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Source: Journal of Wildlife Diseases, 43(4) : 623-634

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-43.4.623

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Journal of Wildlife Diseases, 43(4), 2007, pp. 623–634
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VALIDATION AND NOVEL APPLICATIONS OF THE WHOLE-BLOOD CHEMILUMINESCENCE ASSAY OF INNATE IMMUNE FUNCTION IN WILD VERTEBRATES AND DOMESTIC CHICKENS

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ABSTRACT: The whole-blood chemiluminescence (WBCL) assay is a simple and rapid method of measuring production of reactive oxygen species by circulating leukocytes, particularly heterophils (birds) and neutrophils (other vertebrates). In the interest of substantiating a broadly applicable measure of innate immunity, we investigated the microplate WBCL method for several wildlife species as well as domestic broiler chickens. Lucigenin as a light enhancer was used for all avian blood, wild and domestic, while luminol was used in bear and frog blood. Use of ethylenedinitrilotetraacetic acid (EDTA) as the anticoagulant caused hemolysis of frog blood and decreased WBCL responses in all animals tested. Heparin, even in high concentrations, caused modest to no decrease of WBCL responses. The WBCL response correlated highly with heterophil or neutrophil numbers in all species tested. The WBCL response was tested in freshly collected blood as well as in blood one to five days postcollection in order to determine the utility of this assay for field studies when immediate access to laboratory facilities is not possible. One to three days of delay in performing the test after blood collection caused no, or only a slight, decay of the chemiluminescence response in most animals. Using domestic chickens, we tested the sensitivity of the WBCL method to detect differences between treatment groups and looked for loss of chemiluminescence response over several days using the original blood samples. Significant differences in the WBCL response between experimental groups of broiler chicken were detectable in freshly collected blood, as well as one- to four-day-old blood. Our results show that the innate immune response of populations of wild animals may be successfully compared using this assay, even when blood cannot be tested until a few days after collection.

Key words: Bears, chicken, EDTA, frogs, innate immune response, polymorphonuclear cells, raptors, whole-blood chemiluminescence.

INTRODUCTION

Assessments of the immune status of wild animals are often challenging because many laboratory methods based upon blood samples require rapid access to a laboratory for the purposes of processing the samples (e.g., separation of cell types, proper storage, etc.). In wild animals with nucleated red blood cells (RBC) and all the vertebrates except for mammals, obtaining reliable information on hematology (e.g., leukocyte counts and differentials) is difficult because automated cell counts are not possible.

The whole-blood chemiluminescence (WBCL) assay is a reliable and rapid method of assessing production of reactive oxygen species (ROS) by circulating phagocytes (DeChatelet and Shirley, 1981; Tono-Oka et al., 1983; Nagahata et al., 1991). Besides being a relatively quick and inexpensive assay to perform, a major advantage of chemiluminescence is that it can be performed on low-volume blood samples (1–50 μl) (Marnila et al., 1995; Lojek et al., 1997; Nikoskelainen et al., 2006), which makes it a particularly useful tool in situations when several tests are necessary on a limited amount of blood, e.g., in very small or sick animals. Another key advantage is that the circulating phagocyte status can be evaluated with minimal manipulation of the blood since no isolation of cells is required. The functional state of whole-blood phagocytes may reflect the physiological state of the host better than that of isolated leukocytes because isolation of cells can alter the viability, activity, and receptor expression of cells.

When phagocytes are stimulated, they

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produce ROS in a process called respiratory burst by the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex and myeloperoxidase. When the electronically excited states of the newly produced oxygen species relax to the ground state, photons are emitted. This chemiluminescent light signal is very weak but can be enhanced by using chemicals such as luminol and lucigenin. The luminol-enhanced chemiluminescence (CL) emission is largely dependent on myeloperoxidase activity, whereas the lucigenin-enhanced CL is mainly NADPH oxidase–dependent (Van Nerom et al., 1997; Merrill et al., 2001). Lucigenin is the choice of enhancer in chickens because they appear to lack myeloperoxidase activity (Merill et al., 2001).

The WBCL assay has mostly been applied to human blood and has been used to test differences in innate immune function in trauma, allergy, hyperthyroidism, and other diseases (Ruf et al., 1992; Videla et al., 1993; Kukovetz et al., 1997; Szabó et al., 2000; Vuotto et al., 2000a; Fredriksson et al., 2003; Pallister and Topley, 2004; etc.), but some references exist for other mammals (Nagahata et al., 1991; Lojek et al., 1997; Vuotto et al., 2000b; Letellier et al., 2001; McLaren et al., 2003; Khan, 2004; Pavelkova and Kubala, 2004). Limited information is available for other orders of vertebrates, such as fish (Kubala et al., 1996; Belotsky et al., 2004; Nikoskelainen et al., 2004, 2006; Verho et al., 2005), whereas almost nothing is available about amphibians (Marnila et al., 1995; Gilbertson et al., 2003) and birds (Laudert et al., 1993). Our initial studies for adapting this assay to wild birds were done in chickens because genetic differences are minimal among these inbred birds, and blood samples can be easily collected.

