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ABSTRACT

Measles virus (MV) is being considered for global eradication, which would likely reduce compliance with MV vaccination. As a result, children will grow up without MV-specific immunity, creating a potential niche for closely related animal morbilliviruses such as canine distemper virus (CDV). Natural CDV infection causing clinical signs has never been reported in humans, but recent outbreaks in captive macaques have shown that CDV can cause disease in primates. We studied the virulence and tropism of recombinant CDV expressing enhanced green fluorescent protein in naïve and measles-vaccinated cynomolgus macaques. In naïve animals CDV caused viremia and fever and predominantly infected CD150+ lymphocytes and dendritic cells. Virus was reisolated from the upper and lower respiratory tracts, but infection of epithelial or neuronal cells was not detectable at the time points examined, and the infections were self-limiting. This demonstrates that CDV readily infects nonhuman primates but suggests that additional mutations are necessary to achieve full virulence in nonnatural hosts. Partial protection against CDV was observed in measles-vaccinated macaques, as demonstrated by accelerated control of virus replication and limited shedding from the upper respiratory tract. While neither CDV infection nor MV vaccination induced detectable cross-reactive neutralizing antibodies, MV-specific neutralizing antibody levels of MV-vaccinated macaques were boosted by CDV challenge infection, suggesting that cross-reactive VN epitopes exist. Rapid increases in white blood cell counts in MV-vaccinated macaques following CDV challenge suggested that cross-reactive cellular immune responses were also present. This study demonstrates that zoonotic morbillivirus infections can be controlled by measles vaccination.

IMPORTANCE

Throughout history viral zoonoses have had a substantial impact on human health. Given the drive toward global eradication of measles, it is essential to understand the zoonotic potential of animal morbilliviruses. Morbilliviruses are thought to have evolved from a common ancestral virus that jumped species and adapted to new hosts. Recently, canine distemper virus (CDV), a morbillivirus normally restricted to carnivores, caused disease outbreaks in nonhuman primates. Here, we report that experimental CDV infection of monkeys resulted in fever and leukopenia. The virus replicated to high levels in lymphocytes but did not spread to epithelial cells or the central nervous system. Importantly, like measles virus in macaques, the infections were self-limiting. In measles-vaccinated macaques CDV was cleared more rapidly, resulting in limited virus shedding from the upper respiratory tract. These studies demonstrate that although CDV can readily infect primates, measles immunity is protective, and CDV infection is self-limiting.

Canine distemper virus (CDV) is a member of the family Paramyxoviridae, genus Morbillivirus, and has been described as an important pathogen of dogs since the 17th century (1). Like the closely related measles virus (MV), CDV initially targets lymphoid tissues and causes profound lymphopenia and immune suppression (2, 3). Subsequently, the virus is disseminated to peripheral tissues, resulting in large-scale infection of epithelial cells. Infection of the central nervous system (CNS) is common, while this is a rare complication in measles. This difference, together with the severe immune suppression resulting in impaired CDV-specific immune responses, may explain the high case fatality rates observed in canine distemper compared to the low mortality rates reported for measles.

CD150 or signaling lymphocyte activation molecule F1 (SLAM/F1) is the primary entry receptor for wild-type morbilliviruses on immune cells (4, 5). A recombinant CDV (rCDV) with reduced binding affinity to CD150 was severely attenuated in ferrets, demonstrating that infection of CD150+ lymphocytes and dendritic cells (DC) is vital for entry and virulence (6). Poliovirus receptor-related 4 (PVRL4) was recently described as a cellular receptor for MV on epithelial cells (7, 8). PVRL4 is expressed in the adherens junctions at the basolateral surface of differentiated epithelial cells, explaining why morbilliviruses do not infect epithelial cells apically. Canine PVRL4 was recently demonstrated to be an entry receptor for CDV in the late stages of the disease (9). When ferrets were infected with an rCDV unable to infect epithelial cells, the virus replicated in lymphocytes and caused lymphopenia, but the ferrets did not display fever or rash; CDV could not be isolated from the throat, and epithelial cell infection was...
not detected by immunohistochemistry (10). This suggested that epithelial cell infection is important for clinical disease and virus transmission, as was previously hypothesized for MV (11).

