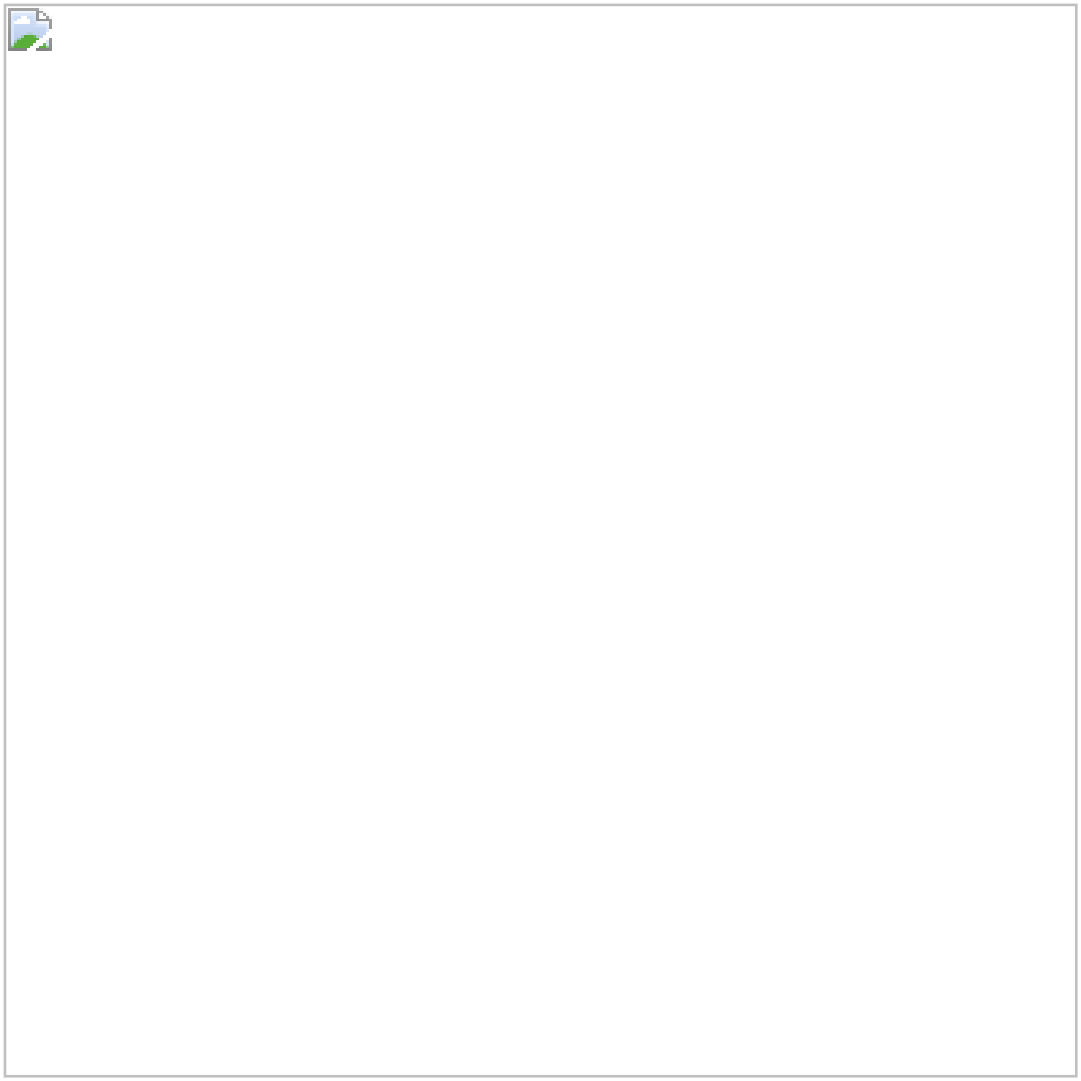

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MORTALITY FROM DUCK PLAGUE VIRUS IN IMMUNOSUPPRESSED ADULT MALLARD DUCKS

