Disease Manifestations of Canine Distemper Virus Infection in Ferrets Are Modulated by Vitamin A Status1,2

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Abstract
The measles virus (MV) causes half a million childhood deaths annually. Vitamin A supplements significantly reduce measles-associated mortality and morbidity. The mechanisms whereby vitamin A acts against MV are not understood and currently there is no satisfactory small animal model for MV infection. We report on the development of a ferret model to study antiviral activity of vitamin A against canine distemper virus (CDV). CDV is closely related to MV at the molecular level and distemper in infected subjects is complex. In well-nourished, vitamin A-replete populations, acute measles results in a rapid decrease in circulating retinol levels that return to normal after the infection resolves (5–7). Isolated vitamin A deficiency is a major risk factor for severe measles disease and death (1). Finally, vitamin A supplements significantly reduce measles-associated mortality and morbidity in populations both with and without endemic vitamin A deficiency (5,8,9). Although the impact of vitamin A supplementation and therapy on measles can be great (10), the mechanisms that underlie these effects are not understood. To date, experiments to explore vitamin A–measles interactions have been severely limited because clinical MV infection occurs only in humans and primates (11–13).

Introduction
Measles virus (MV)6 is no longer endemic in North America, but remains the fifth leading cause of death globally (1) resulting in an estimated 530,000 deaths annually (2). Although death due to MV itself is rare, infection is typically accompanied by immunosuppression and acquisition of secondary infections that can be fatal in 5–15% of cases in many regions of the developing world (3,4). The interaction between vitamin A status and MV in infected subjects is complex. In well-nourished, vitamin A-replete populations, acute measles results in a rapid decrease in circulating retinol levels that return to normal after the infection resolves (5–7). Isolated vitamin A deficiency is a major risk factor for severe measles disease and death (1). Finally, vitamin A supplements significantly reduce measles-associated mortality and morbidity in populations both with and without endemic vitamin A deficiency (3,8,9).

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directly interfere with MV replication in vitro through interaction with type-I interferon-signaling pathways (B. Ward, unpublished results).

Canine distemper virus (CDV) and MV are both members of the Paramyxoviridae morbillivirus subfamily and share a high degree of genetic and functional similarity (18). CDV, like MV, is transmitted via the respiratory route and initially infects lymphocytes and monocytes, leading to systemic infection (19). In dogs and ferrets, CDV causes a disease that is highly reminiscent of measles in humans. Both are clinically defined by a maculopapular rash and fever, appearing 7–12 d after infection, and by at least one of the following symptoms: cough, coryza, conjunctivitis, or diarrhea (3,20,21). Similar to MV infection, CDV results in immunosuppression characterized by lymphopenia, suppression of lymphoproliferation, loss of the delayed-type hypersensitivity response, and the acquisition of secondary infections (1,20,22). Survivors generate high-titer neutralizing antibodies that offer lifelong protection from reinfection (23,24). CDV infection of ferrets has recently been described as a tractable laboratory model to investigate Morbillivirus pathology (20,25).

Ferrets may also be a useful model to investigate the interaction of vitamin A status and health. As in humans, ferret serum and liver vitamin A concentrations are sensitive to changing dietary levels of vitamin A (26–28). Ferrets possess functional retinoic acid receptors and vitamin A metabolic pathways that resemble their human counterparts, although serum and liver vitamin A concentrations in ferrets are ~4- and 20-fold higher than corresponding levels in humans, respectively (28–30). Ferrets have also been used to define the molecular mechanisms of the unexpected increase in cancer risk following β-carotene supplementation in cancer prevention trials in humans (31,32).

In this study, our principal goal was to examine the effects of vitamin A supplements on CDV morbidity in vitamin A–replete ferrets. We also sought to determine whether ferrets could be made vitamin A deficient and to generate preliminary data on the effects of CVD infection with and without supplementation in these ferrets.