Our study had four main purposes. First, we wanted to assess the WBCL method in several wild species and optimize experimental conditions for those species. Second, we needed to determine the best anticoagulant for the WBCL assay in these species. Third, we wanted to know whether this assay was useful in situations when blood had to be shipped long distances, resulting in 2–3 days of delay between sample collection and WBCL testing. Fourth, we wanted to know whether the WBCL method could detect differences between experimental groups of animals with samples that were several days old.

MATERIALS AND METHODS

Animals

Animals used in this study were both male and female mature adults, unless otherwise stated. Northern leopard frogs (*Rana pipiens*) ($n=57$, >25 g each), were purchased from Ward's Natural Science (Rochester, New York) and were housed in tubs with 1-cm-deep water, at room temperature, in 12 hr dark and light cycles. Male American kestrels (*Falco sparverius*) ($n=32$) were bred and housed at the Avian Science and Conservation Centre, McGill University, Montreal, Canada. Male domestic broiler chickens ($n=108$) and leghorn chickens ($n=5$) (*Gallus gallus domesticus*) (all 4-wk old) were raised at the University of Saskatchewan from 1-day-old chicks obtained from Lilydale Hatchery (Wynyard, Saskatchewan, Canada). All broiler chickens, kestrels, and 41 of the frogs included in this study were being used for other research and were sampled opportunistically. Twelve of the frogs were primarily kept for teaching purposes in the biology department. Samples were acquired from five juvenile (<5 yr old) and five adult grizzly bears (*Ursus arctos*), and from three juvenile and seven adult black bears (*Ursus americanus*), that were captured and anesthetized for conservation-related research in Alberta, Canada. Samples from three kestrels were from animals captured in the wild (Saskatchewan, Canada), while two great horned owls (*Bubo virginianus*), three snowy owls (*Nyctea scandiaca*), one golden eagle (*Aquila chrysaetos*), one Swainson's hawk (*Buteo swainsoni*), two lesser scaup (*Aythya affinis*), and one mallard (*Anas platyrhynchos*) were brought to the Western College of Veterinary Medicine (Saskatoon) due to injuries or were permanent residents used for research and teaching.

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Reagents and buffers

Frog ringer solution (116 mM NaCl, 1.2 mM KCl, 1 mM $CaCl_2$, 2.7 mM $NaHCO_3$), pH 7.2, was used for the dilution of frog blood, and Hanks' balanced salt solution (HBSS; Sigma, Oakville, Ontario, Canada) was used for the dilution of blood from all other animals and for preparing working solutions of other reagents. Sodium heparin (LEO Pharma Inc., Thornhill, Ontario, Canada), 1,000 U/ml, and disodium EDTA (ethylene dinitrilo tetraacetic acid disodium salt dehydrate; Merck, Darmstadt, Germany) as a 7.5% solution prepared in HBSS, pH 6.6, were used as anticoagulants in syringes. Zymosan A from *Saccharomyces cerevisiae* (Sigma) was prepared as described by Marnila et al. (1995). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), as a 100-mM stock solution, was prepared in dimethyl sulfoxide, and the aliquots were stored at −20 C. To prepare the 1-mM working solution, the luminol stock was added to HBSS and quickly mixed. (If HBSS was added to stock, a precipitate formed and rendered the reagent useless.) Lucigenin (N,N′-dimethyl-9,9′-biacridium dinitrate; Sigma), as a 10-mM stock solution in HBSS, was prepared, and aliquots were stored at −20 C.

Blood samples

Peripheral blood was collected from all animals using nonlethal methods. All animals used in this study were under current and approved animal care protocols through the University of Saskatchewan's Animal Care Committee. Birds were sampled through the brachial or jugular veins. Frogs were sampled by cardiac puncture. Bear blood was collected from the femoral or jugular veins. For all bears and 36 of the chickens, 2- and 3-ml blood samples, respectively, were collected into 4-ml commercial sodium heparin tubes (Vacutainer®, BD Oakville, Ontario, Canada), which resulted in 34 and 23 U/ml heparin concentration in the blood samples. For 82 broiler chickens, 800–1,000 µl blood was collected into heparinized needles, with approximately 20 U heparin in the hub (half hub). For frogs, 500–1,000 µl blood was collected into heparinized needles, with approximately 20 µl of heparin (20 U) in the hub, resulting in 20–40 U heparin/ml blood. One milliliter of blood from each kestrel and other wild bird species was collected into heparinized needles, with approximately 40 µl of heparin (40 U) in the hub (full hub). From each of eight frogs, 1 ml of blood was collected into syringes containing approximately 20 µl of 7.5% disodium EDTA, resulting in 1.5 mg EDTA/ml blood. From one kestrel, 1 ml of blood was collected with 40 µl of 7.5% disodium EDTA solution in the syringe. Blood from 12 broiler chickens (3 ml each) was collected into commercial K_2EDTA tubes (Vacutainer®, BD), resulting in an EDTA concentration of 2.4 mg/ml blood. All blood samples in our studies were kept at 4 C immediately after collection until tested.