Even though CDV was originally described as an infectious disease of dogs, it naturally infects a wide range of carnivores and has a relatively high propensity to cross species barriers. The virus has been reported to cause disease in members of the carnivore families of Ailuridae, Felidae, Hyaenidae, Mustelidae, Procyonidae, Ursidae, Viverridae, and Phocidae (12). In addition, CDV has also been reported to infect javelinas (13) and was recently detected in rodents (14). A natural outbreak with CDV in nonhuman primates was first reported in 1989 when 22 Japanese macaques (Macaca fuscata) housed in an animal research facility seroconverted to CDV (15). More recently, dramatic outbreaks in breeding colonies of rhesus macaques (Macaca mulatta) in China and cynomolgus macaques (Macaca fascicularis) in Japan were described (16, 17). Collectively these outbreaks included more than 10,000 animals and resulted in case fatality rates of 5 to 30%. The main cause of death was pneumonia, and a few animals displayed neurological signs. The frequent detection of secondary pathogens suggests that the high case fatality rates were probably related to opportunistic infections resulting from CDV-induced immune suppression. It remains unclear whether the viruses that caused these outbreaks had adapted to primates as experimental infection of macaques failed to recapitulate the dramatic clinical signs or lead to the death of the animals.

The World Health Organization is considering measles as a target for global eradication. Although measles eradication would save many lives, it will eventually result in reduced compliance to MV vaccination across the world. In this scenario children would be vaccinated with a high dose of rCDV/SHEGFP (6), low dose of rCDV/SHEGFP (6), or a high dose of rCDV/R252EGFP (6). MV-vaccinated animals had received an intratracheal (i.t.) infection with MV (100% TCD50) 3 months before inclusion in the CDV study as part of another experiment. In the current study, they were infected with either a high (n = 3 group) or low (n = 3 group) dose of rCDV/SHEGFP (6). Animals receiving a high viral dose were infected with 100 TCD50 of CDV, which 50% of the inoculum was administered by the intranasal (i.n.) route, and 10% was administered onto the eyes. Animals receiving a low dose were infected with 10 TCD50 of CDV exclusively via i.t. inoculation. Animals were euthanized at 5, 10, or 14 days postinfection (dpi) (n = 1/group/time point).

Necropsy. Animals were euthanized by exsanguination under deep ketamine/meztominoid anesthesia. Macroscopic detection of EGFP was performed as described previously (22–24). During necropsy, tissues from the upper and lower respiratory tract, including the nasal concha, nasal septum, trachea, primary bronchi, and lungs, were harvested and directly screened for EGFP expression. The lungs were inflated with 2% (wt/vol) low-melting-point agarose before being screened, as described previously (24, 27). After screening, tissues were transferred to buffered formalin (FA). Nonlymphoid tissues were collected directly in FA; lymphoid tissues were collected in either FA for immunohistochemistry or phosphate-buffered saline (PBS) for preparation of single-cell suspensions using cell strainers with a 100-μm pore size (BD Biosciences) and direct use for flow cytometry.

Blood samples. Small-volume blood samples were collected in Vacutette tubes (Greiner) containing K3EDTA as an anticoagulant at 3, 6, 8, 10, 12, and 14 dpi. Total white blood cell (WBC) counts were obtained using an automated counter (pceH100iV; Sysmex). Plasma was separated by centrifugation, heat inactivated (3 min at 56°C), clarified and stored at −20°C. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, washed, and resuspended in complete RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM l-glutamine, 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were counted using a hemocytometer and used directly for flow cytometry and virus isolation. Isolation of CDV was performed on Vero...
dogSLAM (VDS) cells (4) using an infectious center test as previously described (28). Virus isolations were monitored by UV microscopy for EGFP fluorescence after cocultivation with VDS for 3 to 6 days, and results were expressed as number of virus-infected cells/10^6 total cells.

