



ACID EXPOSURE IS AN IMMUNE DISRUPTOR IN ADULT *RANA PIFIENS*

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Abstract—Acidic environments are physiological stressors for amphibians. The objective of the present study was to document the effect of an acidic environment on innate immune system function under controlled experimental conditions in *Rana pipiens*. We developed an in vivo assay, by injecting a suspension of 1- μ m fluorescent beads in fluid thioglycollate, to induce peritonitis. The number of peritoneal exudate leukocytes and their phagocytic activity did not increase with thioglycollate injection when frogs were exposed to pH 5.5 compared to when frogs were exposed to pH 7.0. An environment of pH 5.5 disrupted the inflammatory response of frogs compared to an environment of pH 7.0; at pH 5.5, more nonphagocytic leukocytes and fewer highly phagocytic leukocytes were found compared to those in frogs exposed to pH 7.0. Frogs stimulated by thioglycollate injection and exposed to pH 5.5 had a 50% increase in cells that did not exhibit phagocytosis and a 4- to 10-fold reduction in the number of highly phagocytic cells. This is evidence that acid exposure functions as an immune disruptor in adult *R. pipiens* under laboratory conditions.

Keywords—Amphibians Innate immunity Immune disruptor Environmental toxicants

INTRODUCTION

The initial realization of a potential global decline in amphibian populations occurred at the first World Conference of Herpetology in 1989. Consequently, in 1991, at a joint meeting of the Society for the Study of Amphibians and Reptiles and the Herpetologists' League, a symposium was held to ascertain the possible contribution of environmental acidification on this dramatic decline in amphibian populations [1]. Acidification of aquatic environments (and, therefore, exposure to increasingly acidic conditions in the field) is not a new phenomenon.

The Industrial Revolution in the middle of the 19th century brought about emissions that caused acid precipitation. In 1852, Robert Angus Smith was the first to ascertain the relationship between acid rain and atmospheric pollution and coined the term acid rain. Many sources of acid precipitation exist. However, acid precipitation may be categorized in two ways, naturally occurring and anthropogenic. Naturally occurring contributors include volcanoes and natural processes in both wetlands and oceans [2]. Anthropogenic sources are mainly emissions from fossil fuel burned in power plants and automobiles [3]. The objective of the present study was to document the effects of acid exposure on the innate immune response in adult northern leopard frogs (*Rana pipiens*). We hypothesized that acid exposure disrupts immune function.

Pennsylvania, among other northeastern states, suffered from very acid precipitation (as low as pH 4.67) as recently as 1998 [3]. Although acid precipitation has been reduced in the past decade because of the Clean Air Act (<http://www.epa.gov/oar/caa/contents.html>), many aquatic environments in the northeastern United States have not recovered from acidification. This continuing problem may result from the lack of natural buffering capacity in the soil and water [4], and it has been linked to amphibian mortalities in Sweden [5] and other parts of Europe [6]. The problem of acidification of

aquatic environments has been recognized in the northeastern United States for more than 20 years [7]. The problem still persists today, and the average pH of these habitats has continued to decline and is now less than pH 4.5. Acidification of aquatic environments has not been demonstrated in the western United States [8].

Globally, three documented waves of amphibian mass deaths have taken place. These occurred in Australia, the United Kingdom, and North and Central America. All three waves are associated with two diseases, chytridiomycosis or ranaviral disease [9]. A compromised immune system as a result of exposure to environmental pollutants has been suggested as one of the possible contributors to the emergence of these pathogens [10,11].

We recently published a hypothetical model that explains the interaction between acidification, immune function, and mortality of adult *R. pipiens* [12]. Our model proposes that exposure to acidic conditions adversely affects the natural bacterial defenses of *R. pipiens* by two separate mechanisms, disrupting the proper functioning of the intestinal epithelium as a barrier to bacterial translocation and decreasing both the number and viability of the white blood cells (WBCs). In the present report, we provide further evidence to support that model by examining the acid-induced suppression of the inflammatory response. Suppression of the innate and subsequent adaptive immune response may be the common response of frogs to a variety of environmental pollutants, not just acid. Therefore, compromised immunity may be a factor in the global decline in amphibian populations. As a result of our laboratory's studies, we conclude that acid can be added to the list of environmental stressors that disrupt the innate immune response of adult *R. pipiens*.