Materials and Methods

Ferrets and diets. Six-wk–old male (n = 6) and female ferrets (n = 4) were obtained from Harlan, and an additional 22 male ferrets were obtained from Marshall Farms. Data were pooled from both males and females for vitamin A analyses, but females were excluded from analyses of CDV-induced morbidity because there was no evidence of successful infection (i.e., viremia or CDV disease) in these ferrets. Upon weaning, ferrets were divided into 2 equal groups and consumed, ad libitum, either a vitamin A–deficient diet (TD.04590) or a vitamin A–control diet containing 18,000 international units (IU)/kg (5400 μg/kg) retinol palmitate (TD.04591; Harlan Teklad). These diets were similar to those containing 18,000 IU/kg (5400 μg/kg) retinol palmitate (TD.04591; Harlan Teklad). These diets were similar to those previously published, except that the cottonseed oil was reduced to 75.0 g/kg and replaced with 30.0 g/kg soybean oil (33,34). Ferrets consumed their respective diets for ~48 h, 15–20% weight loss, severe dehydration, severe pneumonia and/or diarrhea, or extreme lethargy and/or depression. Blood was collected by cardiac puncture and liver biopsies were taken for tissue pathology (20,25). CDV infection of vitamin A replete, deficient and supplemented ferrets. Twenty-seven ferrets were born of vitamin A-replete mothers at Harlan and Marshall Farms (1). Upon weaning at 6 wk of age, ferrets consumed either a vitamin A-replete (control, C) diet or a vitamin A–deficient diet (D) for an additional 12 wk (2). At 18 wk of age ferrets were infected with 1.5 × 10⁶ tissue culture infectious doses of 5804P-eGFP/uN canine distemper virus; simultaneously, half the ferrets in each group received 30 mg retinyl palmitate (C+VA and D+VA). The supplemented ferrets were also switched to the control diet (3). CDV pathology was observed for 5 additional wk, or until terminal endpoints occurred, and ferrets were killed (4). Retinol concentrations were determined in serum (weekly after infection) and liver (upon termination). Three additional D ferrets were not infected, but observed for the same period (D, uninfected).

![FIGURE 1] Generation and CDV infection of vitamin A replete, deficient and supplemented ferrets. Twenty-seven ferrets were born of vitamin A-replete mothers at Harlan and Marshall Farms (1). Upon weaning at 6 wk of age, ferrets consumed either a vitamin A-replete (control, C) diet or a vitamin A–deficient diet (D) for an additional 12 wk (2). At 18 wk of age ferrets were infected with 1.5 × 10⁶ tissue culture infectious doses of 5804P-eGFP/uN canine distemper virus; simultaneously, half the ferrets in each group received 30 mg retinyl palmitate (C+VA and D+VA). The supplemented ferrets were also switched to the control diet (3). CDV pathology was observed for 5 additional wk, or until terminal endpoints occurred, and ferrets were killed (4). Retinol concentrations were determined in serum (weekly after infection) and liver (upon termination). Three additional D ferrets were not infected, but observed for the same period (D, uninfected). (10 mg Telazol/kg; Fort Dodge Animal Health) on d 0, 7, 14, 21, and 28 from the jugular vein and divided it into uncoated, EDTA-coated, and heparin-coated tubes. Three D ferrets were not infected, but were bled at the same intervals to control for the effects of the diet and blood collection on ferret survival. All ferrets were killed on d 35 (± 3 d) by pentobarbital overdose unless killed earlier according to the defined terminal endpoints of the study. These endpoints included failure to eat for >48 h, 15–20% weight loss, severe dehydration, severe pneumonia and/or diarrhea, or extreme lethargy and/or depression. Blood was collected by cardiac puncture and liver biopsies were taken for tissue vitamin A measurements. Ferret experiments were approved by the Institutional Animal Care and Use Committee of the Institut-Armand Frappier (Laval, QC).

Clinical symptoms of CDV infection (rash, fever ≥38.5°C, conjunctivitis, dehydration, diarrhea, cough, and coryza) and possible vitamin A deficiency or toxicity were recorded daily (20,35,36). No data on hypervitaminosis A in ferrets are available but, based on observations in humans and domestic animals, redness, flushed skin, desquamation, vomiting, and evidence of increased intracranial pressure might all have been anticipated. The severity of CDV disease was assessed by assigning each symptom a score of 1.0, taking the sum of points for each ferret and calculating the mean.