Bronchoalveolar lavage (BAL). A BAL was performed at 3, 6, 8, 10, 12 and 14 dpi by i.t. infusion of 10 ml of PBS through a flexible catheter. In animals that were sacrificed, a BAL was performed postmortem by direct infusion of PBS (10 ml) into the right-hand side of the lung. BAL cells were resuspended in culture medium with supplements as described above, counted, and used directly for flow cytometry and virus isolation. Virus isolation was performed on VDS cells as described for MV on Vero-hCD150 cells (22).

Throat, nose, and eye swabs. Throat swabs (cytobrush plus; Mediscand Medical) and nose and eye swabs (polyester-tipped minitip urethral swab; Copan) were collected at 3, 6, 8, 10, 12, and 14 dpi in transport medium (Eagle’s minimal essential medium [EMEM] with Hanks’ salts, supplemented with lactalbumin enzymatic hydrolysate, penicillin, streptomycin, polymyxin B sulfate, nystatin, gentamicin, and glycero) and frozen at −80°C. After thawing, samples were vortexed, the swab was removed, and the remaining transport medium was used for virus isolation. Isolation of CDV was performed on VDS cells using an infectious center test as previously described (28). The isolations were screened for EGFP fluorescence at days 3 and 7 posttitration, and results are expressed as TCID₅₀/ml.

Serological analysis of macaque plasma and serum. Virus-neutralizing (VN) antibody responses were measured by an endpoint neutralization assay. Briefly, serial 2-log dilutions (starting at 2⁻³) of heat-inactivated plasma samples were incubated in triplicate with rCDV½EGFP(6), rCDVR252EGFP(6), or the MV Edmonston strain (MV½) for 1 h at 37°C in 96-well flat-bottom plates. Subsequently, titered VDSs (for CDV) or Vero (for MV) cells were added at a concentration of 1 × 10⁵ cells/well. Plates were incubated for 5 to 7 days at 37°C and visually monitored for cytopathic effects. VN titers were calculated as the 50% endpoint of triplicate measurements using the method of Reed and Muench (29). The additional dilution caused by the addition of the virus was included in the titer calculation. MV-F and MV-H glycoprotein-specific serum IgG antibody levels were determined by flow cytometry as described previously (30).

Flow cytometry. CDV-infected cells in PBMC were phenotyped by flow cytometry. PBMC were stained with CD45PE-Cy7 (where APC is allophycocyanin) (clone SP34-2; BD), CD45RAPE-Cy5 (clone L200; BD), CD14PerCP (where PerCP is peridinin chlorophyll protein) (clone M52E2; BD), and CD11cPE-Cy5 (clone A12; BD). Lymphoid organs were stained with the same set of monochromatic antibodies, with the exception of CD14PerCP. Single-cell suspensions were stained with CD3APC-Cy7 (clone SP34-2; BD), CD20PE-Cy5 (clone L27; BD), HLA-DRAPC (clone L243; Biolegend), and CD11cPE (clone BU15; BD). Sorted monocytes were used to identify infected DC in lymphoid organs. The infection percentages within the populations were determined by detection of EGFP. All flow cytometry was performed on a FACSCanto II (BD).

Histological and immunohistochemical analysis. Hematoxylin and eosin (H&E) staining was performed to evaluate histological changes. Immunohistochemical staining was performed using a fully automated BondMax immunostainer with a polymer-based peroxidase detection system. CDV-infected cells were detected using a polyclonal rabbit antibody to EGFP (Invitrogen). Dual-labeling indirect immunofluorescence was performed manually after pressure cooker pretreatment in 0.01 M citrate buffer, pH 9.0, using polyclonal rabbit anti-EGFP and monoclonal mouse antibodies to the macropage/DC marker CD11c (clone 5D11; Novocastra), the T-lymphocyte marker CD3 (clone F7.2.38; Dako), the B-lymphocyte marker CD20 (clone L26; Dako), and the epithelial cell marker cytokeratin (clone AE1/AE3; Dako). In all cases antigen binding sites were detected with a mixture of anti-mouse Alexa 568 and anti-rabbit Alexa 488 (Invitrogen). Sections were counterstained with 4',6'-diamino-2-phenylindole (DAPI) hard-set mounting medium (Vector). Fluorescently stained tissue sections were assessed, and digital fluorescent images were acquired with a Leica DFC350 FX digital camera and processed using Leica FW4000 software.

