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Short communication

Effects of bacterial lipopolysachharide on peripheral leukocytes in the American alligator (*Alligator mississippiensis*)

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Abstract

Alligators were injected intraperitoneally with four different doses (10, 1.0, 0.1, and 0.01 mg/kg body weight) of a mixture of bacterial lipopolysaccharides (LPS) derived from three different types of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*). Injection of the alligators with the LPS mixture resulted in a dose- and time-dependent increase in total peripheral leukocytes Lymphocytes increased at days 3 and 4 post-injection, and decreased back to baseline levels at day 7 for all doses. Alligators that were not treated, and those injected with pyrogen-free saline, did not exhibit statistically significant changes in total leukocytes during the course of the study. Injection of alligators with 0.5 mg LPS/kg body weight derived from one of three bacterial species revealed that the leukocyte increases observed were not statistically different for all three types of LPS. The animals displayed the same increases in total counts and the levels of all circulating leukocyte types were not different between animals treated with a combination of LPS from all three bacterial species.

Keywords: Alligator; Inflammatory response; LPS; Leukocytes

1. Introduction

Lipopolysaccharide (LPS) is a major component of the outer cell wall of Gram negative bacteria. The lipid portion of the LPS contains a toxic substance, Lipid A, which is recognized by the immune systems of most higher eukaryotes (Rietschel et al., 1994). Injection of LPS into higher eukaryotes causes an inflammatory reaction (Smith, 1994).

Little is known about the inflammatory response in the American alligators (*Alligator mississippiensis*). Mateo et al. (1984) showed that subcutaneous injection of juvenile alligators with turpentine, a prototypical inflammatory mediator, caused a local

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inflammatory-like response. In addition, Brown et al. (2001) showed that the American alligator displayed pyogranulomatous inflammation in response to mycoplasmosis. However, attempts in our laboratory to isolate or detect C-reactive protein and haptoglobin from the serum of LPS-injected alligators showed that they failed to produce these common acute phase reactant markers (unpublished results). These proteins are produced in mass amounts by the liver, and secreted into the bloodstream, of higher eukaryotes challenged with LPS (Eckersall et al., 1996; Hiss et al., 2004). This study was conducted to determine if alligator leukocytes respond to bacterial LPS.

2. Material and methods

2.1. Chemicals and biochemicals

Bacterial lipopolysaccharides derived from *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were purchased from Sigma Chemical Company (St. Louis, Missouri). Phosphate-buffered saline (10x concentrate) was purchased from Intermountain Scientific (Kaysville, Utah).

2.2. Treatment of animals

Numerous juvenile alligators, which were hatched in captivity from a single clutch of eggs collected in the wild, were maintained at Rockefeller Refuge in fiberglass-lined concrete tanks approximately 4.57 m long × 1.83 m wide. Several small alligators (up to 50 cm in length) were housed in a single tank. The alligators were transferred to McNeese State University at approximately 8 months of age, and were housed in 3.25 m × 3.25 m outdoor fenced pens each with a subterranean 364 L tank which provided approximately 1.00 m^2 of water surface area. They were fed a dried pelletized food formulation ad libitum. For each experiment, three animals were dosed intraperitoneally with one mL of normal saline, and three each with 0.01. 0.1, 1.0, or 10.0 mg/kg of LPS, while, three animals were left untreated. The alligators were bled via direct cardiac puncture using a 2.5 cm 21 ga. needle and a 3 cc syringe on days 0,1,2,3,4,5,7,9,11 of each experiment. The experimental protocols used to treat the alligators were previously approved by the McNeese State University Animal Care and Use Committee.

2.3. Differential leukocyte counts

Approximately 1 mL of blood was drawn via cardiac puncture using a 2.5 cm 21 ga. needle and a 3 cc syringe. The blood was immediately added to 0.1 volume of 0.5 M EDTA. For differential leukocyte determinations, approximately 5 μL of blood was spotted onto a microscope slide and smeared across the slide using the edge of a second slide. The blood was allowed to dry for approximately five minutes. The slides were stained in Giemsa–Wright stain and viewed under an oil immersion lens at 1000×.

