Immune Function of Cryopreserved Avian Peripheral White Blood Cells: Potential Biomarkers of Contaminant Effects in Wild Birds

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Abstract. Contaminants can cause detrimental effects in wild birds. However, these effects are difficult to measure in all but the most severe cases. Immune function is a sensitive and meaningful biological marker of contaminant-induced effects in captive birds but has more limitations in wild birds due in part to the lack of a proven blood preservation method. We developed methods to assess ex vivo immune function in wild birds using cryopreserved peripheral white blood cells (WBCs). We assessed the effects of cryopreservation on WBC viability and functionality in two immunoassays (concavalin A-induced T lymphocyte proliferation and macrophage phagocytosis) in domestic chickens (Gallus spp.: white Wyandottes and Dominiques) and validated this approach on cryopreserved WBC samples from wild American coots (Fulicia americana). Cryopreservation of chicken WBCs caused a slight but significant decrease in cell viability (99% \pm 0.2 SE for fresh cells versus 84% \pm 2 SE for cryopreserved cells, p = 0.001, Mann-Whitney U, n = 8). No difference was detected in viability between cells that were cryopreserved for less than 10 days (88% \pm 3.7 SE) and more than 50 days (89% \pm 1.3 SE) (n = 6). Overall, there was no statistical difference in the performance of cryopreserved cells compared to fresh cells. Across multiple experiments, cryopreserved T lymphocytes exhibited 200-900% stimulated proliferation above nonstimulated cells, and 40-80% of cryopreserved macrophages ingested yeast. 9,10,Dimethyl-1,2-benz-anthracene (DMBA) reduced proliferation and phagocytosis in cryopreserved cells over an ex vivo exposure range of 0-170 µM DMBA. Tests of immune function on American coot WBCs cryopreserved for up to 10 months (viability of 72% \pm 2.5 SE, n = 24) were similar to the cryopreserved chicken WBCs. This study will facilitate greater use of ex vivo immune function assays as tools to study effects of contaminant exposure in wildlife by demonstrating the viability and functionality of cryopreserved avian cells.

Environmental contamination can negatively affect wild birds in a variety of ways, including reduced fitness, altered immune function, and decreased survival. To understand the magnitude and extent of contaminant effects, sensitive markers are necessary. Biological markers integrate multiple factors (*e.g.*, individual susceptibility, effects from multiple contaminants, chemical bioavailability) that can influence a response to contaminant exposure, and thus have been widely used to assess chemically induced dysfunction (McCarthy and Shugart 1990; Huggett *et al.* 1992; Zelikoff 1998; Fossi *et al.* 1999). Immune function can be altered by contaminant exposure and has great potential as a biomarker in wildlife (Fairbrother 1994; Ross *et al.* 1996b; Keller *et al.* 2000; Zelikoff *et al.* 2000; Grasman 2002).

Numerous studies have evaluated contaminant effects on immune function in domesticated and nondomesticated captive birds, including chickens (Gallus spp.) (Glick 1974; Bridger and Thaxton 1983; Knowles and Donaldson 1997), Japanese quail (Coturnix japonica) (Grasman and Scanlon 1995; Fair and Ricklefs 2002), mallards (Anas platyrhynchos) (Fairbrother and Fowles 1990; Schrank et al. 1990), American kestrels (Falco sparverius) (Smits and Bortolotti 2001), and red-tailed hawks (Buteo jamaicensis) (Redig et al. 1991). In addition, immune function has been assessed in wild birds (Grasman et al. 1996; Saino et al. 1997, 1999; Bishop et al. 1998; Moreno et al. 1998; Raberg et al. 2000; Smits et al. 2000; Grasman and Fox 2001). Notably, the majority of these latter studies assessed measures of in vivo immune function (e.g., hypersensitivity skin test, antibody production) that required recapture of the same individual. Measures of ex vivo immune function (e.g., lymphocyte proliferation, phagocytosis) can be evaluated with a single blood sample. Samples can be transported overnight on tissue culture medium if processed quickly after arrival to the laboratory (Lahvis et al. 1995). Still, for remote locations, the transport of fresh samples from the study site to a laboratory can be problematic. Cryopreservation has been used to overcome the logistical constraints of sample

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transport for marine mammal cells (Ross *et al.* 1993; De Guise *et al.* 1998). However, we are aware of no reported studies on the cryopreservation of avian peripheral white blood cells (WBCs).