Infection of primary normal macaque bronchial epithelial cells. Primary well-differentiated normal macaque bronchial epithelial (wN-MBE) cells were obtained from bronchi of euthanized control macaques, as described previously for humans (31). Undifferentiated NMBE cells were grown in type 1 collagen and fibronectin-coated 775 flasks, trypsinized at 60 to 80% confluence, and seeded into 6.5-mm transwell inserts with a 0.4-μm pore size at 5 × 10⁵ cells per insert as described previously (32). After the NMBE cells had polarized and developed cilia, the monolayers on filters were washed by scratching with the tip of a pipette (25) and subsequently infected apically with 2 × 10⁵ TCID₅₀ of either rMV½EGFP(5), rCDV½EGFP(6), or rCDVR252EGFP(6). The inoculum was removed 90 min after infection. Cells were screened for infection at 72 h postinfection (hpi), fixed, permeabilized, and counterstained for cilia with anti-human β-tubulin IV. Cells were analyzed by confocal laser scanning microscopy using an LSM700 system fitted on an Axio Observer Z1 inverted microscope (Zeiss). Images were generated using Zen software.

Statistical analysis. The initial experiments in which animals were infected with two different CDV strains using high or low inocula were not designed to allow statistical analysis as we used three animals per group. Therefore, we have chosen to present the individual measurements of each animal. The symbols used per animal are identical between the figures, which allows cross-comparison of the data. The comparison of CDV infection in naive or MV-vaccinated animals was performed in 12 animals, of which 6 were vaccinated. The animals all received the same virus, but in each group three animals received a high dose, and three received a low dose. For statistical analysis we ignored the differences in doses. Animals were euthanized at 6, 10, or 14 dpi, prohibiting the use of the area under the curve for statistical analysis. Therefore, we compared the groups at each sampling point, using Student’s t test on log-transformed data. If the data were not normally distributed, we used a non-parametric test (Mann-Whitney U test).

RESULTS

rCDV½EGFP(6) and rCDVR252EGFP(6) efficiently replicate in macaques. Six morbillivirus-naïve cynomolgus macaques were infected i.n., i.t., and onto the eyes with a high dose (10⁵ TCID₅₀) of rCDV½EGFP(6) (n = 3) or rCDVR252EGFP(6) (n = 3). Three additional animals were infected with a 10-fold-lower dose of rCDV½EGFP(6), delivered exclusively by i.t. inoculation. This route was chosen as it is a highly reproducible route of administration for experimental MV infection of macaques. Within each group, one animal was euthanized at 6, 10, or 14 dpi. Flow cytometry was used to detect EGFP directly in blood and BAL samples obtained at 3, 6, 8, 10, 12, and 14 dpi. EGFP-positive (EGFP⁺) cells were detected in the BAL fluid (Fig. 1A) and peripheral blood mononuclear cells (PBMC) (Fig. 1B) of all animals. In PBMC, rCDV½EGFP(6) infected approximately 10 times more cells than rCDVR252EGFP(6) (Fig. 1B). In the low- and high-dose rCDV½EGFP(6) infection groups, similar infection percentages were detected in BAL cells and PBMC although virus replication peaked approximately 1 or 2 days later in the low-dose group (Fig. 1A and B). Such a dose-dependent peak of replication was previously described for MV (33). Macromolecular detection of EGFP during the necropsies of the low- and high-dose rCDV½EGFP(6)-infected macaques at 6 dpi showed EGFP fluorescence in lymphoid tissues (Fig. 1C to G).

