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CYCLOPHOSPHAMIDE EFFECTS ON IMMUNE FUNCTION OF EUROPEAN STARLINGS

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ABSTRACT: We developed and tested a battery of immune function assays on adult European starlings (*Sturnus vulgaris*) exposed to the immunotoxicant cyclophosphamide (CY). Starlings were injected intraperitoneally for three consecutive days with saline or 20 mg/kg CY. Cyclophosphamide did not affect body mass or packed cell volume. However, spleen to body mass ratios and the number of viable spleen cells were lower in CY-treated birds when compared to controls. Peripheral white blood cell numbers were reduced in CY-treated starlings, and the decrease affected all cell types. Phagocytic ability of macrophages cultured from peripheral blood monocytes was impaired in cells from CY-treated birds. Additionally, CY treatment resulted in decreased lymphocyte blastogenesis to the T-cell mitogen Concanavalin A. The hemagglutination response to sheep erythrocytes was lower in birds that had received CY. Thus, these immunological methods detected chemically-induced immune dysfunction in starlings.

Key words: European starling, *Sturnus vulgaris*, cyclophosphamide, immunity, lymphocyte blastogenesis, macrophage phagocytosis, hemagglutination.

INTRODUCTION

Many chemicals are toxic to the immune system (Sharma, 1981) and may increase susceptibility of animals to disease (Exon et al., 1986). Environmental contaminants alter immune function in laboratory animals (Vos, 1977), and free-ranging wildlife may be particularly vulnerable to immune dysfunction caused by exposure to these compounds. However, chemically-induced immunosuppression of a wild species in its natural habitat has not been reported. In laboratory settings, wild species can be immunologically compromised by environmental contaminants. For example, under certain conditions, DDT and dieldrin significantly increase mortality in mallards (*Anas platyrhynchos*) exposed to sublethal chemical concentrations and duck hepatitis virus (Friend and Trainer, 1974). Although these chemicals are banned for general use in the United States, their persistence in the environment and their expanding use in foreign countries maintain their accessibility to wildlife. Additionally, petroleum oil (Rocke et al., 1984) and lead shot (Trust et al., 1990) deleteriously affected immune responses of mallards.

To date, the mallard has been the preferred model for studying immune function of wild birds. However, as interest grows in the field of avian immunology, the mallard will not be representative of all species. Other researchers have begun to explore alternative avian models. Antibody responses to sheep red blood cells (SRBC) have been measured in the red-tailed hawk (*Buteo jamaicensis*) and great horned owl (*Bubo virginianus*) (Lawler and Redig, 1984). Redig et al. (1984) also have adapted methods to stimulate lymphocytes in vitro for immunocompetency studies in bald eagles (*Haliaeetus leucocephalus*), red-tailed hawks and great horned owls.

Our objective was to develop immune function assays for the European starling (*Sturnus vulgaris*). These tests included macrophage phagocytosis, lymphocyte blastogenesis to a T-cell mitogen, and hemagglutination (HA) of SRBC. We chose the starling as a representative passerine be-

cause it is easily captured and maintained in captivity. Moreover, starlings adapt well to the use of artificial nest boxes, allowing both adults and nestlings to be used easily in field studies. We used cyclophosphamide (CY) to determine whether these immune function assays would successfully detect changes in the starling immune system.

MATERIALS AND METHODS

Adult starlings were obtained from the U.S. Department of Agriculture in the state of Washington (USA) in November 1991. Starlings were housed in an indoor flight cage (2 m × 2 m × 3.5 m), with an 8 hr light cycle, at the Wildlife Research Facility, U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, Oregon (USA). The cage was transected by 2 cm dowel perches and reverse osmosis and deionized water was provided from a continuously flowing 5 mm hose into a 5 cm deep rectangular plastic floor pan. Birds were fed ad libitum during their entire captivity from a perch feeder attached to the side of the cage and also from a 10 l ground feeder. Due to the high protein demands of an omnivorous bird such as a starling, the diet consisted of both a game bird maintenance chow (Ralston-Purina, St. Louis, Missouri, USA) and a protein supplement (Animax®, Ralston-Purina) mixed in a 2:1 ratio, respectively. Approximately 1 kg of fresh apples supplemented the diet once each week. Birds resided in the flight cage for at least 1 mo prior to use in experiments (January to February 1992).