Here we investigated ex vivo immune function of cryopreserved avian peripheral WBCs as part of larger ongoing studies to evaluate immune function and contaminant levels in wild avian species. These larger studies required immune function assays that could be performed on a single, relatively small blood sample (2-3 ml). Cell viability and performance in two immune function assays (concavalin A-induced T lymphocyte proliferation and macrophage phagocytosis) were compared for fresh and cryopreserved cells. Assay reproducibility was also assessed. To validate the use of cryopreserved WBCs to assess contaminant effects on immune function, we demonstrated effects on cell proliferation and phagocytic ability from ex vivo exposures to a well-known immunosuppressant, 9,10,dimethyl-1,2-benz-anthracene (DMBA) (Dean et al. 1985; Ha et al. 1993; Trust et al. 1994). Finally, to verify the applicability of these methods in wild birds, we tested our methods on cryopreserved WBCs from wild American coots (Fulicia americana). This research contributes to an expanded use of immune function as a biological marker for contaminant effects in avian species.

Materials and Methods

Blood Collection

Domestic Chicken (Gallus *spp.*): Blood samples were obtained from adult female chickens (Dominique and white Wyandotte) sampled opportunistically from a commercial breeding facility. All birds sampled were banded for identification. Ten milliliters of blood were collected from the jugular vein with a 23-g Vacutainer winged collection kit (Fisher Scientific, Pittsburgh, PA) attached to a 10-ml preheparinized syringe. Blood was then placed in heparinized Vacutainers (Fisher) and stored on ice until processing, which occurred within 6 h of collection.

Wild American Coots: Adult American coots of undetermined sex were captured by mist net from a residential pond in Santa Cruz County and in the South San Francisco Bay. Birds were weighed, and 3–6 ml of blood were collected from the jugular vein with a 23-g needle attached to a 6-ml preheparinized syringe. Blood was transferred to heparinized Vacutainers and stored on ice until processing, which occurred within 10 h of collection. Birds were released immediately following sampling.

WBC Isolation

The methods of Strain and Matsumoto (1991) and Trust *et al.* (1994) were used with slight modifications. Briefly, for lymphocyte and monocyte isolation, whole blood was centrifuged at $1,000 \times g$ for 10 min and the plasma fraction discarded. Roswell Park Memorial Institute 1640 (RPMI, Sigma, St. Louis, MO), with hepes, 10% fetal bovine serum (FBS), and penicillin-streptomycin (200 U–200 µg/ml, Sigma) (C-RPMI), was added to the remaining packed cell fraction in a 1:1 dilution and mixed gently. The whole blood/C-RPMI fraction was then split to recover either lymphocytes or monocytes as follows.

Lymphocyte Isolation: Based on preliminary experiments, we found that a double gradient isolation yielded a mononuclear cell fraction containing a majority of lymphocytes that were sufficient for cryopreservation and subsequent proliferation assays. Therefore, lymphocytes were isolated with the mononuclear cell layer from an aliquot of the blood/C-RPMI mixture layered on top of 3 ml Histopaque 1.119 and 3 ml Histopaque 1.077 (Sigma) and centrifuged 700 \times g for 30 min. The mononuclear cell layer (above 1.077) was collected, and cells were washed twice in 10 ml C-RPMI.