Viruses. After 12 wk of diet (d 0), anesthetized ferrets were inoculated intranasally with 1.5 × 10⁶ tissue culture infectious doses of CDV strain 5804P-GFP/uN, which carries the gene for the enhanced version of the green fluorescence protein (eGFP) in an additional transcription unit upstream of the N gene (25). In healthy, vitamin A–replete ferrets, this virus is not lethal but typically causes a disease that closely mimics measles in humans (rash, fever, conjunctivitis, cough, coryza, diarrhea, immunosuppression, and induction of neutralizing antibodies).
**Results**

**Supplements enhance vitamin A concentrations in control ferrets.** The repeated measures ANOVA demonstrated a quadratic trend in time that was greater in the C+VA group than in the C group \( [F(1,30) = 6.6, P = 0.022] \) (Fig. 2A). Liver retinol concentrations in C+VA ferrets \((2.07 \pm 0.35 \mu mol/g; n = 6)\) were higher than those of C ferrets \((0.69 \pm 0.07 \mu mol/g; n = 6)\) at the end of the 35-d period \((P = 0.002)\). Vitamin A supplementation did not adversely affect the health of C+VA ferrets. Supplemented ferrets behaved and appeared similar to C ferrets, overt symptoms of vitamin A toxicity were not observed, and growth rate of supplemented ferrets did not differ from that of C ferrets (not shown). Thus, vitamin A supplementation concomitant with CDV infection resulted in a sustained increase in tissue storage of vitamin A and a transient increase in circulating serum vitamin A over a 35-d period.

**Infection of female ferrets.** Four female ferrets were initially included in this study, one in each experimental group. Neither the C nor C+VA female ferret exhibited signs or symptoms of CDV disease or detectable CDV in their PBMC at any time during the experiment. As a result, the females were likely not infected at the dose used and were excluded from analysis of CDV morbidity data.

**Supplements reduce CDV morbidity in control ferrets.** We hypothesized that vitamin A supplementation would reduce...
CDV-associated morbidity and we predicted that C+VA ferrets would have less severe CDV disease than nonsupplemented C ferrets. MV infection in humans and CDV infection in ferrets cause an epidermal rash on the face (mouth, chin, ears, and nose), torso, and extremities, pyrexia (fever), conjunctivitis, coryza, cough, diarrhea, and dehydration that result from systemic virus infection as well as secondary infections (3,20,21). As expected, all C ferrets exhibited a whole-body rash and temperature \( \geq 38.5^\circ \text{C} \) by d 12 after infection; 2 ferrets also displayed conjunctivitis or soft stool, and 4 ferrets displayed diarrhea, cough, edema, or dehydration. All C+VA ferrets also exhibited a whole-body rash, but none exhibited an elevated temperature or any other symptoms of CDV infection. The mean CDV morbidity score of C ferrets was higher than that of C+VA ferrets \((P = 0.003)\) (Fig. 2B). Significant differences in temperature were observed over time \([F (11, 100) = 11.0, P < 0.001]\) and between experimental groups \([F (1, 100) = 7.1, P = 0.02]\) in the C vs. C+VA groups following infection (Fig. 2C). An interaction was also observed for experimental group and time \([F (11, 100) = 11.0, P < 0.001]\). The illness displayed by C+VA ferrets was so mild that it did not meet the clinical definition CDV disease (or MV disease in humans) and could not be unambiguously diagnosed as such in the absence of serological confirmation (3,20,21). Consistent with our hypothesis, vitamin A supplementation significantly improved morbidity due to CDV infection in vitamin A–replete ferrets.

**Viremia is independent of vitamin A status.** The mechanism of vitamin A against measles infection in humans is unknown, but retinol significantly inhibits MV and CDV replication in vitro (B. Ward, unpublished data). We predicted that reduced CDV morbidity in C+VA ferrets would be concomitant with reduced viral load in this group. However, we did not detect differences in the peak or pattern of either viral titer per \( 10^6 \) PBMC or in the percentage of CDV-infected PBMC (not shown) from C and C+VA ferrets over the course of infection. Similarly, expression of virally encoded green fluorescent protein was identical in C and C+VA groups (not shown). These data suggest that increasing concentrations of vitamin A in blood and tissue does not have major impact on CDV infection of PBMC or the skin epithelia.