During the dosing experiments, adult starlings were confined to separate but adjoining individual isolation cages (25 cm × 25 cm × 60 cm). Dowel perches and ad libitum food and water were provided throughout each study. Birds were acclimated to these cages for 1 wk prior to experiments.

Twenty-four adult starlings of either sex were divided randomly into two groups of 12, and then randomly assigned to control or treatment subgroups (n = 6 birds per subgroup). Each group of twelve was used in one of two identical studies, referred to collectively as Experiment I. Cyclophosphamide monohydrate (Sigma Chemical Co., St. Louis, Missouri) was dissolved in 0.85% sterile saline to a final concentration of 10 mg/ml. Fresh dosing solutions were prepared prior to each experiment. Starlings were injected intraperitoneally (IP) for three consecutive days with 20 mg/kg/day CY. Control birds were injected with equivalent volumes of 0.85% sterile saline. Birds were killed by carbon dioxide inhalation 24 hr following the last CY administration, and the following endpoints were measured: body and spleen mass, packed cell volume, spleen cellularity, total and differential white blood cell counts, macrophage phagocytosis and lymphocyte blastogenesis.

Blastogenesis data were only available from one of the two replicate studies of Experiment I due to equipment failures during the second study. Moreover, we also wished to study the humoral response of starlings to SRBC. Therefore, in Experiment II, 10 adult starlings (n = 5 birds per treatment) were challenged intravenously (IV) with 4×10^8 SRBC two days prior to beginning CY treatment. Lymphocyte blastogenesis and antibody titers were the only parameters measured in this experiment.

Pre-treatment body mass was measured two days prior to initiation of each study and on each dosing day. An electronic balance was used to weigh birds to the nearest 0.1 g. Final body mass data were collected on the day of necropsy. Spleens were excised, weighed and placed into RPMI 1640 tissue culture medium (Gibco BRL, Life Technologies, Inc., Grand Island, New York, USA) with 100 μ/ml penicillin and 100 mg/ml streptomycin (pen/strep) (Gibco), pH 7.2. Spleen mass was reported as the ratio of spleen mass to body mass.

Spleens were kept on ice approximately 2 hr until total viable spleen cellularity could be determined. Spleens were prepared for testing using the methods of Fairbrother and Fowles (1990). Single-cell suspensions were made by forcing individual spleens through clean nylon mesh with a plastic syringe plunger into RPMI 1640 with pen/strep, pH 7.2. Total viable spleen cell numbers were determined by trypan blue (Sigma) exclusion and a Neubauer hemocytometer (American Optical Instrument, Co., Buffalo, New York).

Blood samples (2 ml) were collected via jugular venipuncture. Starlings immediately were euthanized by carbon dioxide inhalation following blood collection. One ml of blood was placed into 4.5 ml polypropylene snap-cap tubes containing 1 ml of RPMI 1640 tissue culture medium with 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA) with pen/strep and 10 mM Hepes (Gibco), pH 7.2. This sample was kept at approximately 25 C for about 1 hr until it could be evaluated for macrophage phagocytosis. The remaining 0.05 to 1.0 ml blood was transferred into a 2 ml evacuated glass tube (Vacutainer®, Becton-Dickinson) containing sodium heparin and kept at approximately 25 C until evaluation (approximately 1.5 hr). From this sample, the remaining endpoints were measured.

Packed cell volumes were determined using

TABLE 1. Effects of cyclophosphamide exposure (total dose of 60 mg/kg at 20 mg/kg/day) on spleen cellularity, total and differential white blood cell counts in European starlings.