Monocyte Isolation: Monocytes were isolated along with nonadherent leukocytes from an aliquot of the blood/C-RPMI mixture layered on top of 3 ml Histopaque 1.119 and centrifuged at $600 \times g$ for 30 min. The leukocyte layer (above 1.119) was collected, and cells were washed twice in 10 ml C-RPMI. Monocytes were further isolated by adherence prior to performing the phagocytosis assay (see macrophage phagocytosis methods section).

WBC Storage and Assessment of Viability

Cryopreservation was conducted following the procedures of Brousseau *et al.* (1994). Briefly, isolated cells were resuspended in Origen freeze medium containing 10% dimethyl sulfoxide (DMSO) (Fisher). Samples were stored in Nalgene cryogenic vials (Fisher) and placed in a Nalgene cryopreservation container for at least 4 h at -70° C and transferred to liquid nitrogen for storage until analyzed. Cryopreserved cells were thawed quickly by placing the cryovial in a water bath with constant agitation (41°C) and slowly diluting 1:10 with warm C-RPMI. The cell suspension was incubated at 41°C and 5% CO₂ for 4 h. Cells were washed twice and resuspended in C-RPMI to obtain the desired cell concentration for immunoassays.

For evaluation of fresh cells, lymphocytes and monocytes were isolated as described and resuspended in C-RPMI at the desired cell concentration for immune function assays. Cell viability of both cryopreserved and fresh cells was determined with trypan blue exclusion. Cells were counted and identified with Natt and Herrick dilutent (Natt and Herrick 1951) using the hemacytometer method (Gross 1984).

Immune Function Assays

Comparison between Fresh and Cryopreserved Cells: Blood samples collected from chickens were divided into halves prior to manipulation. WBCs were isolated from one half and used for immediate immune function tests, and WBCs isolated from the other half were cryopreserved for at least 24 h before being thawed for tests of immune function.

Mitogen-Induced T Lymphocyte Proliferation: Lymphocyte stimulation and proliferation assays were based on a modified method of Hovi et al. (1978) where a colorimetric 5-bromo-2'-deoxyuridine (BrdU) immunoassay (Boehringer Mannheim, Indianapolis, IN) was used to assess T lymphocyte proliferation. This assay is based on the measurement of incorporation of BrdU (a pyrimidine analog) into DNA during DNA synthesis. One hundred microliters of 3.5×10^6 live lymphocytes/ml (suspended in C-RPMI) were distributed per well in triplicate in 96-well plates with either concavalin A mitogen (Con A, Sigma) (2 and 5 µg/well) or no mitogen (nonstimulated). Plates were incubated for 48 h (41°C, 5% CO₂), after which the BrdU labeling agent was added to each well and the plates were incubated for an additional 22 h. Cells were harvested, and BrdU label absorbance was measured on an ELISA plate reader (Bio-RAD model 3550-UV) according to the manufacturer's instructions. For each individual experiment, lymphocytes from the same chicken (chosen randomly) were

included on all plates, and the proliferation of these samples was used as an internal standard to obtain a normalization factor to correct for interplate bias. Absorbance readings were expressed as percent of nonstimulated cells as follows: Each mitogen-stimulated well was (1) background corrected by subtracting the absorbance of the reagents only; (2) divided by the average absorbance of nonstimulated cells and expressed as a percent; (3) divided by the normalization factor; and (4) triplicate wells were averaged. This average value was used for statistical analysis.

Macrophage Phagocytosis: Fluorescein isothiocyanate (FITC, Sigma) conjugated yeast cells (Ragsdale and Grasso 1989) were opsonized by incubation with FBS for 30 min; yeast cells were then washed and suspended in phosphate buffered saline (PBS).