EDTA and heparin treatment

Whole-blood samples (anticoagulated with heparin) kept at 4 C for less than 24 hr were aliquoted into microcentrifuge tubes, and 7.5% EDTA or 1 U/µl heparin was added to result in a series of EDTA or heparin concentrations. Final EDTA concentration ranged between 0.75 and 6 mg/ml blood, while the heparin concentration, in addition to the heparin used for anticoagulating the blood, ranged between 20 and 80 U/ml. The blood was gently mixed with the EDTA or heparin, incubated at room temperature for 30 min, then diluted with buffer and used in the WBCL assay.

Whole-blood chemiluminescence assay

For the chemiluminescence assay, whole-blood phagocyte samples were routinely run in duplicates, except in broiler chicken trials, where only single wells could be used because of high animal numbers. Whole-blood CL emission was determined at 25 C for frogs, at 37 C for bears, and at 39 C for all bird species. Clear Polysorp microplates (Nunc, Roskilde, Denmark) were used in all experiments (no significant cross-talk was observed between wells). Different blood dilutions, different light enhancers at various concentrations, and several concentrations of zymosan were tested in preliminary experiments to find optimal conditions with good reproducibility. Later, final blood dilutions of 1:30 and final zymosan dilution of 0.3 mg/ml were routinely used. Nonopsonized zymosan was used in all experiments in all species; opsonization of zymosan with frog serum did not enhance the WBCL response of frog whole blood in our preliminary studies. Luminol as a light enhancer at a final concentration of 0.3 mM was used for bears and frogs. Since the use of luminol resulted in very low to undetectable signals in some individuals, lucigenin at a final concentration of 0.3 mM was routinely used in birds.

First, blood was diluted in either frog ringer (for the frog samples) or in HBSS (for all other animals) and added to microplates. Working solution of zymosan was added first to the diluted blood samples followed by the light

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enhancer; both were added within 2 min before starting the test run in a Novostar (BMG Labtech, Offenburg, Germany) luminometer. The final reaction volume was 150 µl in each well. Light emission was recorded over a 2–3 hr period, at 4 min intervals and 1.5 sec/reading. Plates were shaken for 5 sec before each interval. Light-emission results are presented as counts per min (light units measured per min). Background light emission was subtracted. In graphs showing the effect of anticoagulants, the results are expressed as percent of response of controls (no anticoagulant, with only buffer added) because of variation in CL responses between individuals.

Cell viability, buffy coat, and PMN counts

Cell viability was tested with trypan blue (Gibco), 0.4%, in a hemocytometer. Buffy coat thickness as a crude indicator of blood leukocyte numbers in frogs and chickens was measured as a percentage of blood volume in hematocrit tubes. Polymorphonuclear Leukocytes (PMNs) of bears were counted by an automated cell counter and expressed both as absolute PMN numbers/ml and as a percentage of total WBC numbers. The number of PMNs were estimated from Giemsa stained blood smears of frogs and birds by counting 10 fields per animal at 40× magnification, containing approximately 300 RBC/field. Neutrophil numbers in each frog blood sample were adjusted to their RBC counts taken in a hemocytometer from fresh blood because we found great individual variation in hematocrit and RBC numbers between individual frogs.

Statistics

Statistical analyses were carried out using SPSS 13.0 (Chicago, Illinois). The peak CL response (not the calculated percentages) was used in all statistical analyses. Pearson correlation coefficient (r) was calculated to express the correlation between variables throughout this paper, and the P values and sample size (n) are reported. Paired t-tests were used for differences between two treatments for the same animal population (chicken blood collected in EDTA or heparin tubes). Blood samples with any clots were excluded from statistical analysis.

RESULTS

Blood from most animals (grizzly and black bears, owls, hawk, kestrels, broiler chickens, and leopard frogs) had consistent and readily detectable WBCL responses, but only when heparin was used as an anticoagulant. A typical response in a northern leopard frog, a grizzly bear, and an American kestrel is shown in Figure 1. The WBCL response was low in all five leghorn chickens, three ducks, and the golden eagle (response lower than 8000 cpm). High WBCL response was observed in chickens, but not in any of the other species, even in the absence of zymosan (peak usually 50% or less of that observed with zymosan added).

The WBCL peak positively correlated with PMN numbers or buffy coat thickness in all species tested. In frogs, the WBCL response correlated with buffy coat thickness ($P<0.01$, $n=41$) and absolute neutrophil numbers ($P<0.001$, $n=25$). In chickens, the WBCL peak correlated with absolute heterophil numbers and heterophil/mononuclear cell ratio ($P=0.001$, $n=38$). In bears, the WBCL response correlated with absolute neutrophil numbers ($P<0.001$, $n=20$), relative neutrophil numbers ($P=0.01$, $n=21$), and total WBC counts ($P=0.001$, $n=20$). These associations were also significant for the two bear species separately ($P<0.05$, $n=10$).