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ABSTRACT: Environmental contaminants contain chemicals that, if ingested, could affect the immunological status of wild birds, and in particular, their resistance to infectious disease. Immunosuppression caused by environmental contaminants, could have a major impact on waterfowl populations, resulting in increased susceptibility to contagious disease agents. Duck plague virus has caused repeated outbreaks in waterfowl resulting in mortality. In this study, several doses of cyclophosphamide (CY), a known immunosuppressant, were administered to adult mallards (*Anas platyrhynchos*) to determine if a resultant decrease in resistance to a normally sub-lethal strain of duck plague virus would occur, and induce mortality in these birds. Death occurred in birds given CY only, and in birds given virus and CY, but not in those given virus only. There was significantly greater mortality and more rapid deaths in the duck plague virus-infected groups than in groups receiving only the immunosuppressant. A positively correlated dose-response effect was observed with CY mortalities, irrespective of virus exposure. A fuel oil and a crude oil, common environmental contaminants with immunosuppressive capabilities, were tested to determine if they could produce an effect similar to that of CY. Following 28 days of oral oil administration, the birds were challenged with a sub-lethal dose of duck plague virus. No alteration in resistance to the virus (as measured by mortality) was observed, except in the positive CY control group.

Key words: Mallards, *Anas platyrhynchos*, mortality, duck plague virus, oil contamination, immunosuppression, experimental study.

INTRODUCTION

In nature, wild waterfowl may encounter a wide variety of environmental pollutants, which are discharged into aquatic systems either directly, as in oil spills, or indirectly through agricultural runoff to lowland areas. Chemicals that may have immunosuppressive effects can accumulate in wetland vegetation, zooplankton and water where they may be inadvertantly consumed by birds. For example, it has been estimated that approximately 3×10^6 metric tons of petroleum hydrocarbons are deposited annually in the marine environment (National Research Council, 1985). Wild waterfowl can be exposed to oil from both chronic sources (as in discharges from municipal and industrial plants and tanker operations) and from incidental emissions (as in oil spills). Although most documented oil-related mortality of birds occurs from external physical contact with oil, ingestion of petroleum from preening or consumption of contaminated food may cause detrimental systemic effects (Leighton, 1983). Many sublethal effects of ingested oil have been documented for waterfowl, including inhibition of immunological responses (Hodgins et al., 1977).

Duck plague, a herpesvirus infection of ducks, geese and swans, has been responsible for considerable economic losses in the commercial duck industry in the United States since 1967 (Walker et al., 1970). The only known significant epizootic of duck plague in free-ranging waterfowl occurred in 1973 at Lake Andes, South Dakota (USA), where mortality of wild mallards (*Anas platyrhynchos*) was estimated at over 40,000, and several hundred Canada geese (*Branta canadensis*) died (Friend and Pearson, 1973). Deaths from duck plague continue to occur sporadically in free-flying waterfowl, most commonly associated with small outbreaks in domestic and captive-reared birds (Spieker, 1978; Brand and Docherty, 1988).

Some chemical immunosuppressants are known to alter the resistance of animals to herpesvirus infections. Immunosuppressed laboratory mice are more susceptible to acute infection with herpes simplex virus (Rager-Zisman and Allison, 1976; Rajčáni et al., 1974). Immunocompromised humans also are more vulnerable to herpesvirus diseases (Merigan and Stevens, 1971).

The effect of immunosuppression on predisposition of birds to more severe disease from acute duck plague virus (DPV) infection has not been examined previously. The purpose of this study was to determine if mallards given an immunosuppressant would have decreased resistance to infection with a strain of DPV which is normally sublethal in mallards and if it would induce mortality in these birds. Cyclophosphamide (CY), known to alter the immune system of birds (Rouse and Szenberg, 1974; Hashimoto and Sugimura, 1976), was used as a model to test the hypothesis.