Monocytes were prepared following the procedures outlined in Trust *et al.* (1994). Briefly, 4×10^6 leukocytes/ml were suspended in C-RPMI and 1-ml triplicates of each sample were placed in wells of a Costar 24-well tissue culture-treated plate (Fisher) and incubated at 4°C for 24 h. Suspension media was discarded to remove nonadherent cells by inverting plates and flicking, leaving isolated macrophages, and 1 ml of fresh C-RPMI was added to each well. Plates were then incubated for at least 24 h at 41°C 5% CO_2 prior to performing the phagocytosis assay. Suspension media was removed and 50 μ l of 1 \times 10⁷ opsinized FITC-labeled yeast particles were added to each well to obtain an approximate ratio of 1:100 macrophages:yeast cells. Plates were incubated for 15 min at 41°C 5% CO_2 , washed three times with PBS, placed on ice to stop phagocytosis, and examined under an inverted fluorescent microscope (Nikon Diaphot, Nikon) at 400× magnification. Phagocytosis was quantified by counting the number of yeast cells ingested by approximately 100 macrophages per well. A phagocytic index (macrophages ingesting 0, 1-4, > 4 yeast) was calculated (Zelikoff et al. 1991).

Ex Vivo *Exposures:* WBCs were exposed *ex vivo* to DMBA (Sigma) to evaluate the sensitivity of cryopreserved WBCs to a known immunosuppressant. DMBA was dissolved in DMSO (tissue culture grade) and diluted in C-RPMI to obtain desired concentrations (0, 1.2, 5, 12, 50, 170 μ M/well). The final concentration of the DMSO carrier was ~0.125% in the plate wells. Results were expressed as the percent suppression of cell proliferation or phagocytosis of DMBA-exposed cells compared to vehicle-exposed controls.

Statistics

Statistical tests (ANOVA, two sample *t*-test, linear regression) were performed using SYSTAT (version 10, 2000). Nonparametric equivalents (Mann-Whitney U test) were used when necessary. Significance was reported if p < 0.05.

Results

Chickens

Lymphocyte Isolation: The mononuclear cell layer (above Histopaque 1.077) yielded an average of 1.4×10^7 lymphocytes/ml of blood ($\pm 1.5 \times 10^6$ SE, n = 9). The majority of the WBCs recovered were lymphocytes (99.4% lymphocytes, 0.4% monocytes, 0.2% granulocytes), whereas the ratio of lymphocytes to thrombocytes was approximately 1:1 in the cell suspension. The use of a double gradient allowed the isolation of granulocytes (above Histopaque 1.119) in addition to lymphocytes (data not shown).

Monocyte Isolation: The monocyte isolation procedure with a single gradient (Histopaque 1.119) yielded an average of 1.7×10^7 leukocytes/ml of blood collected ($\pm 1.9 \times 10^6$ SE, n = 12). The leukocytes were composed of 95.0% lymphocytes, 2.1% monocytes, and 2.9% granulocytes, and the ratio of leukocytes to thrombocytes was approximately 1:1 in the cell suspension. Single gradient isolation (Histopaque 1.119) yielded approximately five times more monocytes than the mononuclear cell layer in the double gradient isolation (above Histopaque 1.077), most likely because cells were being lost in the 1.077 layer. Nonadherent cells (*e.g.*, granulocytes, lymphocytes) were removed along with the suspension media 24 h after monocytes were placed in the 24-well plates, leaving activated macrophages in the wells (Trust *et al.* 1994).

Cryopreservation of Isolated WBCs: Cryopreservation of isolated WBCs (lymphocytes and monocytes) significantly reduced cell viability (p = 0.001, Mann-Whitney U, n = 8). The average viability for fresh cells was 99% \pm 0.2 SE compared to 84% \pm 2 SE for the cryopreserved cells. However, the relative viability in the cryopreserved cells was more than adequate for the immune function tests (see discussion). The length of time that cells were cryopreserved did not measurably affect cell viability based on a comparison of viability of cells cryopreserved for less than 10 days (88% \pm 3.7 SE viable) versus cells preserved for greater than 50 days (89% \pm 1.3 SE viable) (n = 6).