Treatment[b]	Spleen cellularity[a,b]	Total WBC[b]	Heterophil[b]	Lymphocyte[b]	Monocyte[b]	Eosinophil[b]	Heterophil to lymphocyte ratio
Control	9.5 (2.5)[c]	7.8 (1.1)	3.3 (0.7)	3.7 (0.5)	0.31 (0.06)	0.41 (0.12)	0.92 (0.15)
Cyclophosphamide	3.9 (0.5)[d]	3.1 (0.3)[d]	0.9 (0.1)[d]	1.8 (0.2)[d]	0.11 (0.02)[d]	0.13 (0.04)[d]	0.52 (0.06)[d]

[a] Number of viable leukocytes per spleen.
[b] Expressed as (10^6 cells per ml).
[c] Mean (SE).
[d] Significantly different from control ($P \leq 0.05$).

the methods of Dein (1984). Total peripheral leukocyte (WBC) count was determined using Natt-Herrick's staining solution (Dein, 1984) and a Neubauer hemocytometer. Leukocyte differentiation was performed on blood smears stained with Diff-Quick (Baxter Healthcare Corp., McGraw Park, Illinois, USA) and classified according to Dein (1984) and Campbell (1988). One hundred cells per slide were counted and the percent of each cell type recorded.

Leucocytes were separated from whole blood using histopaque-119 (Sigma) in a density gradient centrifugation (Sorvall Dupont, Wilmington, Delaware, USA). Cells were placed into 24-well tissue culture plates (Corning, Inc., Corning, New York, USA) at 4×10^6 cells per well in a final volume of 1 ml. Plates were incubated at 41 C in 5% CO_2 for 48 hr at which time non-adherent cells were removed and phagocytosis testing was performed. Macrophage phagocytosis testing was conducted using methods of Ragsdale and Grasso (1989) with incubation temperature raised to 41 C. Briefly, yeast cells (*Saccharomyces cerevisiae*) were conjugated with fluorescein isothiocyanate (FITC) and opsonized for 30 min at 41 C with fetal bovine serum; 50 µl of Hanks balanced salt solution (HBSS; Gibco) containing 1×10^7 yeast cells were added to each well. Plates were incubated for 15 min, washed twice to remove noningested yeast particles, and examined under an inverted, fluorescent microscope (Nikon Diaphot, Nikon, Inc., Garden City, New Jersey). Phagocytosis was determined by counting 100 macrophages and recording the number of yeast cells, from 0 to >4, within each macrophage. Results were expressed as percent of macrophages phagocytizing 0, 1 to 3, or ≥4 yeast cells (Zellikoff et al., 1991).

Blastogenesis was performed using the whole blood method of Lee (1977), optimized for the starling with the following modifications. Peripheral whole blood was diluted 1:10 with RPMI 1640 and 100 µl aliquots were dispensed into 96-well, U-bottom microtiter plates (Corning) containing either 0 or 10 µg of Concanavalin A (Con A) (Sigma) per well in 0.1 ml of medium. Plates then were incubated for 24 hr in a 5% CO_2, 41 C humidified atmosphere, followed by a 12 hr pulse of 0.5 micro Currie (µCi) of ^{3}H-thymidine (Amersham, Arlington Heights, Illinois) in 25 µl medium. The cells were harvested onto glass fiber strips for scintillation counting using a 24-well cell harvester (Brandel, Biomedical Research and Development Laboratories, Inc., Gaithersburg, Maryland, USA). The blastogenic response was expressed as a stimulation index (SI) derived by dividing the mean disintegrations per minute (dpm) for triplicate, mitogen-stimulated wells by the mean dpm for unstimulated wells.

Starlings were inoculated intravenously with 4×10^8 washed SRBC (Colorado Serum Company, Denver, Colorado, USA) in 100 µl sterile 0.85% saline. Because titers to SRBC peaked 6 to 7 days after antigen presentation in adult starlings (data not shown), birds in all experiments were challenged with SRBC 6 days prior to study termination. Hemagglutination titers to SRBC were measured from heat inactivated plasma (56 C for 1 hr) using the microtiter techniques of Fairbrother and Fowles (1990) with the following adaptation. Fifty microliters of plasma were serially diluted two-fold in 96-well, U-bottom microtiter plates with HBSS. Each well received 50 µl of a 0.05% suspension of SRBC. The plates were covered and left at approximately 25 C overnight. Titers were expressed as the inverse of the highest dilution showing hemagglutination.