The effects of particular ingested environmental contaminants on resistance of birds to acute DPV infection have not been documented. This paper also presents data on the effect of crude and fuel oil ingestion on the resistance of mallards to acute DPV infection.

MATERIALS AND METHODS

Experimental animals

Game farm-raised adult male mallards were purchased from Pine Cone Farms (Warrenton, Missouri 63383, USA and Park Rapids, Minnesota 56470, USA). The ducks were housed in individual stainless steel cages (36 cm × 53 cm × 46 cm) in the Charmany isolation facility, University of Wisconsin (Madison 53706, USA). Water and a commercial duck feed (University of Wisconsin Agricultural Research Station, Arlington, Wisconsin 53955) were provided ad libitum. A photoperiod of 8 hr daily light was maintained throughout the experiment. Ducks were allowed to adapt to these conditions for at least 2 wk prior to testing.

Before each experiment, all birds were weighed and randomly assigned to treatment groups. Blood samples were taken from the jugular vein of all birds prior to experimentation, and from some of the birds post-challenge. Sera were heat treated (56 C for 30 min) and stored at −20 C. Liver samples were collected from all birds that succumbed to the experimental challenges and were stored at −70 C until DPV isolation was attempted.

Immunosuppressant

Cyclophosphamide (Cytoxan®; Mead, Johnson and Company: now Bristol-Meyers U.S. Pharmaceutical and Nutrition Group, Evansville, Indiana 47721, USA) served as a standard for immune suppression. It is an alkylating agent which functions through the destruction of rapidly proliferating cells. At low doses, CY affects both T-cell and B-cell activities (Bach, 1975). Stock CY was rehydrated with sterile double distilled water and administered orally by intubation. Doses of CY were calculated as mg CY/kg duck based on bird weights at the time of administration.

Environmental contaminants

South Louisiana crude oil (SLCO) and Bunker C fuel oil (BCFO), standard reference oils, were obtained from the American Petroleum Institute (Washington, D.C. 20037, USA) and were used as representative petroleum contaminants. SLCO is carried by tankers along the coastal waters of the eastern United States. BCFO is commonly used as fuel for marine vessels. Doses of oil were delivered into the gizzards of mallards using Teflon tubing and glass syringes, rinsed in acetone and hexane to remove oily residues.

Duck plague virus strains

The DPV stocks were prepared from field isolates from the livers of ducks which died in 1973 outbreaks of duck plague in Lake Andes, South Dakota (USA) and Coloma, Wisconsin (USA). Both the Lake Andes isolate (LA-SD-73) and the Coloma isolate (CO-WI-73) were previously passaged in ducks (two and four times, respectively), and these livers suspended in growth medium and stored at −70 C (Spieker, 1978). Antibodies to DPV in virus neutralization tests were detected with DPV-LA-SD-73, a strain which is highly pathogenic for mallards. Strains of DPV are antigenically identical, but since LA-SD-73 produced plaques more readily than CO-WI-73, it was better suited for neutralizing antibody assays (Burgess and Yuill, 1981). Duck plague virus CO-WI-73 was employed for all viral challenges of the birds. Each duck was inoculated intramuscularly with 1×10^3 plaque-forming units (PFU) of virus in 0.5 ml liver suspension. In healthy adult mallards, DPV-CO-WI-73 is non-lethal, although it has caused mor-

tality in mallard ducklings and other waterfowl species (Spieker, 1978).

