would also have been euthanized, although none did in the present study. Leukocytopenia was detected by enumerating total peripheral blood leukocytes with a Coulter counter. Subjective evaluation of clinical signs was blinded by identifying animals by number only, without reference to vaccine group.

Blood. Blood was collected from anesthetized (5 to 10 mg each of tiletamine and zolazepam per kg) ferrets by venipuncture of the cephalic or saphenous vein or terminally by cardiac puncture.

$TCID_{50}$ assays of CDV. The Onderstepoort strain of CDV was generously provided by Max Appel of the James A. Baker Institute of Animal Health at Cornell University. This strain was grown and titered in Vero cells, essentially as described (4). We used the fifth virus passage after plaque purification. The Snyder Hill strain of CDV was purchased from the American Type Culture Collection (VR-526; Rockville, Md.) and was grown and titered in canine peripheral blood lymphocytes as described previously (6).

Virus-neutralizing antibody. CDV neutralizing titers were determined in Vero cells by using a $TCID_{50}$ format assay based on the method of Appel and Robson (4). In a 96-well plate, duplicate twofold dilutions (from 1:2 through 1:1,024) of heat-inactivated sera were added to a standard inoculum (20 $TCID_{50}$ units) of the Onderstepoort strain of CDV diluted in media (Dulbecco's modified Eagle's medium containing 5% heat-inactivated, newborn calf serum and 25 mM HEPES buffer). After incubation at room temperature for 2 h, 1.2×10^4 Vero cells were added to each well. The plates were then incubated at 37°C in 5% CO_2 for 5 to 6 days. Endpoints were determined by examining plates for syncytia by using phase-contrast optics and an inverted microscope.

PBMC preparation. Ferret peripheral blood mononuclear cells (PBMCs) were separated from 1.5 ml of heparinized whole blood by layering over 1.0 ml of Histopaque-1077 (Sigma Diagnostics, St. Louis, Mo.), and centrifuging at 184 × *g* for 40 min. The PBMCs were then washed twice with cold phosphate-buffered saline (PBS), resuspended in 400 μl of PBS, and frozen at −85°C.

RT-PCR assay. CDV nucleocapsid specific primers from Onderstepoort strain were chosen from the nucleocapsid genome (42). The upstream primer, CDV-15 (5′-GGTCGGAGAATTTAGAATGAAC-3′), and the downstream primer, CDV-23 (5′-CCAAGAGCCGGATACATNG-3′), yielded a 240-bp product spanning nucleotides 588 through 827 in the nucleocapsid genome (GenBank accession number X02000 M10242). RNA was extracted from 50 μl of a suspension of ferret PBMCs by using the guanidinium thiocyanate method developed by Boom et al. (12a) as modified by Park et al. (38a). Reverse transcription (RT) was performed on 8 μl of eluted RNA in a 20-μl reaction volume with 50 U of Moloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies, Gaithersburg, Md.), 1 mM nucleotide triphosphates (dNTPs), 1.25 μM upstream primer (CDV-23) and 2 μl of 0.1 M dithiothreitol in 1× reaction buffer provided by the manufacturer. All cDNA from the RT reaction (20 μl) was used in the 50-μl PCR reaction, with 1.25 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 2.5 mM MgCl, 1 mM dNTPs, and 0.25 μM each CDV-15 and CDV-23 in 1× PCR reaction buffer provided by the manufacturer. The reaction was carried out for 40 cycles with a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.) with the following temperature profile: 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min. Then 15 μl of the product was run on a 3% NuSieve 3:1 agarose gel (FMC, Rockland, Maine) in 1× TAE buffer at 135 V for 54 min and examined by UV transillumination following staining with ethidium bromide. The limit of detection of this assay was 0.02 $TCID_{50}$ units of Snyder-Hill strain CDV in dog PBMCs.