Immune Function of Fresh and Cryopreserved Cells: No statistical difference was detected between the proliferation of cryopreserved lymphocytes compared to fresh lymphocytes (two-way ANOVA, $F_{1,16} = 0.92$, p = 0.35). In addition, no difference was found in proliferation at 2 or 5 µg/well Con-A (two-way ANOVA, $F_{1,16} = 0.01$, p = 0.92) (Figure 1). Across multiple experiments, cryopreserved mitogen-stimulated lymphocytes exhibited between 200–900% proliferation when compared to nonstimulated cells.

Because varying numbers of thrombocytes were isolated along with lymphocytes in the mononuclear cell layer (above Histopaque 1.077) of the double density gradient separation, and the effect of thrombocyte concentration on the BrdU lymphocyte proliferation assay is not clear, proliferation was examined with respect to different thrombocyte concentrations. We found that lymphocyte proliferation did not appear to be affected by varying numbers of thrombocytes in the sample $(3 \times 10^6 \text{ to } 4 \times 10^7 \text{ cells/ml})$, based on a linear regression analysis between thrombocyte concentration and T lymphocyte proliferation (r = 0.3, p = 0.37, n = 11).

Nonstimulated fresh lymphocytes exhibited higher proliferation rates compared to nonstimulated cryopreserved lymphocytes, although the peak stimulated proliferation between the two groups was similar. The BrdU label absorbance for nonstimulated fresh cells was 0.70 compared to 0.05 for nonstimulated cryopreserved cells. This pattern was observed consistently across three experiments (n = 3-5 birds per experiment).

No statistical difference was detected in the phagocytic ability of fresh versus cryopreserved macrophages for the two categories of ingested yeast (1-4 and >4) (p = 0.23 and 0.51, respectively, *t*-test, n = 4) (Figure 2). However, when the total number of yeast ingested was evaluated, a greater percentage



Fig. 1. Mitogen-induced proliferation of chicken lymphocytes. There was no statistical difference in the proliferation response to 2 and 5 µg/well Con A or between fresh and cryopreserved lymphocytes (two-way ANOVA: Con A concentration $F_{1,16} = 0.01$, p = 0.92, fresh versus cryopreserved $F_{1,16} = 0.92$, p = 0.35; error bars are SE)

of cryopreserved macrophages ingested yeast compared to fresh macrophages (77% \pm 2.7 SE for cryopreserved versus $63\% \pm 2.7$ SE for fresh; p = 0.01, *t*-test, n = 4). Across multiple experiments, 40-80% of cryopreserved macrophages ingested yeast.

number of yeast ingested

Reproducibility of Immune Function Assays: The proliferation assay variability (intrasubject) had a 9% coefficient of variation (CV) when the proliferation response was normalized to a designated "internal standard" (i.e., a replicated subsample from the single bird that was analyzed on all plates on all

(error bars are SE)

analysis days). Use of the normalizing factor improved the CV by sixfold (9% versus 53%) based on subsamples analyzed over 4 different days spanning a 10-week period. The phagocytosis assay reproducibility was 13% CV for the 0 yeast ingested category, and 16% CV for the 1–4 yeast and >4 yeast categories. This reproducibility was not improved by normalizing to an internal standard sample. Thus the normalizing strategy proved to be valid for use in the proliferation assay but not necessary for the phagocytosis assay.

Response of Cryopreserved Cells to Ex Vivo Contaminant Exposure: Ex vivo exposure of cryopreserved chicken leukocytes to 0–170 μ M DMBA significantly reduced the ability of those cells to proliferate (one-way ANOVA, $F_{5,12} = 15.23$, p < 0.001) and ingest yeast (one-way ANOVA, $F_{5,12} = 13.41$, p < 0.001) in a dose-dependent manner (Figure 3).