Viral assays

Cultures of primary muscovy (*Cairina moschata*) and pekin (*Anas platyrhynchos*) duck embryo fibroblast cells were used for viral assays and serum neutralization tests. Cell cultures were prepared from 14- to 16-day-old embryos by methods previously described (Rovozzo and Burke, 1973). Medium 199 with Earle's salts (K C Biological, Lenexa, Kansas 66215, USA) was used for cell growth. The medium was supplemented with l-glutamine, 1 g/l; tricine, 1.8 g/l; streptomycin sulfate, 100 mg/l; sodium penicillin, 100 IU/l; gentamicin sulfate, 10 mg/l; and amphotercin B, 2.5 g/l. Heat treated (56 C for 30 min) fetal bovine serum (FBS) was added, to 10%; and the media was buffered with 1 M sodium bicarbonate, to 1%. The same medium, with the FBS decreased to 3%, was used for cell maintenance.

Cell monolayers were prepared in 24-well (16 mm diameter) tissue culture plates (Linbro Scientific Co., Inc. a subsidiary of Flow Laboratories, Inc., McLean, Virginia 22102, USA), using 2.0 ml/well of a 2.0 to 2.5 $\times$ 10^5 cells/ml cell suspension. Plates were incubated at 37 C, in an atmosphere of 5% CO_2, for 2 days prior to use.

Serum samples were tested for antibodies to DPV using the plaque reduction neutralization technique (Schmidt, 1979). Dilutions of sera (1:10) were combined with equal volumes of DPV-LA-SD-73 diluted to 100 PFU/0.1 ml and incubated at 40 C for 1 hr. The medium was removed from the cell cultures and the virus-serum mixtures were inoculated onto the cell monolayers in duplicate (0.2 ml/well) and allowed to adsorb for 1 hr at 40 C. A virus titration with FBS and an untreated cell control (without virus or test sera) were included in each test.

Maintenance medium (1.8 ml/well) was added, and the plates were incubated at 40 C for 3 to 4 days until a cytopathic effect was observed microscopically. The cells were fixed and stained with 1% crystal violet in 10% formalin and the plaques were counted. A $\geq$80% reduction in plaques from the standard was considered indicative of DPV antibody.

At the conclusion of each experiment, an attempt was made to isolate DPV-CO-WI-73 from the livers of all birds. Each liver sample was homogenized with a Ten Broeck tissue grinder (Bellco Glass, Inc., Vineland, New Jersey 08360, USA) and suspended as a 20% mixture in sterile medium. Serial 10-fold dilutions of the suspensions were inoculated in duplicate on cell monolayers (0.1 ml/well). After 1 hr incubation, maintenance medium was added, and the cells were incubated and stained as described previously.

Experimental design

Four experiments were conducted to determine the effect of an immunosuppressant on survival of mallards with sublethal infections of duck plague virus. First, in order to obtain a standard for later comparison, several doses of cyclophosphamide were administered to uninfected birds for 3 days, and mortality was recorded. In the other three studies, CY was given to the mallards on days 2, 3 and 4 post-inoculation with DPV-CO-WI-73. This schedule was based on the findings that resistance to herpes simplex virus in mice is reduced to the greatest extent when CY is administered 3 days after inoculation with the virus (Rager-Zisman and Allison, 1976). Mortality of DPV infected mallards treated with CY was compared to uninfected CY controls that received CY but not DPV, virus control birds that received DPV but not CY, and to a third group receiving no immunosuppressant or virus (Table 1). The uninfected, immunosuppressed control birds were held in a room separate from those inoculated with DPV. Deaths were monitored for 3 wk following inoculation, and then the surviving birds were euthanized by cervical dislocation. Necropsies were performed on all birds that died during the experiment.

To assess the effects of SLCO and BCFO on the resistance of mallards to challenge with a viral agent, each oil was orally administered to ducks (10 per group) for 28 days at 4 ml/kg/day. This dose previously was shown to increase mortality from *Pasteurella multocida* (Rocke et al., 1984). Following oil treatment, the birds were challenged with DPV-CO-WI-73. One group of mallards (8 birds) acted as negative controls; they received the virus but no oil. An additional control group (8 birds) was immunosuppressed with 40 mg/kg/day of CY on days 2, 3 and 4 post-inoculation. Both control groups were sham exposed (intubated, but no oil given) daily during the 28 days when oil was administered to the experimental birds. Mortality was measured for 3 wk post-inoculation. All dead birds were necropsied and the survivors were euthanized at the conclusion of the experiment.