The present study concentrated on the effects of acid exposure on innate immunity. Inflammation is a natural physiological response both to injury and to infection, and it is the hallmark of the innate immune response. Fluid thioglycollate is widely used to induce inflammation experimentally in sev-

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eral animal models, including frogs [13–15]. Unlike many of the assays of innate immunity that are conducted in vitro, we developed an assay using thioglycollate that assesses the phagocytic activity of WBCs in vivo. Moreover, because these frogs were caught in the wild, we could not control for genetic variations, differences in physiological state, and history of exposure to environmental influences. In spite of this, the assay was sensitive enough to account for differences among the experimental groups and was reliably repeated in two consecutive years.

MATERIALS AND METHODS

We developed an assay to examine the effect of acid exposure on the inflammatory response of adult *R. pipiens*. To characterize the inflammatory response, we suspended fluorescein isothiocyanate (FITC)-labeled, 1.0- μ m polystyrene beads (Polysciences, Warrington, PA, USA) in thioglycollate medium. This suspension was then inoculated intraperitoneally, and we assessed the subsequent inflammatory response [16]. We quantified WBC recruitment and assessed the phagocytic capacity of WBC at the inflammatory site. The FITC beads were similar in size to bacteria; therefore, phagocytosis of these beads, which is easily observed using fluorescent microscopy, can be used to assay the phagocytic capacity of WBCs accurately in vivo.

Experimental groups

Rana pipiens of both sexes (length, 10–15 cm), purchased from Amphibians of North America (Nashville, TN, USA), were caught in the northeastern United States by licensed collectors in the fall, after the breeding season and before the frogs entered hibernation.

We conducted two sets of identical experiments in consecutive years. Frogs were randomly allocated into four groups of six to eight animals per group: Thioglycollate-inoculated frogs exposed to a buffer solution adjusted to pH 5.5 (TA group), thioglycollate-inoculated frogs exposed to a buffer solution adjusted to pH 7.0 (TN group), physiological Ringer's solution-inoculated frogs exposed to pH 5.5 (RA group), and physiological Ringer's solution-inoculated frogs exposed to pH 7.0 (RN group). The Widener University Institutional Animal Care and Use Committee approved all protocols for the experiments described in the present paper.

Experimental conditions

Before the 6-d experiments, frogs (experiment 1, $n = 31$; experiment 2, $n = 24$) were acclimated to the laboratory for 7 to 10 d in 39-L aquaria ($n = 6$ frogs/aquarium) filled with aged tap water (depth, 3 cm; water changed daily) containing a Styrofoam raft. Each frog was fed two crickets (age, four weeks) daily. Before the experiments, frogs were randomly assigned to each group. During the 6 d of each experiment, each frog was placed in an individual, autoclaved, Tupperware[®] container (30 \times 20 \times 9 cm) filled with 500 ml of the appropriate sterile-buffered solutions that were changed daily. The pH 5.5 buffer was prepared by adding 4.8 mM citric acid anhydride, 9.5 mM sodium hydroxide, and 0.6 mM sodium citrate to 75 L of tap water. The pH 7.0 buffer was prepared by adding 2.4 mM citric acid anhydride, 4.8 mM sodium hydroxide, and 0.3 mM sodium citrate to 75 L of tap water. Plastic containers were placed in an environmental chamber (Fisher Isotemp[®], Pittsburgh, PA, USA) set at 25°C with a 12:12-h light:dark (lights-on, 6:00 AM) cycle. On day 5, each frog was

injected intraperitoneally early in the afternoon with 2 ml of the appropriate solution. On day 6, the frogs were killed by ether asphyxiation, and peritoneal lavages were performed with 10 ml of an isotonic Amphibian Ringer's Solution (Carolina Biological Supply Company, Burlington, NC, USA). Animals were not fed during the 6-d experiment.