Statistical analysis. Analysis was done with the program SigmaStat for Windows, version 1.00 (Jandel Scientific, San Rafael, Calif.). Binomially distributed variables were analyzed by the Fisher exact test. Normally distributed two-group comparisons were made by the paired or unpaired Student's *t* test. Multiple comparisons of continuous variables (neutralizing titers, leukocyte counts, body temperature, and body weight) were made by analysis of variance (ANOVA) followed by pairwise comparisons with the Student-Newman-Keuls test (only P values of <0.05 are reported as significantly different). Due to the large number of comparisons in some instances (e.g., daily body weights) only the final result (significantly different or not by the Student-Newman-Keuls test) was often reported. Neutralizing titers were transformed to $\log_{10}$ values in order to normalize their distribution for statistical analysis. In many instances the statistical power of the tests to detect the differences actually seen between groups was <0.80. This does not affect positive results (i.e., where a significant difference is found between groups at $P < 0.05$) but indicates that negative results should be interpreted cautiously. In order to correct for the heavier body weight of males (mean ± standard deviation [SD] body weights for the 13 males and 13 females in all groups on the day of challenge were 1,404 ± 112 and 707 ± 58, respectively), body weights were normalized to the weight of each individual on the day of challenge. All animals were the same age within a 3-day range. Control animals receiving the NYVAC-RG ($n = 4$), ALVAC-RG ($n = 3$), and saline ($n = 3$) were grouped together for most analyses as there were no significant differences in their responses to vaccination or challenge.

RESULTS

Neutralizing antibody titers. Ferrets were vaccinated at 14 and 18 weeks of age and challenged i.n. at 22 weeks of age. Vaccinated animals received one of three CDV vaccines: the CDV F and HA proteins expressed in the NYVAC vector (NYVAC-CDV), the same proteins expressed in the ALVAC vector (ALVAC-CDV), or the live attenuated CDV vaccine. Control animals received rabies virus glycoprotein (RG) in the same vectors (NYVAC-RG or ALVAC-RG) or an injection of saline solution. Neutralizing titers were negligible (<1:4) in all animals at the time of the first vaccination and did not change in the controls after vaccination and challenge (Fig. 1). At the time of the second vaccination all the CDV-vaccinated animals had developed neutralizing titers. The inverse geometric mean titer (GMT) for the NYVAC-CDV group (229; range, 128 to

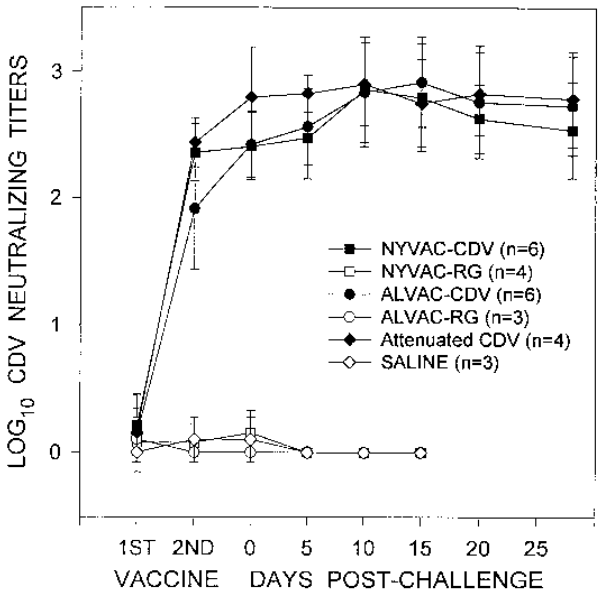

FIG. 1. Serum CDV-neutralizing antibody titers in ferrets vaccinated at 14 weeks (1st vaccine) and 18 weeks (2nd vaccine) of age and challenged i.n. with CDV at 22 weeks of age (0 days postchallenge). Values shown are mean ± SD inverse $\log_{10}$ values. All control animals died by day 17.

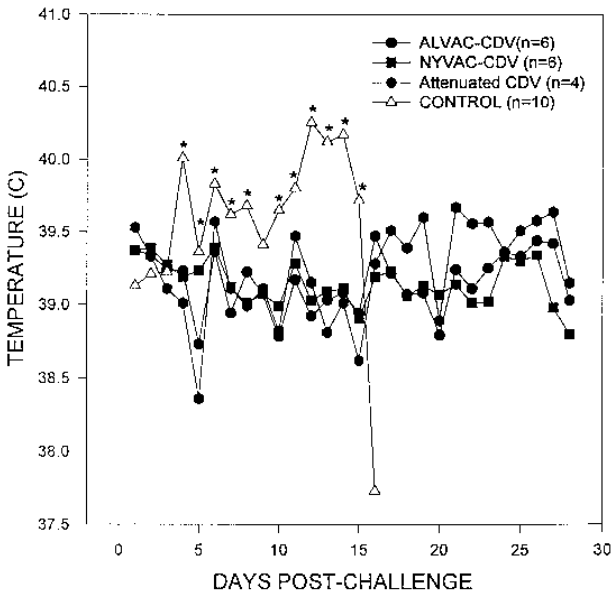

FIG. 2. Mean rectal temperature of ferrets vaccinated with the indicated vaccines and with control preparations (NYVAC-RG, $n = 4$; ALVAC-RG, $n = 3$; saline, $n = 3$) and challenged (i.n.) with CDV. Asterisks indicate days on which the control means were significantly greater than at least one of the vaccine groups by ANOVA and the Student-Newman-Keuls test for pairwise comparisons. All control animals died by day 17.