Statistical analysis

Mortality data was analyzed using stepwise logistic regression procedures (Engelman, 1985; Kahn, 1983). This method tests the effects of categorical independent variables on a binary dependent variable. We used logistic regression to determine the effects of experiment, CY dose

TABLE 1. Design of experiments testing the effects of cyclophosphamide (CY) on resistance to duck plague virus (DPV) in mallard ducks.

Experiment	Number of ducks	CY dose[a] (mg/kg/day)	DPV challenge[b]
CY Toxicity	5	0	−
	6	10	−
	6	20	−
	6	30	−
	6	40	−
	6	50	−
CY/DPV Challenge 1	2	0	−
	5	50	−
	5	50	+
CY/DPV Challenge 2	5	0	−
	5	40	−
	5	0	+
	5	40	+
CY/DPV Challenge 3	5	25	−
	5	35	−
	5	50	−
	4	0	+
	9	25	+
	8	35	+
	5	50	+

[a] CY dose is CY administered orally over 3 days; Days 2, 3, and 4 post-DPV inoculation in those ducks given DPV.
[b] 1×10^4 plaque-forming units of DPV (+) administered intramuscularly; control birds received no virus (−).

and DP virus (independent variables) on mortality (dependent variable) of challenged birds. Analysis of variance and covariance procedures were used to test the effects of DP virus and CY dose on post-inoculation time until death.

RESULTS

Administration of CY prior to DPV-CO-WI-73 infection resulted in mortality of some birds. However, there also were deaths observed in groups treated with CY only. As expected, no mortality was seen in birds infected with DPV but not given CY (Table 2).

The results of these four experiments (as well as the results of control groups of a similar experiment) were combined for analysis. The logistic regression showed a significant ($P < 0.0001$) dose response effect due to cyclophosphamide (Fig. 1). Mortality also was significantly increased

TABLE 2. Mortality of mallards treated with cyclophosphamide (CY) for 3 days or CY for 3 days after challenge with duck plague virus (DPV).

CY dose[a] (mg/kg)	CY-only deaths/number tested[b]	CY/DPV deaths/number tested
0	0/12 (0)	0/17 (0)
10	0/6 (0)	
20	0/6 (0)	
25	0/5 (0)	3/9 (33)
30	0/6 (0)	
35	2/5 (40)	8/8 (100)
40	6/11 (55)	11/13 (85)
50	12/16 (75)	10/10 (100)

[a] CY dose is CY administered orally for 3 days or Days 2, 3 and 4 post-inoculation with DPV.
[b] Number in parentheses is percent mortality.

($P < 0.0001$) by the presence of DPV infection. Differences were not found ($P > 0.3$) among the five experiments conducted.

The influence of duck plague virus infection and CY dose on post-inoculation time until death was tested by analysis of covariance. The dose of CY administered had no significant effect on the time to death of the birds ($P > 0.16$). Infection with DPV caused the ducks to die significantly ($P > 0.0001$) more rapidly than those given CY only. The mean time to death was 8.9 days (±0.35) in CY/DPV birds, compared to 11.9 days (±0.44) in the CY group (Fig. 2).