**Vitamin A deficiency in ferrets.** We next hypothesized that vitamin A deficiency in ferrets could be induced and subsequently rescued with vitamin A supplementation. After 12 wk of control or deficient treatment, serum retinol concentrations in ferrets consuming the vitamin A–deficient diet \((0.47 \pm 0.09 \mu \text{mol/L}, n = 15)\) were much lower than in the C ferrets \((12.50 \pm 1.08 \mu \text{mol/L}, n = 12; P = 0.003)\). Analysis of liver retinol in a single D \((0.055 \mu \text{g/g})\) and C \((0.779 \mu \text{g/g})\) ferret at this timepoint suggested tissue vitamin A stores were also depleted.

These observations demonstrated that 12 wk of the deficient diet is sufficient to cause a profound state of vitamin A deficiency, which was accompanied by negative health and behavioral effects. Prior to CDV infection, 8 of 14 D ferrets developed cataracts or corneal clouding, and 6 of 14 displayed either disorientation or head-bobbing behavior. Additionally, 100% of C ferrets developed corneal clouding during the same period, behavioral and gastrointestinal disorders were otherwise not observed in the C group.

In addition, C ferrets exhibited linear growth during the entire experiment, whereas the growth of D ferrets ceased after 9 wk (Fig. 3A). Differences in body weight occurred over time \([F (16, 151) = 152.7; P < 0.001]\) following infection, and resulting in significantly lower weights in the D ferrets than in the C and D+VA ferrets \([F (1, 151) = 12.7, P = 0.004]\). An interaction occurred for experimental group and time \([F (13, 151) = 12.8, P < 0.001]\). In contrast, similar trends in body weight were observed over time \([F (16, 129) = 153; P < 0.001]\) and for both C and D+VA ferrets \([F (1, 129) = 8.0; P = 0.02]\); although D+VA ferrets remained smaller than C ferrets. An interaction

**FIGURE 3** Vitamin A treatment \((30 \text{ mg})\) on d 0 of infection normalizes the reduced growth rate \((A)\), reduced serum vitamin A concentration \((B)\), and elevated death rate due to CDV \((C)\) in vitamin A–deficient ferrets. Values are means ± SEM, \( n = 6 \) (C and C+VA), \( n = 12 \) (D), and \( n = 5 \) (D+VA). \(A\) Body weights after infection differed between D and C or D+VA. Time \((P < 0.001)\) and treatment \((P = 0.004)\) affected body weight (repeated measures 2-way ANOVA with a significant interaction, \( P < 0.001\) ) \((B)\) There was a linear trend over time that was greater in D+VA than in D \((P < 0.05, \) repeated measures 2-way ANOVA). \(C\) ***Different from C \((P < 0.001, \) log rank test).
occurred between C and D+VA ferrets over time \([F(16, 129) = 7.3; P < 0.001]\). D+VA ferrets stopped displaying disorientation and/or head-bobbing behavior within 24 h of supplementation, but we did not observe a similar improvement of gastrointestinal or ocular symptoms in these ferrets, possibly due to concomitant CDV infection of epithelial tissues.

Vitamin A supplementation completely reversed the deficiency in the D+VA ferrets (Fig. 3B). The repeated measures ANOVA of the D ferrets compared with the D+VA ferrets showed a linear trend in time that was greater among the D+VA ferrets \([F(1, 16) = 16.6; P < 0.05]\). Due to the mortality in D ferrets, the final timepoint (35 d) had to be excluded in these analyses. Consistent with this observation, mean liver retinol concentrations measured at d 35 or upon ferret deaths were significantly higher in D+VA ferrets \((2.12 \pm 0.29 \mu\text{mol/g, } n = 7)\) than in D ferrets \((0.015 \pm 0.004 \mu\text{mol/g, } n = 5; P = 0.003)\). These results demonstrate that profound vitamin A deficiency has an obvious negative impact on ferret health but is rapidly reversible upon high-dose vitamin A supplementation and replenishment of vitamin A in the diet.