Effect of EDTA on whole-blood chemiluminescence

Collecting frog blood into EDTA anticoagulant caused hemolysis of all but one sample. In the one unaffected frog, the cell viability was over 98% on the first day and over 90% even after 2 wk at 4 C. All frog blood collected into heparin maintained over 95% cell viability for 2 wk.

EDTA added to fresh frog blood on the day of collection after samples had been anticoagulated with heparin caused hemolysis in all 19 frogs in a concentration-dependent manner (except at the lowest EDTA concentration [0.75 mg/ml], where hemolysis was often not observed). However, at the lowest EDTA concentration, CL response was consistently inhibited ($P<0.01$) but not completely eliminated (Fig. 2). At higher EDTA concentrations, CL responses were inconsistent. In some cases, the WBCL response in these

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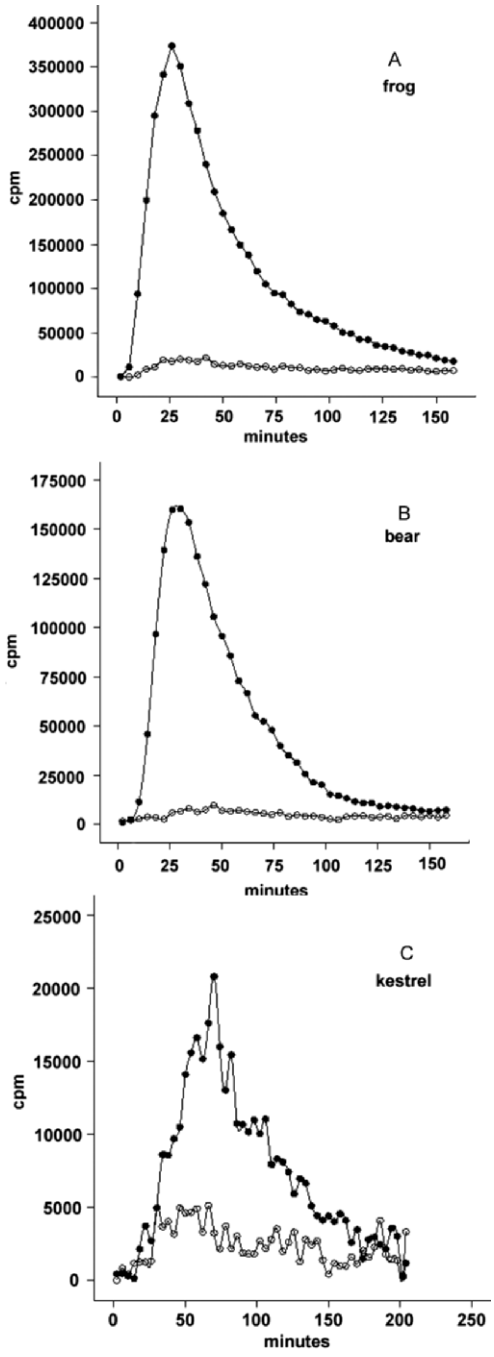


FIGURE 1. Whole-blood chemiluminescence (WBCL) response of a northern leopard frog (a), a grizzly bear (b), and an American kestrel (c). Luminol- (a, b) and lucigenin-dependent (c) WBCL response was stimulated with zymosan (solid circles) or not stimulated (open circles). The results shown are from a single representative experiment for each species.

hemolyzed samples was enhanced, while in others it was inhibited. Additional heparin did not significantly affect the CL peak (Fig. 2; $P>0.05$). A high heparin concentration of 60 U/ml was tested with frog blood to account for possible future situations when less than 0.5 ml frog blood may be collected with heparinized needles. Twenty microliters of heparin (half hub of needle) corresponds to >60 U/ml heparin in frog blood when only 300 µl is collected. In our experience, it is difficult to collect more than 300 µl of blood through cardiac puncture from smaller (<20 g) frogs.

Blood from birds collected into EDTA did not hemolyze. Viability of broiler chicken blood cells was >95% even after 4 days in the EDTA tubes, while blood in heparin tubes varied in cell viability between 90% and 95% by day 4. Hemolysis did not occur when EDTA was added to heparinized blood from chickens or wild birds (0.75–6 mg/ml blood). However, the WBCL response of blood in EDTA was completely diminished in chickens (Fig. 3; $P<0.001$, $n=12$) and in the one kestrel tested. In wild birds and chickens, all experimentally relevant concentrations of EDTA added to heparinized blood samples (>1.5 mg/ml blood) caused dramatic inhibition of the CL response (Fig. 2c, d). However, no inhibition was observed at the lowest, 0.75 mg/ml concentration in the six wild birds tested (Fig. 2c). Additional heparin (20–80 U/ml extra heparin/blood) caused a moderate, concentration-dependent inhibition of the CL response (Figs. 2c, d).