The CY ducks that succumbed to challenge with DPV-CO-WI-73 had lesions consistent with duck plague (Leibovitz, 1971), including necrotic annular bands of the intestine, petechial hemorrhages on the heart, syrinx, and proventricular-esophageal junction, focal necrosis of the liver and general hemorrhagic enteritis. Gross pathologic changes observed in the CY-only group were more varied and not compatible with those of DPV infection. In these birds, the only consistent observation was the presence of bile in the intestine. Duck plague virus was recovered from eight of 32 of the birds in the CY/DPV group. The low percentage of DPV recovery from these birds was probably due to loss of the

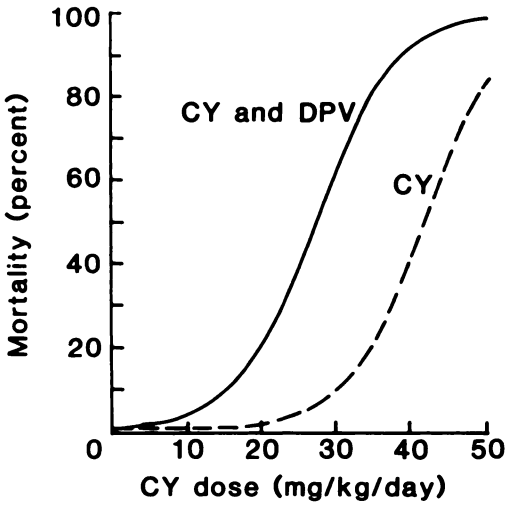

FIGURE 1. Dose response curves in mallards receiving cyclophosphamide (CY) or CY and duck plague virus (DPV) as predicted by computer model.

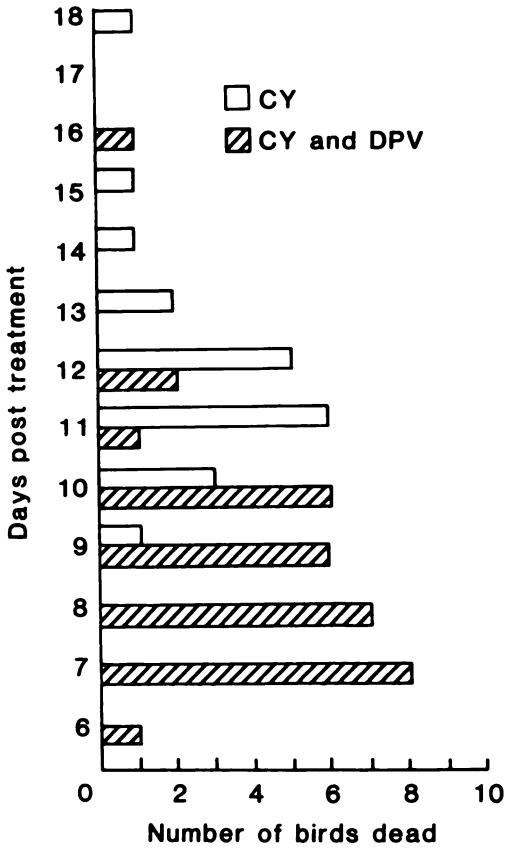

FIGURE 2. Time to death in mallards given cyclophosphamide (CY) or CY and duck plague virus (DPV).

virus during storage of the samples. Virus was recovered from all of the birds tested within 2 mo of sampling, but dropped to 28%, 6% and 0% in those samples tested after 11, 14 and 20 mo of storage at −70 C. Antibodies to duck plague virus were not detected in any pre-inoculation sera of the birds.

Oil ingestion did not appear to alter resistance of mallards to challenge with DPV. There was no mortality in birds treated with SLCO or BCFO, or in the control group which received no oil. There was, however, significant mortality (seven of eight birds) in the group which was immunosuppressed with CY for 3 days following DPV challenge. There was no significant antibody to DPV in the pre- or post-inoculation sera of surviving birds; however, this is not always a very reliable measure of exposure (Burgess, 1978). Duck plague virus was isolated from two of the seven ducks that died during the experiment.

DISCUSSION

A nonlethal strain of DPV caused mortality in immunosuppressed adult mallard ducks. This strain of duck plague virus has only been shown to be lethal in black ducks (*Anas rubripes*), muscovy ducks, Canada geese and 2-day-old mallards (Jacobsen et al., 1976; Spieker, 1978).