256) was nearly identical to that of the group receiving the live attenuated vaccine (276; range, 172 to 512), while the means of both of these groups were somewhat greater than the ALVAC-CDV group GMT (83; range, 12 to 256), although the differences were not statistically significant (ANOVA: $F = 3.72$, $P = 0.0527$). The second vaccination caused a significant increase in the GMT of the ALVAC-CDV group measured 4 weeks later on the day of challenge (266; range, 96 to 512; $t = 3.56$, $df = 5$, $P = 0.016$ by paired Student's t test), while the titers in the other groups did not increase significantly. Thus, on the day of challenge, there were no significant differences among the GMTs of the three vaccine groups (ANOVA: $F = 2.40$, $P = 0.1297$). Neutralizing titers after challenge did not change in the live attenuated vaccine group. In the NYVAC-CDV and ALVAC-CDV vaccine groups, titers increased slightly and peaked on day 10 or day 15 postchallenge. The increase was not statistically significant in the ALVAC-CDV group, but there was a significant increase from day of challenge to day 10 in the NYVAC-CDV group ($t = 2.67$, $df = 5$, $P = 0.046$ by paired Student's t test).

Survival. Ferrets in the control groups became symptomatic beginning on day 8 postchallenge. All 10 animals developed CNS signs and were euthanized. One animal succumbed on day 14, followed by five on day 16 and the remaining four on day 17. All of the animals in the three vaccine groups survived. The difference in survival was statistically significant when results for the 10 control ferrets (3 ALVAC-RG, 4 NYVAC-RG, and 3 saline treated) were compared to those for either of the recombinant vaccine groups (6 of 6 survivors in either NYVAC-CDV or ALVAC-CDV vs. 0 of 10 for all controls; $P = 0.000125$ by the Fisher exact test) or to those for the live attenuated CDV vaccine group (4 of 4 survivors vs. 0 of 10; $P = 0.0010$).

Fever. As shown in Fig. 2, control animals became febrile on day 4 postchallenge and their group mean temperature remained higher than the mean temperatures for the CDV vaccine groups through day 15 (with the single exception of day 9). The peak temperature differences were seen on days 12 through 14. The first death occurred on day 14. The peak differences were not large (1.2 to 1.5°C) and were not obvious when raw data from individual animals were examined during the course of the clinical examinations. On days when blood samples were drawn (days 0, 5, 10, 15, 20, and 28) temperatures were taken while the animals were anesthetized. This presumably accounts for the systematically lower temperatures seen on these days.

Leukocytopenia. The total number of peripheral blood leukocytes in the control group and the live attenuated vaccine group dropped steadily from the day of challenge through day 15 (Fig. 3). The number of leukocytes remained steady in both the NYVAC-CDV and the ALVAC-CDV groups throughout the study. While there were no significant differences among the groups on days 0 and 5, there were significant differences on day 10 (ANOVA: $F = 7.08$, $df = 25$, $P = 0.0017$), with both the control and live attenuated vaccine groups having significantly lower numbers than either of the recombinant vaccine groups ($P < 0.05$ by Student-Newman-Keuls test). Even greater differences were observed between the same groups on day 15 ($F = 30.3$, $df = 24$, $P < 0.0001$). Although the differences among the survivors in the vaccine groups did not reach statistical significance on day 20, the live attenuated vaccine group was still significantly leukocytopenic on day 28 (comparison among the three groups by ANOVA yields $F = 7.46$, $df = 15$, $P = 0.0069$; significantly lower than either recombinant group at $P < 0.05$ by Student-Newman-Keuls test). Differential analysis of peripheral blood smears from the ALVAC-RG group revealed that the decrease in total leukocytes was due entirely to a decrease in the total number of lymphocytes. For example, the total number of lymphocytes dropped from 6,873 ± 3,099 on day 0 to 970 ± 697 on day 10 while the total number of polymorphonuclear leukocytes remained constant.