**Profound vitamin A deficiency is lethal in ferrets.** All vitamin A–deficient ferrets were killed prior to d 35 after infection whether or not they were concomitantly infected with CDV because they lost 15–20% of their maximum weight, failed to eat for >48 h, or were severely dehydrated. The D ferrets had a median survival rate of 13 d after infection, whereas both D+VA and C+VA ferrets and all but one C ferret recovered from CDV infection and survived until d 35 (Fig. 3C; \(P < 0.0001\)). The survival of the D+VA ferrets is particularly striking given their profound state of deficiency at the time of infection (Fig. 3B). Infected and uninfected D ferrets had equivalent mean survival times (not shown). Although the CDV-infected D ferrets had signs of severe CDV morbidity (not shown), the relative roles of deficiency itself compared with CDV infection were difficult to determine because of the overlap of signs and symptoms (e.g., weight loss, anorexia, and neurologic changes).

### Discussion

Despite the efficacy of the live attenuated vaccine, measles infection causes ~500,000 deaths per year (2). Although no 100% curative therapy exists, high-dose vitamin A supplements dramatically reduce mortality and morbidity rates, especially in young children at the greatest risk of infection (8,9,37). Because infection depletes vitamin A, even in populations where deficiency is rare, and deficiency itself is a risk factor for severe disease and mortality, high concentrations of circulating and tissue vitamin A may be beneficial (4–7). We describe here, to our knowledge for the first time, a tractable animal model to study the impact of vitamin A on *Morbillivirus* infection.

High-dose vitamin A supplements transiently increased circulating vitamin A concentrations and caused a sustained increase in liver concentrations in the vitamin A-replete (C) ferrets (Fig. 2) without observable toxicity. The mean serum vitamin A concentration observed in C ferrets \((12.50 \pm 3.75 \mu\text{mol/L})\) is consistent with previously published values \((28–30)\) and 400–2000% greater than the normal range in humans \((1.05–3.05 \mu\text{mol/L})\) (38).

Because vitamin A supplementation decreases MV–associated morbidity and mortality in humans, we predicted the same result in CDV-infected ferrets. Consistent with our hypothesis, un-supplemented ferrets exhibited a febrile illness with conjunctivitis, cough, diarrhea, and/or dehydration that closely parallels the clinical manifestations of measles in humans (3,20,21). In contrast, supplemented (C+VA) ferrets developed only a transient illness with a mild rash and no fever. Given the genetic similarity between MV and CDV, as well as the remarkable consistency in the influence of vitamin A on measles in humans and on CDV in ferrets in this study, it follows that this model will be useful for determining the mechanism of vitamin A against *Morbillivirus* infections in vitamin A-replete individuals.

We suspected that vitamin A reduced morbidity by directly inhibiting CDV replication in vivo, as occurred in vitro for HIV \((41–44)\), herpes simplex virus (15), and MV (B. Ward, unpublished results). However, we did not detect any differences in viremia, the percentage of infected PBMC, or viral load (eGFP expression) in the skin of C and C+VA ferrets (not shown). As these assays do not directly measure kinetics of viral replication, vitamin A supplements could have had other effects such as delaying secretion from infected cells or blocking replication or clearance from secondary target tissues. Consistent with this last hypothesis, C+VA ferrets did not display respiratory, ocular, or gastrointestinal symptoms, whereas C ferrets displayed frank conjunctivitis, respiratory distress, and/or diarrhea. Despite repeated attempts, we were unable to collect adequate conjunctival impression samples to assess histopathologic changes in epithelial and goblet cell distribution described in humans with vitamin A deficiency (45).

Rodent models demonstrate that vitamin A deficiency leads to a T<sub>H</sub>1–biased immune response, whereas vitamin A supplements result in stronger T<sub>H</sub>2 responses (46–52). In humans, vitamin A deficiency during infection may promote T<sub>H</sub>1 responses in some conditions (53,54). Although we have not yet measured the T<sub>H</sub>1/T<sub>H</sub>2 balance in the response to CDV in ferrets, our preliminary data suggest that vitamin A supplements did not significantly alter the kinetics of cell-mediated immune responses such as the rash.