rCDV½EGFP(6) and rCDVR252EGFP(6) are virulent in the macaque and cause lymphopenia and fever. Virus isolations were performed from eye, nose, and throat swabs, from BAL cells,
and from PBMC to confirm the pathogenicity of rCDV$^{SHE}$EGFP(6) and rCDV$^{R252}$EGFP(6) in macaques and demonstrate that the EGFP$^+$ cells produced infectious virus. Peak virus replication was reached at 6 to 8 dpi with both strains (Fig. 2A to E) although, in general, rCDV$^{SHE}$EGFP(6) replicated to higher levels than rCDV$^{R252}$EGFP(6). Furthermore, all groups developed transient leukopenia, but white blood cell (WBC) levels returned to normal from day 8 onward, except in the low-dose rCDV$^{SHE}$EGFP(6) infection group (Fig. 2F). All animals developed fever, which peaked earlier in animals infected with rCDV$^{SHE}$EGFP(6) (Fig. 2G). CDV-specific virus-neutralizing (VN) antibodies against either CDV strain were detected from 8 dpi onward (Fig. 2H to I), but no MV$^+$-specific VN antibodies could be detected in these animals (Fig. 2J).

**Tropism of rCDV in lymphoid tissues.** The phenotype of CDV-infected cells in PBMC and lymphoid tissues was assessed by flow cytometry and immunohistochemistry. Helper and cytotoxic T lymphocytes and B lymphocytes were the predominant CDV-infected cell types in PBMC, and infection percentages in these subpopulations exceeded those observed previously in experimental MV infection (22, 23, 34). Virtually no CD14$^+$ monocytes or CD3$^+$ CD8$^+$ natural killer (NK) cells were infected by CDV (Fig. 3A). Five key lymphoid tissues were examined, and highest infection levels were detected in B lymphocytes, followed by helper and cytotoxic T lymphocytes. Analogous to the virus isolations (Fig. 2D and E), macaques infected with a high dose of rCDV$^{SHE}$EGFP(6) reached higher infection percentages on 6 dpi, and low-dose-infected macaques reached higher infection percentages at 10 dpi (Fig. 3B). Flow cytometric staining with CD150 indicated that the CDV-infected EGFP$^+$ cells were CD150$^+$ (Fig. 3C).

Many EGFP$^+$ cells with the phenotype of DC were detected in the lymphoid tissues (Fig. 3D, left panel), and dual indirect immunofluorescence using anti-EGFP and anti-CD11c monoclonal antibodies showed that EGFP$^+$ CD11c$^+$ cells were present in these tissues (Fig. 3D, right panel). This was confirmed using flow cytometry to examine single-cell suspensions prepared from lymphoid tissues which also contained CDV-infected DC. After lymphocytes were excluded on the basis of forward and side scatter (Fig. 3E, red plot, left panel), large cells were gated as nonclustered...
FIG 2 rCDVSHEGFP(6) and rCDVR252EGFP(6) viruses are virulent in macaques and cause lymphopenia, fever, and VN antibody responses. The animals and legends correspond to those of Fig. 1. (A to E) Virus was isolated in VDS cells, and results are expressed in TCID<sub>50</sub>/ml of transport medium (eye, nose, and throat swabs) or as numbers of infected cells/10<sup>6</sup> total cells (BAL cells and PBMC). (F and G) White blood cell (WBC) counts and body temperature are shown relative to the starting values of the individual animals. All groups showed leukopenia and fever. (H to J) VN antibody responses to CDV strains SH (H) and R252 (I) were detected from 8 dpi onward, but MVEd-specific VN antibodies were not detected (J). VI, virus isolation.
ing cells that were CD3^+ CD20^- with a high level of HLA-DR expression (HLA-DR<sup>high</sup>). CDV-infected DC were shown as CD11c<sup>-</sup> EGFP<sup>-</sup> cells within this population (Fig. 3E, right panel).

**rCDV-infected epithelial cells were not detected in macaques.** During necropsy, the upper and lower respiratory tracts were sampled extensively; tissues were processed and paraffin embedded, and sections were analyzed for the presence of EGFP by indirect immunofluorescence and immunohistochemical staining. Submucosal infection of lymphoid and myeloid cells was detected in all tissues of the respiratory tract, including the adenoids, tonsil, nasal septum, trachea, primary bronchus, and lung (Fig. 4A to F). Notably, epithelial cell infection was not detected in any of the tissues at the time points examined. Although in several cases CDV-infected cells were detected within the epithelial layer, dual immunofluorescence consistently identified these as CD3<sup>-</sup>, CD11c<sup>-</sup>, or CD20<sup>-</sup> (Fig. 4G to I, insets) and cytokeratin negative (data not shown).