Body weight. The body weights of the 10 control animals began to decrease on day 6 and continued to decline precipi-

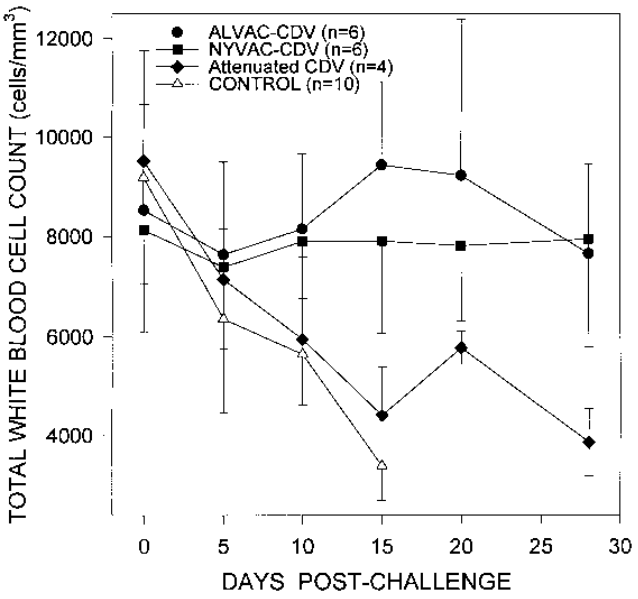

FIG. 3. Mean ± SD total peripheral blood leukocyte counts of ferrets vaccinated with the indicated vaccines and with control preparations (NYVAC-RG, $n = 4$; ALVAC-RG, $n = 3$; saline, $n = 3$) and challenged (i.n.) with CDV. All control animals died by day 17.

tously until these animals were euthanized (Fig. 4). In total, the control animals lost 15 to 20% of their initial body weights. On day 6, the body weights of the controls were significantly less than in the NYVAC-CDV group, and after day 6 the control body weights were consistently less than in both the NYVAC-CDV and ALVAC-CDV groups ($P < 0.05$). Surprisingly, the weight loss in the live attenuated vaccine group was similar to that seen in control groups (15% of their initial weight). More specifically, weight loss in the live attenuated vaccine group began earlier (day 2), reached a nadir at day 15, and then

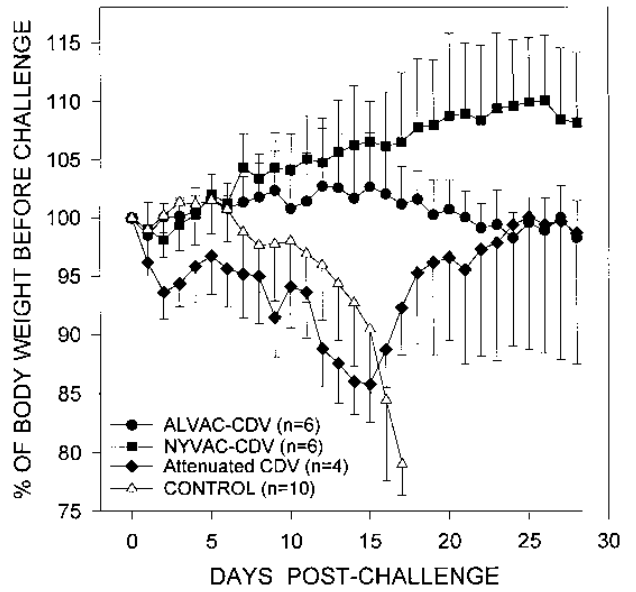

FIG. 4. Mean ± SD body weights of ferrets vaccinated with the indicated vaccines and with control preparations (NYVAC-RG, $n = 4$; ALVAC-RG, $n = 3$; saline, $n = 3$) and challenged (i.n.) with CDV. All control animals died by day 17.

rebounded. From day 3 through day 17 the ferrets which had received the live attenuated vaccine weighed less than those in both the NYVAC-CDV and ALVAC-CDV groups ($P < 0.05$). By day 18, the live-attenuated-CDV-vaccinated animals no longer differed in weight from the ALVAC-CDV animals, but they still weighed less than the NYVAC-CDV animals through day 26 ($P < 0.05$). Neither the NYVAC-CDV nor the ALVAC-CDV groups demonstrated a significant weight loss. However, the NYVAC-CDV animals continued to grow unabated after challenge and, by day 18 postchallenge, had a significantly higher average weight than the ALVAC-CDV animals. This observation was consistent through day 26 ($P < 0.05$). The growth curve of the ALVAC-CDV animals increased slightly through day 15 (when they were 102.6% of their day 0 weights) but drifted downwards thereafter so that by day 28 they were at 98.2% of their day 0 weights, while the body weights of the NYVAC-CDV animals had increased to 108.2% of the day 0 weights. These differences are not explained by having more males (which are heavier) in the NYVAC group (four males, two females) than the ALVAC group (four females, two males) because the patterns of growth within each group did not differ by sex (data not shown).

