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Characterization of the Serum Complement Activity of the Broad-Snouted Caiman *Caiman latirostris* (Crocodilia: Alligatoridae)

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Pablo A. Siroski, Mark Merchant, María Virginia Parachú Marcó, Carlos I. Piña, and Hugo H. Ortega (2010) Characterization of the serum complement activity of the broad-snouted caiman *Caiman latirostris* (Crocodilia: Alligatoridae). *Zoological Studies* **49**(1): 64-70. The sheep red blood cell (SRBC) hemolysis assay was used to detect and characterize complement-system activity of broad-snouted caiman (*Caiman latirostris*) serum. The hemolytic activity of caiman serum was inhibited by 2 classic inhibitors (EDTA and heat) suggesting the existence of complement-system activity. In addition, we found that the capacity of *C. latirostri*s serum to disrupt SRBCs was concentration, temperature, and kinetics dependent. Hemolytic activity was detected from a very low concentration (< 10%) of caiman serum and increased until 100%. Temperature influenced the activity of the serum by disrupting SRBC membranes. The serum showed a peak of hemolysis between 30 and 40°C, within which lies the optimal temperature caimans prefer during thermoregulation for normal physiological processes. Hemolytic activity rapidly occurred at 2 min, and maximum activity was detected at 60 min. These observations reflect previously reported findings in other crocodilian species (*Alligator mississippiensis*, *Crocodylus johnstoni*, and *Cro. porosus*), thus adding to the knowledge of the role of the complement system in immunological activities of crocodilians. http://zoolstud.sinica.edu.tw/Journals/49.1/64.pdf

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Animals are exposed daily to millions of potential pathogens through contact, ingestion, and inhalation (Hancock and Scott 2000). Immunity to infection is mediated by 2 general systems, innate (or natural) and acquired (or adaptive). Such mechanisms have evolved and diversified in response to many factors, including the environments in which organisms live, body complexity, distinct physiology, and lifespan (Zarkadis et al. 2001).

The adaptive immune system, with its randomly generated and vast antigen-receptor diversity, allows organisms the prospect of evading microbial insults (Flajnik and Du Pasquier 2004). In contrast to adaptive immunity, which is restricted to vertebrates, innate immunity is more ancient and is used by invertebrates such as insects and echinoderms, as well as by higher animals (Song et al. 2000).

Components of the innate immune system are markedly conserved between insects and mammals, indicating a common ancestral origin

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for this branch of immunity (Hoffmann et al. 1999). The serum complement is an important element of the innate immune defense of animals against infectious agents. It appears to be highly conserved in vertebrates, although research on reptiles and amphibians is scarce.

The complement system consists of a group of plasma proteins that play critical roles in host defense by interacting with components of both the innate and adaptive immune systems. It represents an important arm of the innate system in vertebrates and invertebrates (Song et al. 2000), which can be sequentially activated in a multi-step cascade reaction by classical, alternative, and lectin pathways (Götze and Müller-Eberhard 1976, Loos 1982).

Studies showed that the complement system, as part of the innate mechanism of fish and other poikilothermic vertebrates, is more diverse than that of higher vertebrates, so a broader range of antigens can be recognized (Sunyer et al. 1998). Poikilothermic species, ranging from teleosts to reptilians, appear to contain a well-developed complement system resembling that of homeothermic vertebrates (Zarkadis et al. 2001).

Some studies reported the presence of complement-system components in a variety of reptiles (Koppenheffer 1986). Merchant et al. (2005b) detected alternative pathway serum complement in the blood of the American alligator *Alligator mississippiensis*, and similar results were found by Merchant and Britton (2006) in freshwater (*Crocodylus johnstoni*) and saltwater (*Cro. porosus*) crocodiles. Both studies suggested the existence of potent complement systems in those species.

The sheep red blood cell (SRBC) hemolysis assay was developed to detect complement-system activity. Complement activity is primarily measured and quantified using hemolytic assays with sheep erythrocytes, a rabbit anti-sheep erythrocyte antibody, and a complement source, usually guinea pig serum (Shevach 2005). Merchant et al. (2006) modified the SRBC lysis assays described by Mayer (1967) to characterize American alligator complement-system activity. These assays were used herein to detect and characterize the serum complement activity of *Caiman latirostris*.