2.4. Total peripheral leukocyte counts

Blood to be used for total leukocyte counts was diluted ten-fold in phosphate-buffered saline (pH 7.4) and the cells were counted using a hemacytometer under 400× magnification.

2.5. Statistics and controls

Alligators for each treatment group were analyzed in triplicate. Twelve animals were used in each experiment. The results represent the means ± standard deviations for three independent determinations. The results from each treatment group were compared to those from other treatment groups using analysis of variance and Sheffe's post hoc comparisons.

3. Results

Fig. 1 shows the total (Fig. 1A) and differential (Fig. 1B–F) peripheral leukocyte evaluations from alligators treated with different doses of LPS, pyrogen-free saline or untreated animals. Alligators treated with 0.01, 0.1, 1.0 and 10 mg/kg LPS exhibited 244%, 283%, 305%, and 470% increases in total peripheral leukocytes, respectively, 24 h after treatment. These increases were statistically significant ($p < 0.01$). In contrast, untreated alligators or alligators injected with 1 mL of pyrogen-free saline showed no increase ($p > 0.05$) in total leukocyte values. Differential leukocyte analyses revealed that

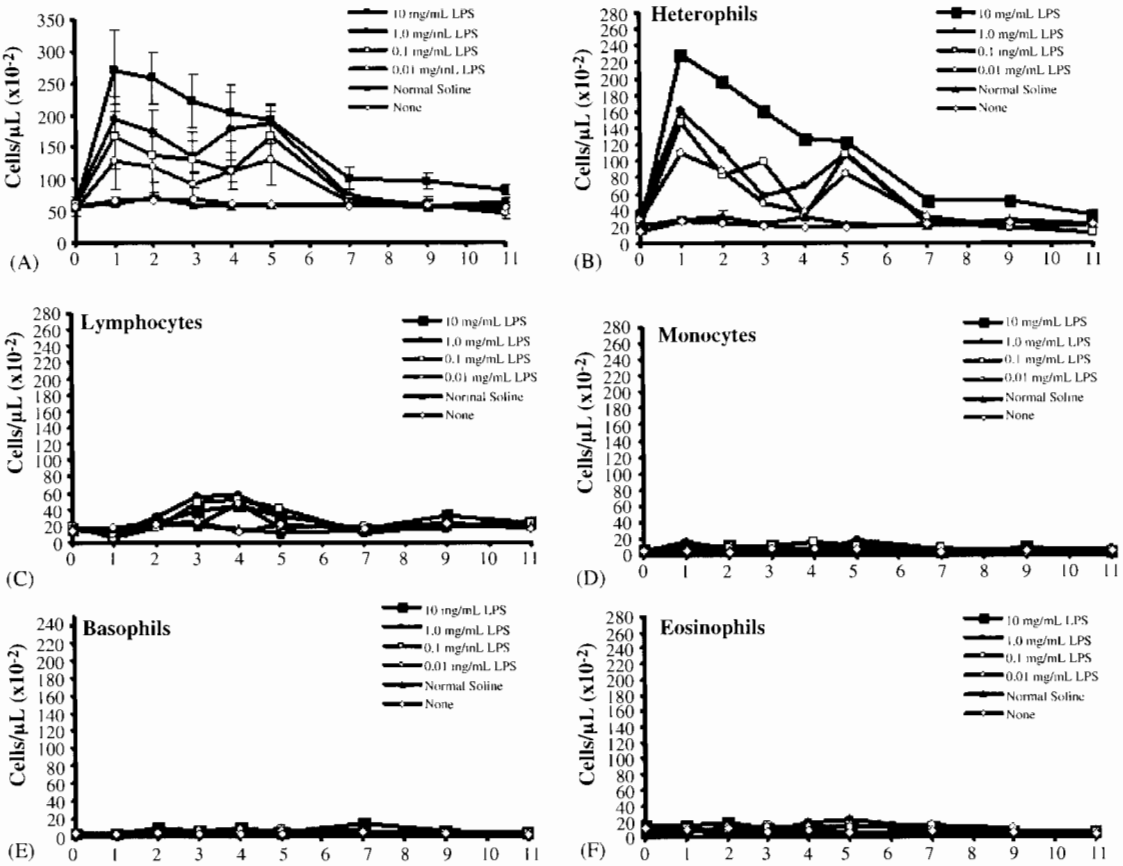

Fig. 1. Kinetic response of five types of alligator peripheral leukocytes to intraperitoneal injection of LPS. Alligators were injected intraperitoneally with three different doses of bacterial LPS. The animals were bled at various time points and the levels of circulating leukocytes were manually determined by Geimsa–Wright staining followed by phase contrast microscopy.