Clinical signs. Control animals developed clinical signs of distemper as early as day 8 postchallenge (Fig. 5). A typical chin rash develops early in distemper in ferrets and was evident in one control on day 8 and in most controls by day 11, when the prevalence in the controls was significantly higher than in the NYVAC-CDV or ALVAC-CDV groups, which never developed the chin rash (6 of 10 vs. 0 of 6; $P = 0.0338$ by Fisher's exact test). The chin rash is a prototypical manifestation of a generalized erythema which is readily discernable because the skin of ferrets is a light gray. The prevalence of erythema became significantly greater in the controls than in either the NYVAC-CDV or ALVAC-CDV group, which did not develop erythema, on day 12 (6 of 10 vs. 0 of 6; $P = 0.0338$). Interestingly, the attenuated CDV vaccine animals also developed the chin rash and generalized erythema. This difference did not reach the level of statistical significance for chin rash, but the prevalence of erythema in the attenuated vaccine group was significantly greater than in either the NYVAC-CDV or ALVAC-CDV group by day 13 (3 of 4 vs. 0 of 6; $P = 0.0333$). Conjunctivitis also developed in the controls but in none of the vaccinated animals. The difference, as compared to results for NYVAC-CDV and ALVAC-CDV groups, became significant on day 12 (7 of 10 vs. 0 of 6; $P = 0.0114$). Control animals but none of the vaccinated animals, were also significantly more likely to have a decreased level of activity by day 12 postchallenge (7 of 10 vs. 0 of 6; $P = 0.0114$). CNS signs did not develop in any vaccinated animal and were significantly more common in the controls by day 16 (6 of 10 vs. 0 of 6; $P = 0.0338$).

Detection of CDV RNA in PBMCs by RT-PCR. PBMCs were analyzed for the presence of CDV viral RNA by RT-PCR assay 5, 10, 15, 20 and 28 days after infection. As shown in Fig. 6, the 240-bp, CDV-specific PCR product was found on days 5, 10, and 15 after infection (this animal died before the next time point) in a ferret immunized with the control NYVAC-RG vaccine construct, but no evidence of CDV RNA was found in animals receiving the NYVAC-CDV vaccine, the ALVAC-CDV vaccine, or the live, attenuated CDV vaccine. The results for other animals in each vaccine and control group were identical to those of the representative animals, shown in Fig. 6. No evidence of CDV RNA was found in any of the vaccinated animals, but all control animals were positive at all time points tested (Table 1).