All statistical calculations were performed using the StatView 512+ statistical program for Apple Macintosh personal computers (Brain-Power, Inc. Calabasas, California, USA). Results were analyzed for each endpoint using an unpaired Student's *t*-test after block effects between the two replicate studies in Experiment I were determined to be non-significant. Although data were available from two blastogenesis assays (Experiments I and II), they were considered to be independent experiments and analyzed separately. Only one data set was

TABLE 2. Effects of cyclophosphamide exposure (total dose of 60 mg/kg at 20 mg/kg/day) on selected European starling immune functions.

Treatment	Spleen/ body mass	Macrophate phagocytosis		Stimulation index	Hemagglutination titers
		<4 yeast/macro-phage	4 yeast/macro-phage		
Control					
Experiment I	0.72 ± 0.11[a]	33.0 ± 3.8	69.5 ± 3.6	23.5 ± 5.4	—[c]
Experiment II	—	—	—	21.2 ± 6.6	10.7 ± 0.7
Cyclophosphamide					
Experiment I	0.57 ± 0.08[b]	70.6 ± 2.2[b]	29.4 ± 2.2[b]	4.4 ± 1.1[b]	7.0 ± 1.0[b]
Experiment II	—	—	—	3.3 ± 1.2[b]	7.0 ± 1.0[b]

[a] Mean ± SE.
[b] Cyclophosphamide treated birds had significantly different values from control birds ($P < 0.05$).
[c] Parameter was not measured in this experiment.

available for the calculation of HA titers and it was analyzed using an unpaired Student's t-test. Differences between control and treatment groups were considered to be significant when $P < 0.05$.

RESULTS

Cyclophosphamide affected all immune function endpoints but not body mass or packed cell volume. Mean (±SE) body mass decreased slightly in both the control (from $\bar{x} = 83.5 \pm 1.7$ to 79.5 g ± 1.9, $n = 12$) and treatment group (from $\bar{x} = 81.8$ g ± 2.2 to 77.2 ± 2.4, $n = 12$) over the course of the study. However, body mass was not significantly different between the two groups either before ($P = 0.57$) or after ($P = 0.47$) treatment. Packed cell volumes also did not differ ($P = 0.71$).

Cyclophosphamide had marked effects on splenic parameters. The total number of viable spleen cells decreased 59% ($P = 0.03$) (Table 1), and mean spleen to body mass ratios decreased 21% ($P = 0.04$) in treatment birds (Table 2). Peripheral white blood cell numbers were reduced by 60% ($P = 0.004$) in CY-treated starlings. Mean heterophil, lymphocyte, monocyte and eosinophil numbers were also significantly lower in CY birds ($P < 0.05$) (Table 1). Heterophil to lymphocyte ratios decreased 43% in the CY group as compared to the control group (Table 1) ($P = 0.02$).

Macrophages from CY-treated birds ingested fewer fluorescent yeast than macrophages from control birds (Table 2) ($P = 0.0001$). The percent of macrophages from CY-treated birds with four or more yeast was 59% lower as compared to macrophages from control birds.

Proliferation of starling lymphocytes in vitro by Con A was reduced in the CY-treated birds by 81% ($P = 0.006$). Results from the blastogenesis assay were only available from one group of test animals ($n = 6$ birds per control and 6 birds per treatment). Therefore, the experiment was repeated with 10 additional starlings ($n = 5$ birds per control and 5 birds per treatment), and an 85% depression of the blastogenic response was demonstrated (Table 2).

Cyclophosphamide inhibited the antibody HA response to SRBC. Mean HA titers were 35% lower in the CY-treated starlings when compared to controls ($P = 0.04$) (Table 2).

DISCUSSION

These methods were successful for measuring impairment of immune function in starlings after exposure to the immunosuppressive agent, CY. Doses of 20 mg/kg/day CY for 3 days suppressed some aspects of non-specific and humoral immune responses in adult starlings. Cyclophosphamide also inhibited the lymphocyte proliferation response to the T-cell mitogen, Con A.