the heterophil values were increased 392%, 435%, 736%, and 1211% after 24 h, compared to baseline levels measure prior to treatment. The circulating lymphocytes, monocytes, basophils and eosinophils showed little or no change ($p < 0.05$) during the first 24 h after LPS injection (Fig. 1C–F). The heterophil/lymphocyte ratios (H/L) increased from 1.1 to 24.7 in animals treated with 10 mg/kg LPS. Likewise, the H/L values for animals treated with 0.01, 0.1, or 1.0 mg/kg LPS were increased ($p > 0.01$) from near 1.0 to 15.0, 24.2, and 11.4, respectively. Untreated alligators and those injected with pyrogen-free saline maintained H/L values of near 1.0 throughout the 11-day study, and were statistically different from untreated controls ($p < 0.05$).

The heterophil counts in the LPS-treated alligators decreased slowly until day 4–5, when another significant rise in heterophils population was noted (Fig. 1B). After the secondary increase, the heterophil population decreased slowly back to control values. Alligators treated with the lowest three doses (0.01, 0.1, and1.0 mg/kg) returned to control levels at 7 days post-treatment, while animals treated with 10 mg/kg still exhibited heterophils values slightly higher than baseline (1.5-fold) at the end of the 11-day study. A rise in the lymphocyte population (Fig. 2C) was observed beginning on day 2, peaking at days 3–4, and declining back to near baseline levels at day 7 post-injection. Circulating quantities of monocytes, basophils and eosinophils did not change substantially ($p < 0.05$) during the course of the 11-day study (Figs. 1D–F).

Fig. 2 displays the total (Fig. 2A) and differential (Fig. 2B–F) leukocyte evaluations from untreated