**Limited spread of rCDV in well-differentiated normal macaque bronchial epithelial cells.** Primary NMBE cells were obtained from the bronchus of a euthanized macaque and differentiated on the air-liquid interface (32). The monolayer of wd-NMBE cells was scratched and infected with cell-free MV<sup>K52</sup> EGFP(3), rCDVSHEGFP(6), or rCDVR<sup>K252</sup>EGFP(6) virus. MV<sup>K52</sup> EGFP(3), which served as a positive control, caused foci of infection around the scratch (Fig. 5A). No MV infection was observed outside the scratched area of the wd-NMBE cell culture. rCDVSHEGFP(6) and rCDVR<sup>K252</sup>EGFP(6) infected a minimal number of single cells in the wd-NMBE cell cultures around the scratch, but the infection did not spread or cause fusion (Fig. 5B and C, respectively). The top images of panels A, B, and C show a scan of a complete well; the images below are an enlargement of the dotted square box shown above. Staining of human β-tubulin IV clearly demonstrated the presence of ciliated cells in the wd-NMBE cell cultures (Fig. 5D, upper and lower panel, respectively).

**MV vaccination induces partial protection against CDV challenge infection in macaques.** Six macaques, vaccinated 10 months earlier with MV<sup>40</sup>, were challenged with either a low (n =
3) or high (\(n=3\)) dose of rCDV\(^{\text{H1005}}\)EGFP(6). Prior to challenge we confirmed that vaccinated macaques had MV-specific VN antibody levels that are considered protective against measles (mean, 2.4 IU/ml; range, 0.84 to 6.7 IU/ml). To compare CDV loads in vaccinated and unvaccinated macaques, virus was isolated from nose (Fig. 6A) and throat (Fig. 6B) swabs, from BAL cells (Fig. 6C), and from PBMC (Fig. 6D). All vaccinated macaques were productively infected by rCDV\(^{\text{H1005}}\)EGFP(6) independent of the dose. However, virus replication levels were significantly reduced, and the infection was more rapidly cleared in all samples tested from the vaccinated macaques (Fig. 6A to D). Interestingly, virus loads detected in the upper respiratory tract of vaccinated macaques were substantially reduced compared to those of unvaccinated animals, suggesting that MV vaccination might not offer sterile immunity but still reduces transmission of CDV between macaques during an outbreak, as observed in the breeding colony in China.

**Correlates of cross-protection in macaques.** In agreement with the finding that experimental CDV infection of macaques failed to induce MV-specific VN antibodies (Fig. 2J), MV vaccination failed to induce CDV-specific VN antibodies (Fig. 7A to C, 0 dpi). However, CDV-specific VN antibodies appeared approximately 8 dpi in both vaccinated and unvaccinated macaques although antibody levels were higher in vaccinated than in unvaccinated animals (Fig. 7A and B). Interestingly, MV-specific VN antibody levels of vaccinated macaques were boosted by the CDV infection (Fig. 7C), suggesting the existence of cross-neutralizing B-cell epitopes. Serum IgG antibody levels to the MV-F or -H glycoproteins were measured to determine the specificity of the antibodies mediating this secondary immune response. Although both curves showed a slight increase, boosting of F-specific IgG antibodies was more pronounced (Fig. 7D). At 10 dpi all vaccinated animals showed a more than 4-fold increase in MV-F-specific IgG antibody levels, while for MV-H-specific IgG, an increase was noted for only two out of four animals (data not shown). Accelerated clearance of CDV in MV-vaccinated macaques may also have resulted from cross-specific cellular immune responses. Whereas CDV infection of unvaccinated macaques resulted in severe leukopenia, with lowest WBC counts measured at 8 dpi, leukopenia in MV-vaccinated animals was less pronounced and was followed by a rapid increase in WBC counts from 6 dpi onward (Fig. 7E).

**DISCUSSION**

In the present study, we show that two recombinant CDV strains readily infected cynomolgus macaques and caused fever and leukopenia. The viruses predominantly replicated in CD150\(^+\) lymphocytes and DC, indicating that CDV was capable of using macaque CD150. Partial immunity in MV-vaccinated macaques from CDV challenge was observed, resulting in restricted virus shedding from the upper respiratory tract.