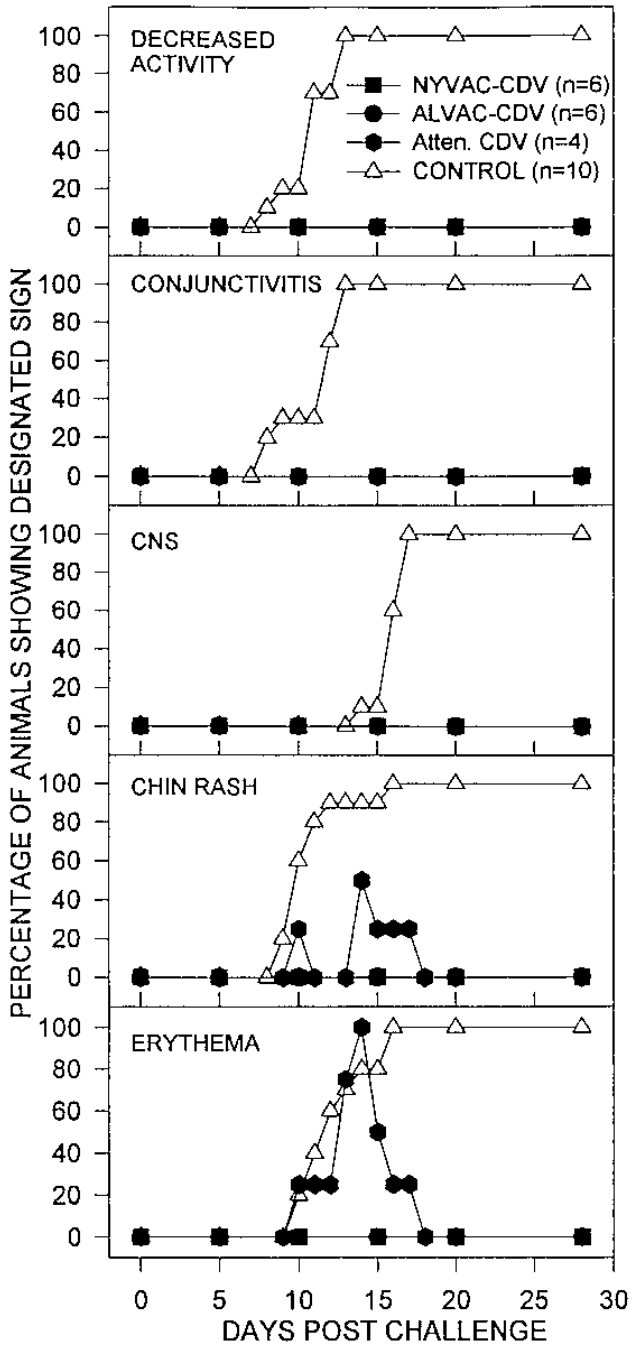

FIG. 5. Prevalence of indicated clinical signs of distemper among ferrets vaccinated with the indicated vaccines and with control preparations (NYVAC-RG, $n = 4$; ALVAC-RG, $n = 3$; saline, $n = 3$) and challenged (i.n.) with CDV. Animals which died (deaths occurred only in the control group) were counted in both the numerator and denominator when prevalences were calculated.

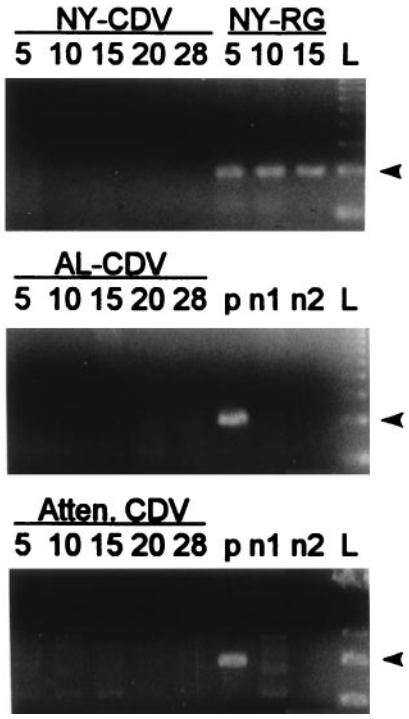

FIG. 6. Results of RT-PCR assay for CDV in PBMCs of immunized and control ferrets after i.n. challenge with the Snyder Hill strain of CDV. PBMCs were collected from ferrets immunized with the indicated vaccines 5, 10, 15, 20 and 28 days after challenge. The positive control (p) was tissue culture supernatant containing infectious CDV (Onderstepoort strain). The first negative control (n1) consisted of PBMCs from uninfected ferrets carried through the RT and PCR steps. The second negative control (n2) was water carried through the PCR step only. A 123-bp ladder (L) was used as for molecular size markers. The arrow at right indicates the 240-bp CDV-specific product near the 246-bp size marker.

DISCUSSION

All three vaccines used in this study elicited high, protective titers of neutralizing antibody after two immunizations. After one immunization the GMT in the ALVAC-CDV group was lower than in the other vaccine groups but increased to equivalent levels after the second immunization. These data confirm earlier studies which demonstrate that vaccines including the HA of CDV and other morbilliviruses induce significant neutralizing antibody titers. Both the NYVAC and ALVAC vectors have been shown to elicit MV-neutralizing antibody in dogs (48, 49). The small increase in titer after challenge with virulent CDV that was seen in the NYVAC group suggests that virus replication may have occurred after challenge. However, no viral RNA was detected in PBMCs of any vaccinated animal, suggesting that significant virus replication, if it occurred, was limited to the respiratory tract or associated lymphoid tissue.

All three vaccines protected against death due to distemper, but the live attenuated vaccine failed to protect against all clinical and laboratory signs of infection. The animals receiving

TABLE 1. Number of ferrets positive for CDV RNA by RT-PCR analysis of PBMCs following i.n. inoculation with 1,000 $TCID_{50}$ units of CDV

Vaccine group	No. of ferrets positive/total no. of ferrets at time (days) after infection				
	5	10	15	20	28
NYVAC-CDV	0/6	0/6	0/6	0/6	0/6
ALVAC-CDV	0/6	0/6	0/6	0/6	0/6
Attenuated CDV	0/4	0/4	0/4	0/4	0/4
Controls[a]	10/10	10/10	10/10	—[b]	—[b]

[a] Including NYVAC-RG ($n = 4$), ALVAC-RG ($n = 3$), and saline sham vaccination ($n = 3$).