White blood cell count

White blood cells were counted to assess the extent of the inflammatory response. The WBC counts were performed on peritoneal lavage fluid using a hemocytometer [16]. Data for total leukocyte counts were analyzed by analysis of variance (ANOVA) and post hoc Tukey–Kramer test and reported as number of WBCs per milliliter.

Phagocytic activity assay

One-micrometer fluorescent beads are easily counted, and these were used to assess phagocytic activity of peritoneal WBCs. The FITC-labeled beads were diluted into the inoculation medium to a final concentration of 2.5×10^7 beads/ml. Each frog was inoculated intraperitoneally with 2 ml of the FITC suspension. Using a fluorescence microscope, the number of beads inside each WBC was counted. One-hundred leukocytes were counted per allocation, and based on the number of phagocytosed beads in each cell, cells were placed into two categories: Cells with zero beads (nonphagocytic), and cells with more than 10 beads (highly active phagocytic leukocytes). Data for phagocytic activity were analyzed with a three-way chi-square test (pH, thioglycollate, and phagocytic activity) [17]. The Mann–Whitney *U* test was used to test specifically for differences between pH 5.5 versus pH 7.0 in the thioglycollate-treated frogs regarding the number of nonphagocytic and highly phagocytic cells.

RESULTS

The number of peritoneal exudate leukocytes and their phagocytic activity did not increase with thioglycollate injection when frogs were exposed to buffer adjusted to pH 5.5 compared to frogs in the control group exposed to buffer adjusted to pH 7.0. An environment of pH 5.5 disrupted the inflammatory response of frogs compared to an environment of pH 7.0; at pH 5.5, more nonphagocytic leukocytes and fewer highly phagocytic leukocytes were found compared to those in frogs at pH 7.0. In both experiments, the number of WBCs per milliliter of peritoneal exudates was decreased significantly (4- to 10-fold) in the thioglycollate-stimulated, acid-exposed animals (ANOVA: experiment 1, $df = 3, 27$; experiment 2, $df = 3, 20$; $p < 0.01$) (Fig. 1). The thioglycollate-stimulated group held at pH 7.0 had a significantly higher peritoneal WBC influx compared to the other three groups (Tukey–Kramer: $p < 0.05$). The number of WBCs in the thioglycollate-stimulated, pH 5.5, TA group was similar to that in the two non-stimulated groups (RN and RA groups, no thioglycollate stimulation; Tukey–Kramer: $p < 0.05$).

Similarly, in both experiments, a significant association was found among the three variables: pH, thioglycollate treatment, and phagocytic activity (χ^2 test: $df = 4$, $p < 0.001$). The percentage of nonphagocytic WBCs in the pH 5.5-stimulated group was approximately 1.5-fold greater than that in the stimulated group exposed to pH 7.0 (TN group; Mann–Whitney *U* test: $p < 0.05$) (Fig. 2). Conversely, the percentage of highly phagocytic WBCs was two- to threefold greater (Mann–Whitney *U* test: $p < 0.05$) (Fig. 3) in the thioglycollate-stimulated