Although virus was not recovered in all cases, mortality of DPV-exposed birds probably ensued ultimately from viral disease, rather than from the possible toxic effect of CY. Lesions seen at necropsy were indicative of duck plague virus infection, and virus was recovered from all birds that were tested within 2 mo.

There were significantly more deaths in groups of birds that were CY-treated and challenged with the virus than in those administered CY only. Time to death of birds in the DPV/CY group was much shorter than in the CY-only group, and within a range consistent with acute duck plague virus infection. In natural infec-

tions of duck plague virus, death has been reported to follow exposure to the virus by 4 to 12 days (Leibovitz, 1971). Other species of waterfowl experimentally inoculated with DPV-CO-WI-73 had mean times to death ranging from 6.7 to 7.4 days (Spieker, 1978).

The toxicity of CY for mallards has not been reported previously. In our experiment, mortality was observed in uninfected ducks treated with CY at doses >30 mg/kg for 3 days. The LD_{50} values for a single oral dose of CY in laboratory mice, rats and dogs are 350, 94 and 44 mg/kg, respectively (Wheeler et al., 1962). The most common pathologic changes associated with CY toxicity in these species include hemorrhages of the lungs and lymph nodes, and accumulation of bile in the intestine (Wheeler et al., 1962). However, the lethal effects of CY may be caused by opportunistic infection due to decreased resistance to natural flora of the animal. Following CY administration, respiratory infections are common in experimental rats (Wheeler et al., 1962). The cause of death in the CY-exposed birds was not determined. It is possible that the ducks died of overt CY toxicity, but mortality may have been due to bacterial or viral agents.

No effects on resistance to duck plague virus challenge were observed for mallards chronically exposed to either SLCO or BCFO. Past studies have shown that these contaminants decrease resistance of ducks to *Pasteurella multocida*, a bacterial agent (Rocke et al., 1984). Immunological protection against herpesvirus infections is thought to be primarily cell mediated (Openshaw et al., 1979). The immune suppression observed previously for SLCO and BCFO did not appear to be antibody-mediated (Rocke et al., 1984), but different functions of the cellular immune system may be involved in herpesvirus and bacterial infections. Protection in avian species against *P. multocida* is thought to be primarily T-lymphocyte-mediated, but with a large cellular component, particularly involving macrophage activating factor-stimulated macrophages, which directly destroy the bacteria (Curtis, 1981). Typically, resistance against herpesviruses in mammals focuses more on cellular mechanisms such as antibody-dependent cell cytotoxicity and natural killer cell activity (Rouse and Babiuk, 1978). In similar studies of the effect of immunosuppressants on DPV infection, lead and a polychlorinated biphenyl were not able to alter DPV infection in carrier mallards (Barga, 1980), although polychlorinated biphenyls have been shown to decrease resistance to duck hepatitis virus (Friend and Trainer, 1970).

Different results may have been obtained if oil administration had been continued beyond viral challenge. The timing of exposure to immunosuppressants relative to herpesvirus challenge has been shown to be important in mice (Rager-Zisman and Allison, 1976). It is possible also that resistance to challenge might have been decreased if oil had been administered over a longer period of time prior to challenge, or with higher doses.

Although our findings do not indicate that oil exposure increases host susceptibility to DPV, our results with cyclophosphamide suggest that other immunosuppressants could contribute to mortality from this virus infection in natural waterfowl populations. Many potentially immunosuppressive compounds, including polychlorinated biphenyls, pesticides and heavy metals, may be found to persist in habitats occupied by wild birds. Exposure to immunosuppressive environmental contaminants could lead to decreased resistance of waterfowl to DPV, and cause mortality in birds infected with otherwise nonvirulent strains of the virus. The role of environmental contaminants in immunological suppression in the wild should be ascertained. Chemical surveillance for such contaminants in areas associated with outbreaks of duck plague would be useful to determine if any correlations exist.

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