Change of WBCL response over time

The CL response in northern leopard frogs was remarkably stable over 2 days. For 2-day-old blood, the peak WBCL correlated very highly with those at day 0 ($P<0.001$, $n=41$) (Fig. 4a). For a subsample of frog blood, the CL response was measured after 2 wk of refrigeration, and only <40% decay of the WBCL response was observed (data not shown).

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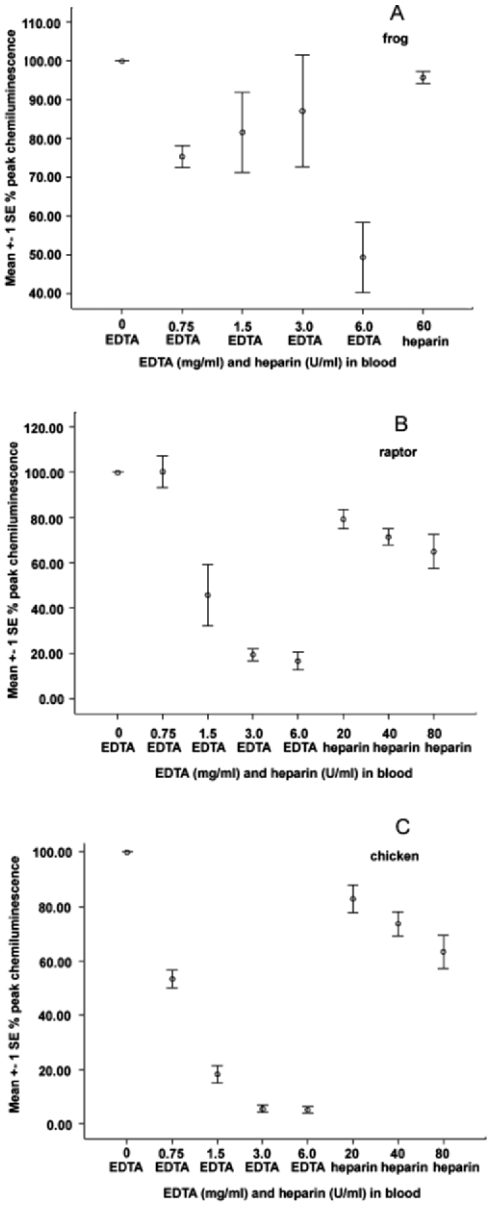


FIGURE 2. Effect of ethylene dinitrilo tetraacetic acid (EDTA) and heparin on relative chemiluminescence (CL) intensities of whole blood of frogs (a), raptors (b), and broiler chickens (c). Different concentrations of EDTA and heparin were added to lightly heparinized whole blood. The WBCL response is expressed as a percentage of the untreated control blood (collected with only a heparinized needle), which was considered to be 100%. The WBCL responses (mean±SE) from four frog (n=19), two raptor (n=6; 4 owls, 1 eagle, and 1 hawk), and two chicken (n=7) experiments are shown.

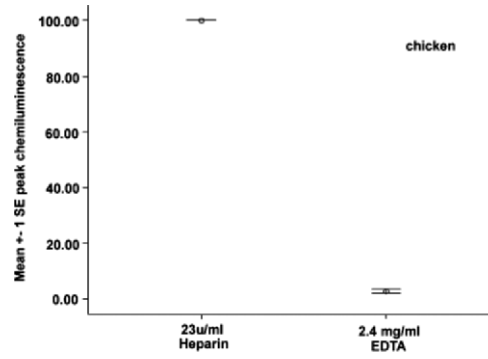


FIGURE 3. WBCL response (mean±SE) of broiler chicken blood anticoagulated with heparin or EDTA. Blood from the same animals (n=12) was collected in commercially heparinized (23 U/ml) or EDTA-treated tubes (2.4 mg/ml) using Vacutainer® needles.

In 10 wild birds tested, the WBCL response was maintained over 3 days without significant declines (Fig. 4b), and it was quite stable for up to 5 days for the six birds that were retested (data not shown). In kestrels, the WBCL activity was relatively stable in ~80% of the birds between days 2 and 5 (Fig. 4c). Results from days 2 and 5 were significantly correlated (P<0.001, n=32). However, ~20% of the samples had begun to decay by day 5. Blood samples from kestrels were not available to be tested at day 0 (because of shipping distance), except for the two birds captured separately (those included in Fig. 4b). In broiler chickens, although a minor and consistent decay occurred, differences between experimental groups were still readily detectable 24 hr and 96 hr later (Figs. 5a, b). Differences between experimental groups were no longer detectable by day 7 because of variable and significant declines in the WBCL activity (data not shown).