Cryopreservation and Immune Function of Wild American Coot WBCs

Cell Isolation and Cryopreservation: Lymphocyte isolation with a double gradient (Histopaque 1.077 over 1.119) yielded an average of 4.1×10^6 lymphocytes/ml of blood collected ($\pm 7.9 \times 10^5$ SE, n = 10), with samples containing 98.5% lymphocytes, 1% monocytes, and 0.5% granulocytes. The ratio of lymphocytes to thrombocytes was approximately 2:1 in the cell suspension. Monocyte isolation with a single gradient (1.119) yielded an average of 4×10^6 leukocytes/ml of blood collected ($\pm 8.3 \times 10^5$ SE, n = 9), with samples containing 96% lymphocytes, 3.5% monocytes, 0.5% granulocytes, and a 1:1 ratio of leukocytes:thrombocytes. American coot WBCs cryopreserved for over 10 months had an average viability of 75% ± 3 SE (n = 10) for lymphocytes and 70% ± 3.8 SE (n = 14) for leukocytes (from the monocyte isolation).

Immune Function Assays on Cryopreserved American Coot WBCs: The mitogen-induced lymphocyte proliferation response to Con A was evaluated in cryopreserved samples from five American coots. Mitogen-stimulated proliferation in these birds ranged from 220% to 580% above the nonstimulated cells. The percentage of cryopreserved American coot macrophages that engulfed yeast ranged from 15% to 88%. The birds that had the greatest phagocytosis of 1-4 yeast also had the greatest phagocytosis for >4 yeast (Figure 4). Results from both assays were comparable to results from the domestic chicken samples.

Discussion

Immune function is a complicated process; many different elements of the immune system must work together to elicit an effective immune response. Therefore the ideal assessment of immune function requires a suite of tests that measure several different components. Although *in vivo* measures of immune function are ideal, they are not feasible for wildlife populations where individuals are difficult to recapture. Cryopreservation of cells allows *ex vivo* assessment of certain aspects of immune function for species residing in remote locations when it is not possible to rapidly transport samples back to the laboratory. In addition, cryopreservation facilitates sample analysis by allowing samples from a number of individuals collected over time to be run in a single batch, thereby potentially decreasing interexperiment variation. This method also enables stored blood to be used as a baseline against which to monitor wild populations that may be changing over time in response to natural or anthropogenic stressors.

Our results indicate that cryopreservation does not adversely affect the *ex vivo* evaluation of avian immune function. Cryopreserved avian leukocytes were able to maintain viability and functionality in immunologic assays and appeared not to be affected by length of time cryopreserved. Consistent with our results, Miller *et al.* (1996) demonstrated that human lymphocytes cryopreserved for up to 100 weeks retained functional activity and thus could be used to serve as controls to track changes in immune function. In addition, Ross *et al.* (1993) examined mitogen-induced lymphocyte proliferation on cryopreserved cells from free-ranging harbor seal (*Phoca vitulina*) mothers and their pups. They reported a suppression of lymphocyte function in the mothers at the end of lactation compared to an increase of lymphocyte function in their pups.

Laboratory studies have revealed that both cellular and humoral immunity are affected by contaminant exposure in birds (Fairbrother *et al.* 1994; Grasman and Scanlon 1995; Knowles and Donaldson 1997; Smits and Williams 1999). We found that cryopreservation did not affect macrophage or T lymphocyte function for two different immunoassays: phagocytosis and mitogen-induced proliferation. Although we did not investigate B lymphocyte proliferation, T lymphocyte proliferation is an important step in the activation of B cells for T cell-dependent antibody production. The similar results we obtained with cryopreserved wild American coot WBCs demonstrate the applicability of this approach in wild birds.

We found that a cryopreserved subsample used as a normalizing internal standard on all plates substantially improved the reproducibility of the lymphocyte proliferation assay (9% CV using the internal standard versus 53% CV without, based on samples analyzed over 4 different days spanning a 10-week period). Therefore, a cryopreserved chicken internal standard was used in all proliferation assays, including assays on American coots. The use of this internal standard also provided an informative method to monitor dramatic changes in assay performance that may be due to problems associated with reagent quality or cell culture conditions. This approach to ensure assay reproducibility and hence data quality may be especially important when analyzing wildlife samples at multiple, protracted intervals.