In recent years, we have studied the tropism of MV in ma-
caques (22–24). The predominant infection of CD150/H11001 lymphocytes and DC in macaques we report here for CDV corresponds well with the reported tropism of MV in macaques (22). In addition to being lymphotropic, in its natural host species CDV readily infects epithelial and neuronal cells (2, 3, 26). The epitheliotropic nature has also been described for MV in nonhuman primates (22, 25, 35) although CDV infects higher percentages of epithelial cells in carnivores than MV does in primates. Morbillivirus entry into epithelial cells requires the cellular receptor PVRL4. This receptor is present in the adherens junction on the basolateral side of epithelial cells, and entry into these cells is postulated to be important for viral shedding (7,36). In contrast to previously reported experimental infections with rMV or rCDV modified to prevent PVRL4 binding (10,11), we isolated significant amounts of CDV from BAL cells, throat swabs, and nasopharyngeal swabs, demonstrating shedding of CDV in the absence of detectable infection of epithelial cells. This observation suggests that recently proposed hypotheses explaining transmission of morbilliviruses (37) may not tell the complete story.

Even though both CDV strains infected macaque lymphocytes and DC, at the time points examined, we were not able to detect CDV in either epithelial cells or the CNS. Moreover, in vitro infection studies in primary wd-NMBE cells showed that cell-free rCDVSHEGFP(6) and rCDVR252EGFP(6) could infect single cells when the monolayer was scratched to expose the basolaterally expressed PVRL4, but both CDV strains were unable to spread to the neighboring cells. In contrast, these cultures were readily infected with wild-type MV. In vivo large numbers of CDV-infected immune cells were seen within the respiratory epithelium or in the respiratory submucosa, demonstrating that epithelial cells were exposed to CDV. In a recent report Sakai et al. described experimental infections of macaques with the CDV strain isolated dur-

FIG 5 Limited spread of rCDV in scratched well-differentiated normal macaque bronchial epithelial cells. (A) As shown in the upper panel, infection of wd-NMBE cells with cell-free rMVSHEGFP(3) (MV) caused several foci of infection around the scratch. The lower panel shows an enlargement of the boxed area. (B and C) Infection of scratched wd-NMBE cells with cell-free rCDVSHEGFP(6) or rCDVR252EGFP(6) resulted in single EGFP+/H11001 cells without cell-to-cell spread. (D) Staining with anti-human β-tubulin IV (red) identified the presence of ciliated epithelial cells in the wd-NMBE cell cultures infected with MV or CDV. CDVSH, rCDVSHEGFP(6); CDVR252, rCDVR252EGFP(6).

FIG 6 MV vaccination induces partial cross-protection against CDV. Comparison of virus isolation data from the six unvaccinated and the six MV-vaccinated rCDVSHEGFP(6)-infected macaques, expressed as geometric means ± standard errors of the means. All macaques were productively infected by rCDVSHEGFP(6), but virus replication levels were significantly reduced in the nose (A), throat (B), BAL cells (C), and PBMC (D) of MV-vaccinated macaques. Statistically significant differences are indicated with an asterisk (*P < 0.05). VI, virus isolation.
ing an outbreak among cynomolgus macaques in China. The authors showed that this virus not only could efficiently use both macaque CD150 and PVRL4 in vitro but also infected both lymphocytes and epithelial cells in vivo (17). These observations suggest that the continuing chain of CDV transmissions in macaques has resulted in adaptation of the virus, enabling cell-to-cell spread in macaque PVRL4-positive (PVRL4/H11001) epithelial cells. However, based on our data it is impossible to conclude whether the absence of viral spread in epithelial cells was based on poor binding affinity for macaque PVRL4 or on an intracellular block of virus replication.