No gross signs of toxicity, such as emaciation, lethargy, or mortality were observed subsequent to CY exposure. Goldberg (1988) reported mortality in adult male mallards after exposure to >30 mg/kg/day CY for three consecutive days. However, in developing a battery of immune function assays for broiler chickens, Baecher-Steppan et al. (1989) did not observe mortalities until a single IP dose of CY was injected at 300 mg/kg; an immunosuppressive dose in the broiler chicken was 100 to 200 mg/kg CY injected in a single IP exposure. In a pilot study, adult starlings were given 50 mg/kg/day CY for 3 days (total dose = 150 mg/kg) which resulted in 17% mortality. Thus, it appeared that starlings were more sensitive than chickens but similar to mallards in susceptibility to CY toxicity.

Cyclophosphamide inhibits rapidly proliferating cells, including those of the immune system (Calabresi and Parks, 1975). Therefore, it was not surprising that splenic mass, spleen cellularity, and peripheral leukocyte numbers were lower in CY-treated starlings than in control birds. Suppression by CY of peripheral WBC numbers affected all cell types as indicated by differential WBC determination.

In chickens, higher heterophil to lymphocyte ratios occurred when these birds were subjected to stress such as increased dietary corticosterone (Gross and Siegel, 1983); these ratios were less variable and had a greater range of values than did individual cell numbers. In contast, heterophil to lymphocyte ratios were lower in CY-treated starlings due to a greater decrease in heterophil numbers than in lymphocytes. Assuming birds are similar to mammals, the recruitment of granulocytes is greater than lymphocytes in the periphery due to the granulocyte's status as a terminal, quick response cell type (Schalm et al., 1975). Moreover, mitotoxins such as CY are known to result in agranulocytosis (Bach and Strom, 1985), presumably through action upon the stem cells responsible for granulocyte formation. Cyclophosphamide also damages proliferating lymphocytes, as well as lymphocytic stem cells. However, non-proliferating lymphocytes present in circulation may not have been affected by CY. With the rapid depletion of granulocytes from the periphery, these non-dividing lymphocytes could have caused a higher number of this cell-type than heterophils.

Data are scarce regarding the effects of CY on non-specific immunity, particularly macrophage function. Cyclophosphamide induces monocytopenia but does not affect phagocytosis or bactericidal functions in mice (Buhles and Shifrine, 1977). It depresses antigen trapping in the spleen and inhibits antigen retention in the lymphoid organs of mice (Nettesheim and Hammons, 1970). Effects of CY on phagocytic functions of macrophages in birds have not been well-characterized. In starlings, CY decreased the number of circulating monocytes and also phagocytic ability of these cells after they had become macrophages in vitro. Starling macrophage phagocytic ability appears to be more sensitive to CY toxicity than macrophages from other animals. According to Bach and Strom (1985), macrophage number and function was not significantly altered in mammals given therapeutic doses of CY. In chickens exposed to a single IP dose of 150 mg/kg, phagocytic activity of peritoneal macrophages was not affected 3 days after exposure and slightly increased two days post-CY (Baecher-Steppan et al., 1989). The ability of starling monocytes to fully differentiate into functioning macrophages may have been impaired by CY.

In vivo exposure to CY suppressed the in vitro proliferation response of lymphocytes stimulated with the T-cell mitogen Con A. Cyclophosphamide suppresses this response in other species, such as mice and chickens (Dean et al., 1979; Baecher-Steppan et al., 1989). At elevated doses, CY is cytotoxic to T-lymphocytes (Dean et al., 1979). Sharma and Lee (1977) demonstrated a transient inhibitory effect of CY on the T-cell system of chickens. Reduction

of blastogenic responses to the T-cell mitogen PHA and morphologic degeneration of the thymus were evident after neonatal administration of CY. Linna et al. (1972) also demonstrated an initial decrease in the lymphocyte population of the thymus after exposing newly hatched chickens to high doses of CY.

Humoral responses of starlings were impaired by CY exposure as indicated by decreased HA titers to SRBC. These results were expected because studies from both mammalian and avian species have reported decreased antibody titers to a particulate antigen after CY exposure (Glick, 1971; Linna et al., 1972; Rouse and Szenberg, 1974).

Cyclophosphamide is a widely used immunosuppressive and chemotherapeutic agent. It is a potent immunotoxicant that suppresses some aspects of immune function in most animals in which it has been researched. In this study, CY suppressed all immunological endpoints measured in starlings. Thus, these immunological methods can detect chemically-induced immune dysfunction in starlings.

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