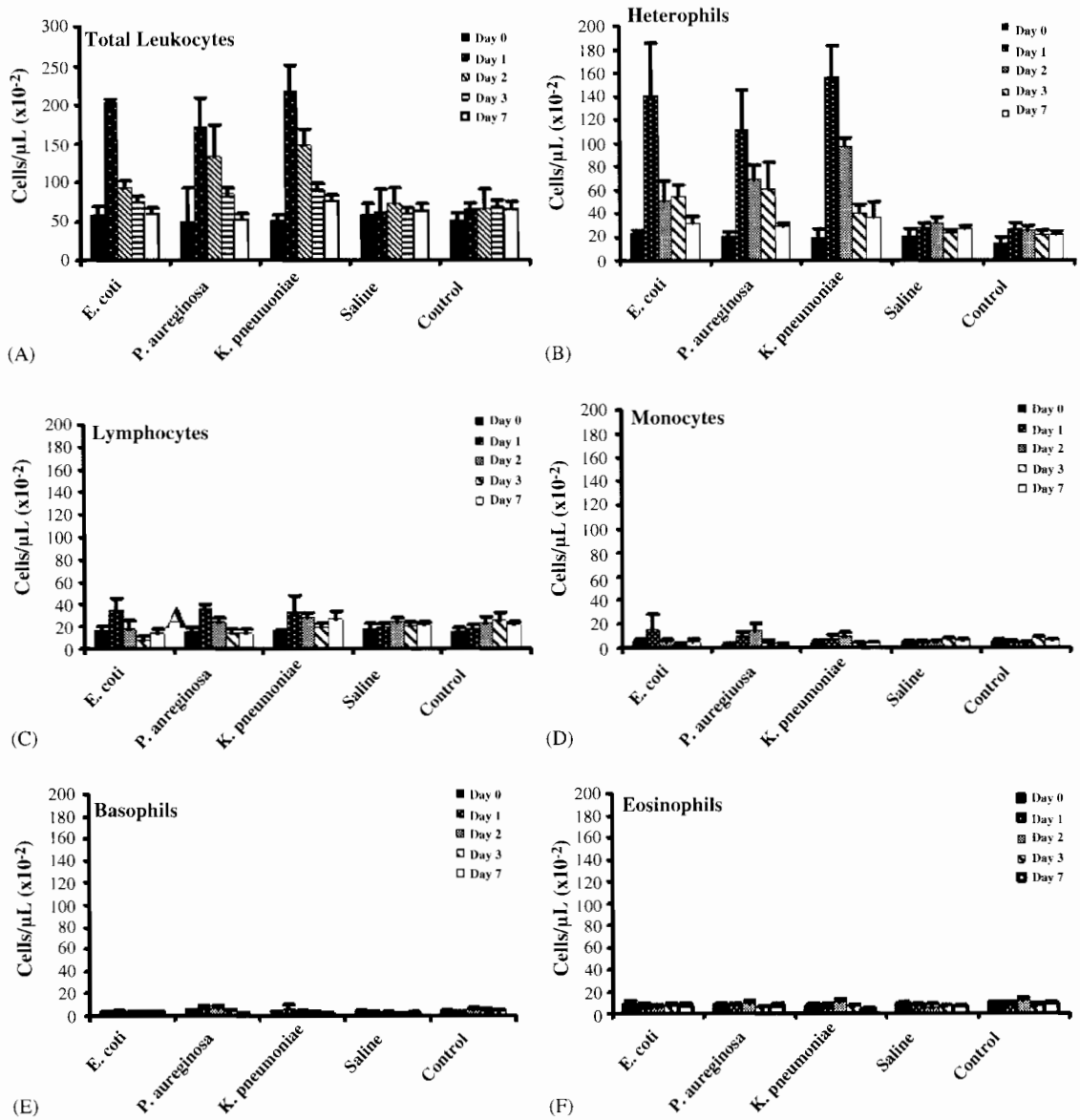

Fig. 2. Kinetic response of five types of alligator peripheral leukocytes to intraperitoneal injection of LPS. Alligators were injected intraperitoneally with derived from bacterial LPS from three different diverse species of bacteria. The animals were bled at various time points and the levels of circulating leukocytes were manually determined by Geimsa–Wright staining followed by phase contrast microscopy.

alligators, alligators injected with pyrogen-free saline, or animals injected with 0.5 mg/kg LPS derived from one of three different bacterial sources. Animals treated with LPS derived from *Escherichia coli*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* displayed 346%, 351%, and 436% increases in total peripheral leukocytes at 24 h post-injection. These values slowly declined and were near baseline one week after LPS injection. Untreated alligators and those injected with pyrogen-free saline did not display increases in peripheral leukocyte values during the seven day experiment ($p < 0.05$). Differential leukocyte evaluations revealed that heterophils were increased 613%, 533%, and 780% 24 h after intraperitoneal

administration of LPS derived from *E. coli*, *P. aeruginosa*, and *K. pneumoniae*, respectively. The heterophil values declined rapidly after 24 h and were near control values one week after LPS injection. In contrast to the results in Fig. 1, lymphocytes showed a slight, but statistically significant ($p < 0.05$), increase 24 h post-injection. Alligators injected with *E. coli*, *P. aeruginosa*, and *K. pneumoniae* LPS displayed a 225%, 247%, and 213% increase in peripheral lymphocytes, respectively (Fig. 2C). As a result of the early increase in lymphocytes, the H/L ratios were not as high as observed in Fig. 1 (1.44 for *E. coli* LPS, 1.40 for *P. aeruginosa* LPS, and 1.25 for *K. pneumoniae* LPS). Circulating levels of monocytes, basophils, and eosinophils did not appear to change substantially ($p > 0.05$) in response to LPS treatment at any dose or time point after injection (Figs. 2D–F).