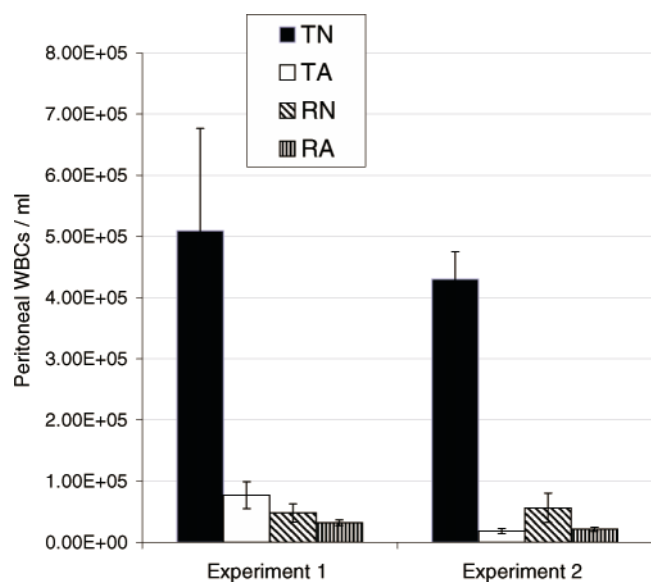
Immune disruptors in *Rana pipiens*

Fig. 1. Number of peritoneal exudate white blood cells (WBCs) per milliliter recovered from the four treatment groups. The TN group (experiment 1, $n = 8$; experiment 2, $n = 6$) were frogs stimulated with thioglycollate and exposed to a neutral pH (7.0). The TA group (experiment 1, $n = 8$; experiment 2, $n = 6$) were frogs stimulated with thioglycollate and exposed to an acidic pH (5.5). The RN group (experiment 1, $n = 8$; experiment 2, $n = 6$) were frogs stimulated with Ringer's solution and exposed to a neutral pH (7.0). The RA group (experiment 1, $n = 7$; experiment 2, $n = 6$) were frogs stimulated with Ringer's solution and exposed to an acidic pH (5.5). In both experiments, the number of WBCs per milliliter of peritoneal exudates was decreased significantly in the thioglycollate-stimulated, acid-exposed animals (TA group) as compared to the thioglycollate-stimulated, neutral-exposed animals (TN group).

group held at pH 7.0 compared to the stimulated group exposed to an acidic pH of 5.5. In both experiments, the percentage of both nonphagocytic and highly phagocytic cells in the stimulated, pH 5.5 group was similar to the percentage of cells in both unstimulated groups.

DISCUSSION

We measured *in vivo* the inflammatory response and phagocytic activity of WBCs in outbred adult northern leopard frogs (*R. pipiens*). Both responses are parts of the innate immune response. The assay measured the phagocytic activity of both resident and recruited WBCs in peritoneal exudates. We have shown, as have others [18], that phagocytosis of FITC microspheres can be used to assess the activity of phagocytic WBCs.

Suppression of innate immune system function (inflammatory response, antigen phagocytosis, and presentation) could compromise adaptive immune response and, therefore, may lead to immune suppression and death. Compromising the innate immune response by exposure to an acidic environment could unleash a cascade of physiological events leading to mortality or low reproduction. In the present study, an acidic environment decreased the influx of leukocytes to the peritoneal cavity, thus weakening the inflammatory response of the frogs. Furthermore, the acidic environment decreased the phagocytic function of the leukocytes. Acid exposure decreased the number of leukocytes at the inflammatory site by 4- to 10-fold. This decrease is evidence of decreased cell recruitment. We also have demonstrated that the activity of these phagocytic cells is diminished under acidic conditions. Frogs stimulated with thioglycollate and exposed to pH 5.5 (TA

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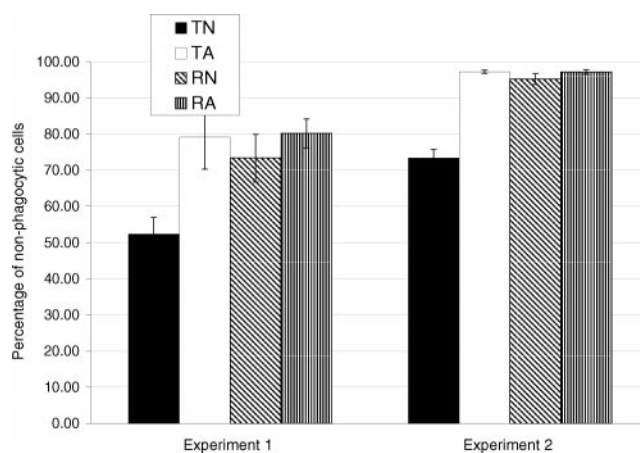


Fig. 2. Percentage of nonphagocytic peritoneal exudate white blood cells (WBCs) as determined by fluorescence microscopy in the four treatment groups. The percentage of nonphagocytic cells was significantly higher in the thioglycollate-stimulated, acid-exposed group (TA group) as compared to the thioglycollate-stimulated, neutral-exposed group (TN group). See Figure 1 for the definitions of RN and RA.

group) did not mount as strong an inflammatory response as frogs exposed pH 7.0 (TN group); in fact, they had a similar inflammatory response to unstimulated controls. In essence, acid exposure reduced the inflammatory response to background levels (RN group). This may be caused either by a decreased activity of phagocytic cells (i.e., macrophages and neutrophils) or by a replacement of phagocytic cells with nonphagocytic lymphocytes.