Blood clots, even small ones, negatively affected the WBCL response even in fresh blood. Clots in blood stored for 1–4 day at 4 C always caused a dramatic decrease or complete elimination of the WBCL response (data not shown).

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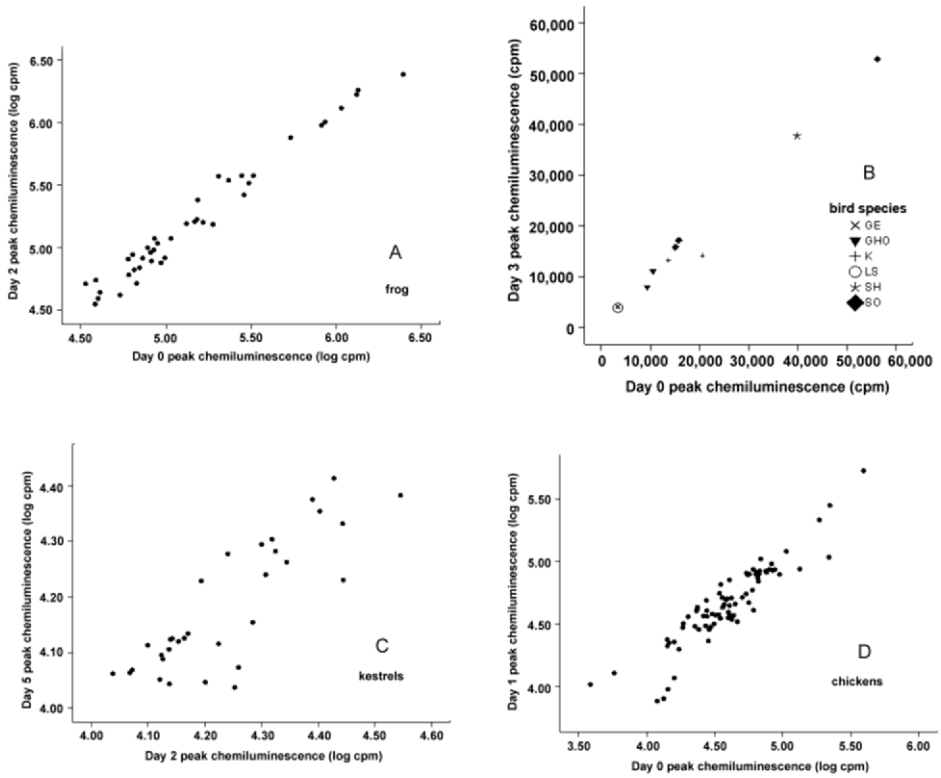


FIGURE 4. Effect of storage time on the WBCL response in frogs (a; 0 vs. 2 days), wild birds (b; 0 vs. 3 days), captive kestrels (c; 2 vs. 5 days), and broiler chickens (d; 0 vs. 1 day). Wild birds included golden eagle (GE), great horned owl (GHO), American kestrel (AK), lesser scaup (LS), Swainson's hawk (SW), and snowy owl (SO). Blood samples were stored at 4 C.

In bears, only a few blood samples were tested on both day 2 and day 5, but WBCL response was significantly correlated between these two days ($P<0.05$, $n=7$). Day 0 samples were not available for bears. Two samples from the same bear (one grizzly bear captured twice with a 1-wk interval between captures) had almost

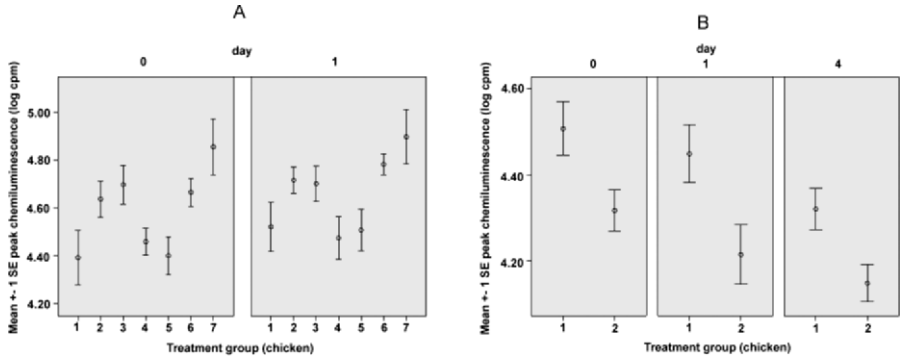


FIGURE 5. Reproducibility of WBCL response (mean±SE) on different days after collection using the same blood samples from broiler chickens from seven treatment groups (a) or two treatment groups (b). The blood from each animal, stored at 4 C, was analyzed on day 0 and 1 (a) or days 0, 1, and 4 (b). Group sizes varied between 12 and 22 birds.

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identical WBCL responses (data not shown).