4. Discussion

Little research has been focused on the basic immunology of crocodilians. Shaharabany et al. (1999) showed that crude tissue extracts of the Nile crocodile (*Crocodylus niloticus*) displayed antibacterial properties. Results from other studies in our laboratory have shown that blood from the American alligator (*Alligator mississippiensis* (Merchant et al., 2005a) and the saltwater (*Crocodylus porosus*) and freshwater crocodile (*Crocodylus johnstoni*) (Merchant and Britton, 2006) exhibit potent serum complement activity. Furthermore, the antibacterial activities can be used to distinguish the phylogenetic relations between the living crocodilian species (Merchant et al., 2006a). Other studies have focused on the antimicrobial actions of leukocyte extracts from *A. mississippiensis* (Merchant et al., 2006b). The results from these studies support the idea that crocodilians display potent and broad acting innate immune systems.

The results displayed in Fig. 1A show that intraperitoneal injection of bacterial LPS in alligators results in a concentration- and time-dependent increases in total peripheral leukocytes. The rapid rise in total leukocytes can be attributed solely to the increase in heterophils (Fig. 1B). As the total leukocytes decline after 24 h, another small population increase is observed at 4–5 days post injection. This increase is attributed to a secondary increase in heterophils (Fig. 1B) and an increase in the population of lymphocytes (Fig. 1C). Like higher eukaryotes, monocytes, basophils and eosinophils comprise a small portion of the total leukocytes. Circulating levels of monocytes exhibit a small change in response to LPS (Fig. 1D) but do not contribute substantially to the increase in total leukocytes. Basophil and eosinophil populations remain largely unchanged in response to LPS challenge (Fig. 1E–F).

Mammals can be extremely sensitive to the source of LPS. For instance, injection of Balb/c mice with LPS derived from *E. coli* HB101 results in maximal induction of acute phase gene transcription in the liver. The induction of hepatic acute phase gene expression is a sensitive indicator of acute-phase inflammation. In contrast, treatment of Balb/c mice with LPS from other strains of *E. coli* results in little or no induction of hepatic acute phase gene transcription (Dr. Jeff Rabek, personal communication). However, the results in Fig. 2 illustrate that alligators respond similarly to LPS challenge from three different diverse species of bacteria. It is interesting that alligators exhibit nearly identical responses to two different types of diverse opportunistic human bacterial pathogens (*E. coli* and *K. pneumoniae*) and a common soil bacterium (*P. aeruginosa*). These results support those of other studies in our laboratory that have indicated that alligators respond to a wide variety of microbial insults (Merchant et al., 2003, 2004, 2005b).

Intraperitoneal administration of LPS in higher eukaryotes usually results in a migration of large numbers of neutrophils to the peritoneal cavity within 24–48 h post-injection. Although the peripheral leukocyte values changed drastically in response to intraperitoneal LPS challenge in this alligator study, multiple attempts to isolate leukocytes from the peritoneal cavity failed. At this time, we are uncertain if alligator leukocytes lack the ability to migrate to the site of infection.

Another interesting observation is that alligators can obviously tolerate high doses of LPS without mortality. In contrast the LD_{50} of LPS derived from *E. coli* in mice has been reported to be 0.045–0.500 mg/kg body weight (Guglielmotti et al., 1997; Su et al., 1999). However, no mortality was observed with alligators at dose of 1.0-10.0 mg/kg. Alligators were able to withstand 40- to 222-fold the reported LD_{50} of

mice without mortality. These results seem to indicate that *A. mississippiensis* responds rapidly to toxic challenges with an increase in leukocytes, and can potentially handle a much higher bacterial burden than that of higher eukaryotes. This is supported by our results that show that the highest dose (10 mg/kg) of LPS did not change the LPS serum protein electrophoresis profile in any of the three alligators at any time point (data not shown).

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