Frogs differ in their sensitivity to acid exposure. *Rana pipiens* appear to be especially sensitive during all stages of development [19–24]. Previously, we demonstrated that adult *R. pipiens* exposed to pH 5.5 suffer a 72% mortality within 10 d [25], and recently, we proposed a model for the effects of acid on adult *R. pipiens*. We demonstrated that acid-stressed frogs may suffer from a systemic infection caused by transit of endogenous gut bacteria into the vascular system [12]. This systemic infection demonstrates the vulnerability of acid-exposed frogs, and it may explain the high mortality rate they suffered. These endogenous bacteria colonized the spleens of acid-exposed frogs [12]. These data suggest that a compro-

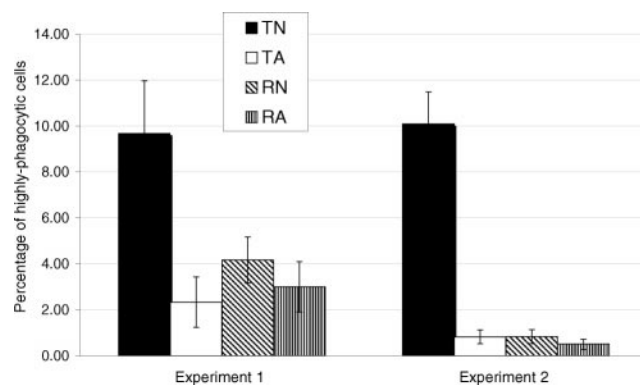


Fig. 3. Percentage of highly phagocytic peritoneal exudate white blood cells (WBCs) as determined by fluorescence microscopy in the four treatment groups. The percentage of highly phagocytic cells was significantly higher in the thioglycollate-stimulated, neutral-exposed group (TN group) as compared to the thioglycollate-stimulated, acid-exposed group (TA group). See Figure 1 for the definitions of RN and RA.

mised inflammatory response, in conjunction with an increased ability of gut bacteria to transit the intestinal epithelium because of acid exposure, may provide a partial explanation for the recent decline in *R. pipiens* populations in the northeastern United States.

Our observations provide empirical support not only for our model [12] but also for the hypotheses of other investigators [26–28] that environmental stress may be the initiating factor leading to immunosuppression of the innate and adaptive immune response, resulting in systemic distribution of opportunistic and virulent bacteria. Ultimately, this may contribute to the death of adult frogs. Glorioso et al. [26] isolated and identified bacteria (including *Aeromonas hydrophila*) from Louisiana bullfrogs and tadpoles (*Rana catesbeiana*) exhibiting red-leg disease, a bacterial septicemia. The bacteria identified from cardiac blood were both pathogens and opportunistic pathogens. Some of these same organisms also have been isolated from healthy frogs. It therefore was suggested that environmental factors could function as “stressors” that predispose animals to lethal infection with opportunistic pathogens. Carey [27] suggested a working model to explain the disappearance of 11 populations of boreal toad between 1974 and 1982 in Colorado, USA. The apparent cause of extinction was infection with *A. hydrophila*. In that model, she proposed that environmental factors function as stressors, leading to immunosuppression, systemic distribution of bacteria, and death. Carey et al. [28] further refined this working model by examining the effects of cold temperature on immune function.

The effect of stress on immune function and the interaction between the endocrine and immune systems have been the subject of intensive study. Previous work in domestic animals showed the connections between environmental stressors on immune function and host–pathogen interactions [29]. Stress, both physiological as well as psychological, has been shown to alter immune function in humans [30]. We therefore have coined the term immune disruptors to describe the effects of environmental toxicants on immune function (*SETAC Globe*, vol. 5, no. 3, pp 48–49). Acid exposure clearly functions as an immune disruptor in *R. pipiens* and may have similar effects on other acid-sensitive amphibians and aquatic vertebrates [3]. Therefore, acid exposure may play a role in population declines of acid-sensitive amphibians, such as *R. pipiens* in the northeastern United States.

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