The strain S804P-eGFP/iN CDV used herein is a recombinant virus expressing eGFP as a separate transcriptional unit upstream of the N gene (25). In ferrets this virus typically causes rash, fever, and a range of other symptoms as well as immunosuppression. However, ferrets rapidly make neutralizing antibodies and recover fully from infection with this strain, unlike the parent CDV strain, which is rapidly lethal (severe symptoms typically appear within 1–2 d). Although the mechanism of attenuation of S804P-eGFP/iN is not yet defined, we elected to use this nonlethal strain to detect possible effects of altered vitamin A status in either sense: i.e., decreased morbidity in C+VA vs. C ferrets or increased morbidity in D vs. D+VA ferrets. In light of our findings, future experiments will include a trial of vitamin A supplementation in wild-type CDV infection.

In humans, hyporeteninemia (<0.7 \mu\text{mol/L} serum retinol) occurs in 20–92% of all acute measles cases even in populations where vitamin A deficiency is rare, although serum concentrations return to normal as the infection resolves (5,7,39). In contrast, serum retinol concentrations in C ferrets during the CDV infection remained relatively constant throughout infection. It is unknown whether retinol binding protein synthesis is suppressed during the acute phase of CDV infection in ferrets, as it is in humans with acute measles (40). Circulating vitamin A in ferrets consists of a higher proportion of retinyl esters than in humans (26), which could influence turnover rates. Here, we measured total vitamin A concentration and not relative concentrations of retinol and retinyl esters. It may be necessary to measure retinol and retinyl esters separately to fully assess
changes in vitamin A circulation during infection and supplementation.

Our final hypotheses predicted that profound vitamin A deficiency could be induced in ferrets and could be rescued with high-dose vitamin A supplementation. Twelve weeks of the vitamin A deficient diet was sufficient to severely deplete serum retinol and 17 wk was sufficient to deplete liver retinol stores (Fig. 3). Both serum and liver retinol were restored completely with 30 mg vitamin A and return to a vitamin A-replete diet. These data are consistent with the observation of a small but significant decrease in ferret serum retinol after 5 wk of consuming a vitamin A-deficient diet (26). We also observed that profound depletion of serum and tissue vitamin A is ultimately lethal in ferrets. Survival time of D ferrets after CDV infection varied with the supplier (~7 d for Marshall Farms and ~26 d for Harlan), raising the possibility that baseline vitamin A concentrations in the 2 groups differed. Vitamin A deficiency led to anorexia and arrested growth, disorientation or head-bobbing behavior, cataract development, soft stool, diarrhea, and lethargy within 9 wk of starting the deficient diet. Similar signs of deficiency have been observed in cows, rats, and mice fed suboptimal quantities of vitamin A (16,49,55,56). Vitamin A supplementation, however, rapidly restored even the most severely affected ferrets to apparent normal health.

Most ferrets were killed before the ultimate cause of death could be determined. In gnotobiotic rats, vitamin A deficiency is associated with increased bacterial translocation from intestine to kidney, which is not observed in deficient rats with a normal complement of intestinal bacteria (16). We predict that vitamin A deficiency rendered ferrets susceptible to a physiological challenge that would not normally be lethal (i.e., underlying infection), as observed in deficient humans who are more susceptible to bacterial, viral, and parasitic infections (17,38). Currently, we are measuring the kinetics of development of vitamin A deficiency in ferrets to identify when ferrets lack overt symptoms of deficiency but are at increased risk of severe morbidity and mortality from CDV.

To our knowledge, this is the first report of an animal model that mimics specific aspects of the interplay between vitamin A status and measles in humans. Vitamin A supplements can enhance vitamin A status in vitamin A-replete ferrets and reduce the severity of Morbillivirus infection, relative to nonsupplemented ferrets. This result closely replicates the observation that high-dose vitamin A supplements can reduce measles morbidity and mortality even in well-nourished populations (5,8,9). We also show, for the first time, that profound vitamin A deficiency can be induced in ferrets and is rapidly and completely reversed with vitamin A supplementation. We think that this model will allow detailed investigation of the cellular and molecular mechanisms of vitamin A against Morbillivirus infection in vivo.

**Literature Cited**


13. Retinoid signaling and activator protein-1 expression in ferrets given vitamin A in CDV-infected ferrets