In previous experimental morbillivirus cross-species infection studies, viruses were typically less pathogenic in the new host than in their natural host. Experimental infection of pigs showed that they were susceptible to CDV infection although the virus replicated exclusively in lymphoid tissues, and there were no clinical signs of disease (38, 39). Experimental infection of cats resulted in subclinical infection, and again the virus did not infect epithelial or neuronal cells or transmit from cat to cat (38–40). However, large outbreaks among felids in Africa and North America caused by CDV were reported in the 1990s (41–43), and an outbreak among tigers in a Japanese zoo was recently reported (44). Strikingly similar to these observations, experimental infection of seals with CDV led to an exclusively lymphoid and not epithelial infection, which did not transmit to sentinel hosts (45). However, in 1987 and 2002 CDV caused massive lethal outbreaks in Baikal and Caspian seals (46–49). Taken together, these observations are reminiscent of what we observe as a self-limiting nonlethal experimental infection in macaques, contrasting with recently reported lethal CDV outbreaks in macaques in China and Japan (16, 17). Apparently, a morbillivirus that crosses the species barrier and is transmissible within the new host species can readily adapt to achieve full virulence.

Immunological relationships between morbilliviruses have long been reported (50–52). Cross-reactivity of sera was shown in the 1980s using monoclonal antibodies. However, the cross-neutralizing capacity of specific antibodies is not fully understood. Örvell et al. reported that convalescent-phase sera from CDV-infected dogs could not cross-neutralize MV (53). Furthermore, it was shown that MV-monospecific antibodies against the H and F glycoproteins failed to cross-neutralize CDV (54). These data are surprising since vaccination with attenuated morbilliviruses cross-protects from infection with heterologous morbilliviruses. In fact, live attenuated and recombinant MV vaccines have been used to immunize dogs against CDV in the presence of maternal antibodies (19, 55, 56), resulting in partial protection from CDV even though CDV-specific antibodies could not be detected. In macaques we found that MV vaccination did not lead to detectable CDV cross-neutralizing antibodies, but MV-specific antibodies were boosted upon CDV challenge, indicating that cross-reactivity on an antibody level is present. In addition, we observed a rapid proliferation of WBC in MV-vaccinated macaques upon CDV challenge, reminiscent of a secondary cellular immune re-

![Graphs and diagrams illustrating correlates of protection in MV-vaccinated macaques.](http://jvi.asm.org/ downloads/4431.png)

**FIG 7** Correlates of protection in MV-vaccinated macaques. (A to C) VN titers against rCDVSHEGFP(6) (A), rCDVR252EGFP(6) (B), and MVEd (C) in MV-vaccinated and unvaccinated macaques before and after infection with rCDVSHEGFP(6). MV vaccination did not induce detectable CDV-specific VN antibodies, and CDV-specific VN antibodies appeared in both vaccinated and unvaccinated macaques at approximately 8 dpi (A and B). MV-specific VN antibody levels in sera from vaccinated macaques were boosted by the CDV infection (C). (D) Serum IgG antibody levels to the MV-F and -H glycoproteins were measured by a flow cytometric assay (30), demonstrating a boosting of antibodies specific to MV-F and MV-H. (E) WBC counts showed severe leukopenia in unvaccinated macaques, whereas leukopenia in MV-vaccinated animals was less pronounced and followed by a rapid increase of WBC counts from 6 dpi onward. All plots show geometric means ± standard errors of the means. Statistically significant differences are indicated with an asterisk (P < 0.05).
spontaneous. Combined, these data suggest that MV vaccination can induce CDV cross-immunity on both humoral and cellular levels. In conclusion, we have demonstrated that nonhuman pri- mates are susceptible to infection with two different recombinant CDV strains and that measles immunity provides partial protection and limits viral shedding. Thus, morbillivirus cross-specific immunity of the human population restricts the possibilities of CDV or other animal morbilliviruses replicating in or adapting to humans. However, if measles eradication is followed by significant drops in MV vaccination coverage, such adaptation cannot be excluded. Although this should not be used to argue against measles eradication, it reinforces the general idea that sustained MV vaccination and serological and virological surveillance of morbillivirus infections will remain essential. Potentially, the use of MV strains as vectors for vaccination against heterologous pathogens could provide the necessary incentive to sustain compliance to MV vaccination, retain morbillivirus seropositivity, and take advantage of what is one of the safest and efficacious live attenuated virus vaccines.

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