The observation that proliferation of nonstimulated fresh lymphocytes was significantly greater than nonstimulated cryopreserved lymphocytes should be noted. A possible explanation may be that the chickens used here were not housed under laboratory conditions but were free ranging in a small commercial breeding facility. As a result, the chickens' T lymphocytes may have been stimulated *in vivo* prior to blood sampling, causing those lymphocytes to continue to proliferate *ex vivo* in our assay (even in the absence of mitogenic stimulation). Alternatively, cryopreservation may have damaged activated T cells, thus lowering the apparent proliferation of nonstimulated cells in our *ex vivo* assays. Following cryopreservation, recovery of cells activated prior to cryo-





Fig. 3. (a) Chicken lymphocyte proliferation was suppressed following ex vivo exposure to DMBA. Mitogen Con A = 5 μ g/ well. Overall effect of treatment was significant based on one-way ANOVA ($F_{5,12} = 15.23$, p < 0.001) (letters indicate a significant difference between specific treatments, Fisher LSD; error bars are SE, n = 3).(b) The number of macrophages that ingest yeast was reduced following ex vivo exposure to DMBA. Overall effect of treatment was significant based on one-way ANOVA ($F_{5,12} = 13.41$, p < 0.001) (letters indicate a significant difference between specific treatments, Fisher LSD; error bars are SE, n = 3)

preservation is lower than recovery of cells in a resting state prior to cryopreservation. This is particularly evident for immune cells containing lytic granules (Plebanski 2000).

Cryopreserved WBCs responded predictably to a known immunosuppressant, DMBA. T lymphocyte proliferation and phagocytosis was decreased in a dose dependent manner over the exposure range 0–170 μ M DMBA. Notably, both proliferation and phagocytosis were significantly reduced at 50 μ M DMBA, which is similar to results reported in *ex vivo* exposure studies on mouse lymphocytes, where 40–50 μ M DMBA produced significant reductions in cytotoxic T cell function and antibody response (House *et al.* 1989; Ha *et al.* 1993). Here, proliferation was reduced at 50 μ M DMBA, and phagocytosis was reduced at 12 μ M DMBA. Because of the small number of chickens used (n = 3), these results do not necessarily reflect a difference in sensitivity between the phagocytosis and proliferation assays. Although cytotoxicity was not quantified, DMBA may have caused a general cytotoxic effect rather than a specific suppression of proliferation and phagocytosis. Burchiel *et al.* (1992) found that *ex vivo* exposure of 10–30 μ M DMBA increased intracellular calcium in mouse thymic cells. DMBA also has been shown to induce apoptosis in murine B-cell lymphoma by calcium-dependent pathways (Burchiel *et al.* 1993), though whether this occurs in chicken peripheral WBCs is not known.

The need to monitor wild populations for immune function is clear. Epizootics have been associated with contaminant-induced immunosuppression in marine mammals (Aguilar and Borrell 1994; Ross *et al.* 1996a) but have yet to be documented for wild avian species. In addition, contaminant exposure can exacerbate the effects of other environmental stressors on the immune system (Porter *et al.* 1984). By demonstrating the viability and functionality of cryopreserved cells this study expands the possible application of immunotoxicology as a tool in studies of environmental toxicology. Specifically, our results show that (1) cryopreservation is possible to provide effective



Fig. 4. Phagocytosis of macrophages from five wild American coots. There was a wide range of phagocytic ability from the wild birds. In addition, birds that had the greatest phagocytosis for 1-4yeast also had the greatest phagocytosis for > 4 yeast

long-term storage of avian peripheral WBCs collected under field conditions; (2) cryopreserved cells retain sufficient viability and functionality to perform *ex vivo* immune function tests; and (3) cryopreserved cells respond to a known immunosuppressant (DMBA) with a measurable decrease in immune function.

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