DISCUSSION

We applied the WBCL assay to several wild animal species and to the domestic broiler chicken. This assay has been successfully used to test differences in innate immune responses measured through PMN status of peripheral blood between experimental groups or populations of some wild species, namely fish (Nikoskelainen et al., 2004, 2006), frogs (Marnila et al., 1995; Gilbertson et al., 2003), badgers (McLaren et al., 2003), and mallards (Laudert et al., 1993). To our knowledge, this paper is the first to adapt the whole-blood assay for bears, raptors, and the domestic chicken, although the CL responses from isolated poultry leukocytes have been evaluated (Kogut et al., 1998, 2001; Merrill et al., 2001). In addition, we provide practical information regarding this technique in frogs (i.e., the choice of anticoagulant, the longevity of the WBCL activity of stored blood, the WBCL response's relation to PMN numbers in the blood, etc.) and in all the other animals tested. We not only tried this assay in several species but also confirmed the findings of Laudert et al. (1993), which state that the technique can be used to detect differences in the WBCL response between treatment groups in experimental settings.

Blood from most animals (bears, frogs, owls, hawk, kestrels, and broiler chickens) had consistently strong WBCL responses with our methods when heparin was used as an anticoagulant. The WBCL peak, a measure of the functional capacity of circulating PMNs, correlated highly with numbers of both PMNs and total WBCs in all species tested, which agrees with other reports (Tono-Oka, 1983; Nagahata et al., 1991). Interestingly, high WBCL response was observed in broiler chickens even in the absence of zymosan, which is thought to be necessary to stimulate the CL response, similar to the phenomenon in fish (Nikoskelainen et al., 2006). This finding is likely due to spontaneous degranulation of heterophils as a consequence of adherence and subsequent activation of cells from contact with the microplate. Eliminating regular shaking of the plate did not eliminate this response (unpublished results). According to our observations, the shaking action used in our assays was not enough to keep cells in suspension; they inevitably settled to the bottom of the wells.

We had low WBCL responses from leghorn chickens ($n=5$), adult lesser scaup, and the mallard, but since these were not species of major interest to us, we did not pursue the optimization of the WBCL assay for them. Our finding of low responses in ducks ($n=3$ only) is in contrast with high cpm reported by Laudert et al. (1993), but they studied ducklings (3–4-wk old), not adults, measured responses in a scintillation counter, used luminol as an enhancer, and observed peaks at 4–6 hr after starting the reaction. It is possible, that luminol is a better light enhancer in some species of birds. Luminol is known to cross biological membranes and enhance light produced by both intracellular and extracellular production of ROS. There may be great differences in the ability of birds from different taxa and of different ages to produce ROS, which could only be confirmed with further studies.

The chemiluminescence response of a phagocyte is a direct reflection of its ability to produce ROS (Ginsburg et al., 1993; Merrill et al., 2001), which are meant to destroy invading organisms and thus provide first-line immunologic defense. When the CL assay is used with isolated leukocyte populations, the CL activity/cell can be accurately measured (DeChatelet and Shirley, 1981; Kournikakis and Simpson, 1995). Under such controlled conditions, the number (and type) of leukocytes in the reaction is known, and the reaction mixture only

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contains buffers and reagents the researcher determines. However, when the CL assay is applied to whole blood, the CL intensity measured is related to several factors. First, as with isolated cells, the WBCL assay is expected to be related to CL activity/phagocyte. The contribution of CL activity of monocytes to the WBCL response is likely negligible due to their low numbers normally present in blood and the fact that they show lower CL activity than do PMNs (Lojek et al., 1997; Desmidt et al., 1996). Therefore, this assay primarily measures the activity of PMNs, as has been suggested by others (Conlon et al., 1991). Second, the WBCL assay is related to PMN numbers in the blood as demonstrated in our work and supported by others (Tono-Oka et al, 1983; Krause et al., 1989). With isolated phagocytes, measured CL activity is proportional to cell number, as expected (Kournikakis and Simpson, 1995; Lojek et al., 1997), while this relationship usually is not proportional between WBCL activity and whole-blood volume (DeChatelet and Shirley, 1981; Kubala et al., 1996; Lojek et al., 1997; Nikoskelainen et al., 2004; Pavelkova and Kubala, 2004). In frogs, however, CL intensity proportionally correlated with blood volume (Marnila et al., 1995; Papp and Smits, unpublished observation) similar to the relationship between CL activity and the number of isolated frog spleen leukocytes (Papp and Smits, unpublished observation).

In addition to the activity and number of PMNs in the blood, other factors that may influence the WBCL response are the light absorption by the hemoglobin in RBC (Redl et al., 1983; Tono-Oka et al., 1983) and the zymosan-opsonizing ability of plasma (amount of antibodies, complement or lectin) (Nikoskelainen et al., 2004; Pavelkova and Kubala, 2004; Verho et al., 2005). Although the WBCL signal also is dependent upon factors other than the release of ROS by PMNs, which somewhat complicate the interpretation of the WBCL assay results, we believe that, in most situations, the WBCL method is the method of choice for monitoring the respiratory burst activity in blood. Often, isolation of PMNs is neither feasible nor preferable (due to high number of samples and time-consuming separation procedures, too little blood available, potential loss/change of activity during the isolation procedure, etc.).