[b] All control ferrets died between days 15 and 20 after infection.

the attenuated CDV vaccine became leukocytopenic, lost weight, and developed the erythematous rash typical of distemper. While weight began to recover by day 15 postchallenge and the rash disappeared by day 18 postchallenge, these animals still had significant leukocytopenia 4 weeks after challenge. Leukocytopenia is a hallmark of morbillivirus infections (37) and occurs in ferrets infected with CDV (32). A decrease in lymphocytes is principally responsible for the decrease in total leukocytes. The extent of the decrease in this study was less severe than was seen by Kauffman et al. (32) after administration of a canine tissue culture-derived CDV vaccine strain. Animals in that study became lethargic and febrile, which did not happen with ferrets in this study, and several animals in the previous study developed secondary bacterial pneumonia and died. All animals in this study survived. In addition, none of the animals which received the attenuated CDV vaccine in the present study showed evidence of viremia in PBMCs. This finding is somewhat surprising because the leukocytopenia, weight loss, and rash suggest that active virus replication occurred in these animals. We feel that the sensitivity of our assay (0.02 $TCID_{50}$ units) was sufficient to detect a clinically significant viremia. Alternatively, virus replication in the respiratory tract could have triggered an inflammatory response and production of cytokines such as tumor necrosis factor and interleukin-1 which could account for the decreased weight (through decreased appetite) and possibly the leukocytopenia (by recruiting lymphocytes into regional lymph nodes). The erythematous rash of measles is presumed to be caused by a cell-mediated immune response to infected endothelial cells (37). If also true with CDV, this would suggest that limited dissemination of virus to the skin (and perhaps other tissues) did occur but was not detected by our RT-PCR assay. It is also possible that strain differences between the live attenuated CDV vaccine and the challenge strain (Snyder Hill) account for the lack of complete protection conferred by the attenuated vaccine. However, such strain differences have not previously been shown to affect resistance to challenge infection. Furthermore, the HA and F genes expressed by the NYVAC- and ALVAC-based recombinant vaccines were also derived from a different strain (Onderstepoort) than was used for the challenge, but these animals were completely protected against developing signs of infection.

The results of the RT-PCR for detection of viremia in PBMCs indicate that if a vaccine protects against the development of viremia after i.n. challenge then it will also protect against death. Viremia developed by day 5 after challenge in the 10 control animals, all of which subsequently died of distemper. Day 4 was the first day that controls were febrile, and by day 5 a downward trend in the leukocyte count was also evident (although the difference was not statistically significant until day 10). Previous work has shown that initial replication of CDV occurs in the lymphoid tissue in the lungs, followed by local replication in lymph nodes and epithelial cells, which then leads to a generalized viremia (19, 34). All three vaccines used in this study successfully protected against the development of viremia, although the clinical data in the attenuated vaccine group, and the slight increase in antibody titers in the NYVAC and ALVAC groups, suggest that virus replication did occur at the site of inoculation.

Both recombinant vaccines protected completely against clinical and virologic evidence of infection. The ferrets receiving the NYVAC-CDV vaccine were afebrile, had no faltering in weight gain, and showed no overt clinical or laboratory evidence of infection. This strain of vaccinia virus has been specifically attenuated by deletion of host range and virulence genes and has been constructed for use as a human vaccine vector (46). Similarly, the ferrets receiving the ALVAC-CDV vaccine were afebrile and were solidly protected against clinical and laboratory signs of infection. That these animals showed little weight gain in contrast to the steady growth of the age-matched NYVAC-CDV animals suggests that a mild infection may have occurred, although this evidence is equivocal. This vector has also been developed for use in human vaccines and has already been through phase I trials (15, 47). This growth plateau through day 28 postchallenge, and the persistent leukocytopenia seen in the attenuated CDV vaccine group, suggest that future vaccine studies should continue beyond 28 days.

An animal model for evaluating the protective efficacy of measles vaccines should meet the following three criteria. First, since all *Morbillivirus* infections in their natural hosts have significant case fatality rates, the challenge infection should also present a significant risk of mortality. This criterion is particularly useful because protection against death (or euthanasia, when the animal is moribund) is an unambiguous measure of vaccine efficacy. The mortality rate of European ferrets after challenge with the Snyder Hill strain of CDV is essentially 100%. This is significantly higher than the mortality from measles, which is greatest among infants and very young children with complicating factors such as crowding, malnutrition, and poor access to supportive medical care, which can produce mortality rates of from 10 to 15% (1). Thus, the higher mortality may present a greater challenge for candidate vaccines than would be faced in eventual human trials.