Blood has been collected previously with EDTA as the anticoagulant for assessing leukocyte function (Kogut et al., 1998, 2001; Gilbertson et al., 2003; Pallister and Topley, 2004; Nikoskelainen et al., 2006). However, we found that EDTA caused hemolysis in the blood of northern leopard frogs. Others (Hattingh and Smith, 1976; Harms et al., 2000) have reported the same problem in some reptiles, birds, and fish. Hemolysis is probably related to EDTA increasing the osmotic fragility of red blood cells (Kafka and Yermiahu, 1998). When added to heparinized blood, EDTA inhibited the WBCL response of frogs, even at the lowest concentration. We also observed dramatic inhibition by EDTA of the CL responses of leukocytes isolated from frog spleens (unpublished observation). Although interspecies and individual differences occur in sensitivity to EDTA, we have shown that EDTA is not a suitable anticoagulant for frog blood to be used in the WBCL assay.

Although hemolysis was not a problem in other species, EDTA inhibited the WBCL response in all species tested. In agreement with our observations, EDTA used for human blood has been noted to decrease or eliminate CL and oxidative burst of both whole-blood and isolated leukocytes (Bohmer et al., 1992; Ruf et al., 1992; Ginsburg et al., 1993; Elsner et al., 1995). In another study, EDTA also significantly decreased adherence of chicken heterophils (Andreasen et al., 1990), which would compromise their function. EDTA has been shown to be inferior to heparin as an anticoagulant in maintaining respiratory burst activity in

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fish whole blood and isolated human eosinophils (Nikoskelainen et al., 2006; Prince et al., 1995), although the authors of the first paper speculated that this was due to the enhancement of CL by heparin and not to the inhibition by EDTA. Based on our results and the literature cited, the weight of evidence points to EDTA as the inhibitor of PMN activity. The negative effect of EDTA on oxidative burst by PMNs is likely due to the inhibition of Ca^{2+}-dependent binding of stimulator particles to PMNs or the inhibition of Ca^{2+}-dependent enzymatic events, since EDTA is a chelating agent. However, no effect of EDTA or heparin on WBCL activity in rats has been found (Lojek et al., 1997). Possibly, the nature of the salt solutions employed to dilute the whole blood and to measure CL in activated PMNs is important (Ginsburg et al., 1993). Ethylene dinitrilo tetraacetic acid seemed to be a suitable anticoagulant when cells were isolated and washed before use in a CL assay (Kogut et al., 2001). Although heparin can also cause a modest, dose-dependent decrease in respiratory burst activity (our results; Salih et al., 1997), it remains the anticoagulant of choice when conducting the WBCL assay in birds and other wildlife including frogs.

To be practical for use in wild animal research, sample quality related to a particular assay should not degrade for a minimum of 2 days; such delays between sample collection and testing are common due to shipping delays or other field-related events. Our studies show that cooled blood is stable enough for 2–3 days to perform this assay with consistent results in frogs, wild birds, and domestic chickens. In frogs, longer delays may be acceptable because cells remain viable and maintain >60% of their original CL activity for up to 2 wk. Since WBCL activity differences between individual frogs can reach two orders of magnitude (Fig. 4a), even a moderate decrease in CL activity may be detectable between experimental groups or populations. More studies are necessary to test this hypothesis.

Our chicken studies confirmed that this assay was sensitive enough to detect differences in innate immunity between experimental groups of birds. Ideally, samples from the study groups would be collected on one day and shipped together to a laboratory within two days. Establishing a decay-curve for particular species of interest would allow comparisons of samples collected at different time points. Although genetic diversity in wild animals and environmental variability may affect the WBCL response, this test of innate immunity offers a practical, meaningful, and noninvasive addition to a suite of in vivo tests of immune function that may be applied to wildlife.

This assay of innate immune function is particularly useful in small-bodied animals because minute amounts of blood will suffice (<50 μl), and for animal orders with nucleated RBC (fish, amphibians, reptiles, and birds) because it provides a practical means of determining their PMN status, which is otherwise a very laborious due to the inability to use automated leukocyte counts. This WBCL assay can be used in conjunction with tests of specific T and B cell immunity to assess immune function in wild and captive species.

ACKNOWLEDGMENTS

We thank M. Drew, T. Warren, J. Dahiya, and R. Gonda for access to blood samples from experimental broiler chickens, and M. Olsgard and B. Hersikorn for assistance in collecting blood from different animal species. We thank the Foothills Model Forest Grizzly Bear Research Program for providing blood from grizzly and black bears. We thank B. Trask, University of Saskatchewan, for running WBCL tests on bear and kestrel blood. This work was financially supported by a National Sciences and Engineering Research Council Discovery grant to JS.

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Received for publication 18 October 2006.

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