Second, the challenge infection should produce an illness similar to measles when given via the natural, intranasal, route of infection. The clinical course of infection should produce clear clinical and laboratory indicators of severity which can be objectively measured in individual animals. CDV infection of ferrets and dogs is quite similar to measles in many key aspects. Both viruses are spread via the respiratory route and are highly infectious. MV replicates first in the respiratory epithelium and then spreads, via infected macrophages, to regional lymphoid tissue. CDV may bypass the initial replication in the respiratory epithelium, although this is unclear (19, 34), and directly infect macrophages in the lung or, perhaps, nasal epithelium. In either case, CDV, like MV, is found in regional lymph nodes within 2 to 4 days of infection. Virus replicates in this site and spreads, with intracellular carriage in cells of the lymphoid system being important in both cases, to other lymphoreticular tissues. Immune suppression and lymphopenia are features of both infections and contribute to opportunistic secondary bacterial infections, particularly pneumonia (32, 35, 37). After further replication, a secondary (cell-associated) viremia disseminates virus to many sites, particularly epithelial cells, throughout the body. The CMI response to MV infection of endothelial cells in subepithelial capillaries produces the typical measles rash. A more diffuse erythematous rash occurs with CDV in ferrets. At this point the immune response to measles is usually able to clear the infection, but in ferrets challenged with virulent strains of CDV, CNS involvement typically develops, leading to seizures and death. With more-attenuated CDV strains, virus clearance also occurs in ferrets (32).

Third, the ideal model system would involve protection by a measles vaccine against a homologous challenge infection (i.e., MV itself). Such an animal model is not available. The model described here is also homologous, employing CDV vaccines and a CDV challenge. Useful information can be provided from such experiments because of the close relationship among morbilliviruses. Many studies utilizing polyclonal sera and monoclonal antibodies have demonstrated that the nucleocapsid (N), matrix (M), and F proteins, and the HA pro-

tein to a lesser extent, have significant antigenic cross-reactivity (29, 43, 44). These relationships have also been confirmed by cDNA sequence analysis, showing that CDV and MV structural proteins F, N, M, and HA are 67, 66, 76, and 36% identical, respectively, at the amino acid level (9, 20). Since CDV and MV are so closely related, heterologous protection against CDV challenge can be elicited by immunization with measles vaccines. Heterologous MV vaccination of puppies has long been used by veterinarians as a way of overcoming maternal antibody inhibition of direct vaccination with CDV (7, 17, 25, 40, 45). MV vaccination of ferrets can also protect against CDV challenge (3). CDV and MV share at least one cytotoxic T-lymphocyte epitope on N (12), and heterologous protection studies suggest that such epitopes may be present on M and F as well (55). Protection studies also suggest that immunization with MV may prime the immune system to respond rapidly upon exposure to CDV. While MV does not induce CDV-neutralizing antibody, MV-immunized dogs do respond with a rapid, apparently secondary, neutralizing antibody response to CDV (7). Since T-helper epitopes have been identified on conserved structural proteins (N and F) (28), MV may prime these T-helper cells to respond when challenged with CDV. Thus, heterologous protection studies can also be carried out in the CDV-ferret model, using MV vaccines to protect against heterologous challenge with CDV.

What other small-animal models are available for measles vaccine development? Intracerebral inoculation of selected MV strains in rats, mice, and ferrets produces encephalitis and has been used as a model of subacute sclerosing panencephalitis (8, 51, 55). However, the intracerebral route of inoculation severely limits the utility of such models in testing measles vaccine strategies. Membrane cofactor protein (also called CD46) was recently identified as the cellular receptor for MV. Expression of CD46 on the surface of murine cell lines renders them to susceptible to MV infection (23, 36). This raises the possibility that transgenic mice expressing CD46 may also be susceptible to MV infection. Such a model would have the advantages inherent in the use of mice for immunologic studies. However, the first published report on such animals indicates that transgenic mice created with a cDNA clone of the C-CYT2 isotype of CD46 were resistant to MV replication in vivo, although cells cultured from these mice were susceptible to infection in vitro (30). It is thus far from certain that transgenic mice will sustain significant replication of MV in vivo. Another proposed model for measles research is intranasal infection of cotton rats with MV (56). While limited MV replication apparently occurs in cotton rats, no dissemination or clinical disease develops. For studies of protection against the development of symptomatic infection, the CDV-ferret model offers clear advantages over any of the other small-animal models thus far developed or proposed.

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