MATERIALS AND METHODS

Animals

Four (*n* = 4, 3 females and 1 male, 142-168 cm in total length) adult caimans from a semi-natural environment in Proyecto Yacaré (Convenio Gobierno de Santa Fe/MUPCN) Santa Fe Province, Argentina were captured. These animals were fed chicken heads and fish 3 times per week. At the end of the summer, blood was taken from each animal using a spinal vein technique (Tourn et al. 1994, Zippel et al. 2003). The blood was allowed to clot at room temperature and then centrifuged at 2500 ×*g* for 15 min. The serum was removed and pooled for subsequent analysis.

SRBC hemolysis assay

Fresh SRBCs were obtained from heparinized whole blood collected from Merino sheep (*Ovis aries*) at Estación Zoológica Experimental, Santa Fe City, Argentina. Sheep blood was washed with phosphate-buffered saline (PBS, pH 7.4) several times until the supernatant was clear, and then a 2% SRBC (v/v) solution was prepared.

To characterize the *C. latirostri*s complement system, we used the SRBC hemolysis assay. This assay is based on the hemolytic disruption of SRBCs by means of the immunological proteins of the serum. Frequently, it is used to evaluate complement systems in clinical laboratories (Kirschfink and Mollnes 2003), and it was adapted to crocodilian serum by Merchant et al. (2006).

The hemolytic assay was performed to detect complement-system activity of broad-snouted caiman serum, including 2 classical inactivators of the serum complement, heat and ethylenediamine tetraacetic acid (EDTA). Untreated caiman serum, preheated serum (56°C for 30 min), and serum treated with 50 mM EDTA were exposed to 2% (v/v) SRBCs. In all 3 cases, 1 ml of serum was added to 1 ml of 2% SRBCs (v/v) for 30 min.

Another assay was performed to evaluate the effects of temperature on the complement system of broad-snouted caiman serum by preheating aliquots of caiman serum and SRBCs at different temperatures (5-45°C, at 5°C intervals) for 15 min prior to SRBC exposure. Then 0.5 ml of preheated serum and 0.5 ml of SRBCs were combined and incubated at the desired temperature. After that, the mixture was put on ice to stop the reaction. To characterize the kinetics of SRBC hemolysis by caiman serum, 25 ml of serum was mixed with

25 ml of 2% SRBCs (v/v). After incubation, 0.5 ml of this combination was removed at different time intervals (2, 5, 10, 15, 20, 25, 30, 45, and 60 min). To study the concentration dependence of *C. latirostri*s complement-mediated SRBC hemolysis, different amounts of caiman serum (from 0% to 100%) were incubated with 2% SRBCs for 30 min.

All assays were carried out at room temperature except the temperature study to be able compare with results reported by previous studies. In all assays, except in the kinetic assay, where the incubation time points varied, serum was incubated with SRBCs for 30 min. Following incubation, the mixture was centrifuged at 2500 ×*g* for 5 min. Then 300 μl of supernatant was transferred to a microtiter plate, and the optical density was measured at 540 nm in a microplate reader (Labsystem Multiskan RC, Helsinki, Finland).

Complement hemolysis was obtained by the method proposed by Mayer (1967). The CH_{50} value represents the volume of serum required to produce 50% maximum hemolysis. In this study, it corresponded to concentrations of serum required to produce 50% hemolysis of 1% SRBCs in a 1-ml-volume reaction.

Statistical analyses and controls

With the aim of obtaining a positive control, a solution of 1% SRBCs containing 1% (v/v) Triton X-100 was aggressively injected and ejected several times through a tuberculin syringe until complete hemolysis had been achieved. Full hemolysis was confirmed by means of an optical microscope (Olympus BH-2, Tokyo, Japan) at 400X. Positive-control aliquots were centrifuged, and the optical density of the supernatant was measured in a microplate reader at 540 nm. All experiments were conducted in triplicate to obtain a valid statistical evaluation of the results. The results from SRBC hemolysis after exposure to caiman serum were divided by the positive control hemolysis for each experiment, and all results are expressed as the percent maximum hemolysis (MH%; mean ± standard error).

Statistical analysis was performed using SPSS 16.0 software (SPSS for Windows 2007). Data were tested for normality with the Kolmogorov-Smirnov test, and homogeneity of variances between groups was verified by the Levene test (Levene 1960). One-way analysis of variance (ANOVA) was used to test for differences between treated (heat and EDTA) and untreated groups. Regression analyses were used to describe the effects of temperature, kinetics, and concentration dependence. A p value of < 0.05 was considered statistically significant.

RESULTS

Different assays with unsensitized SRBCs were conducted to characterize the complement system of *C. latirostri*s. Data depicted in figure 1 demonstrate the ability of broad-snouted caiman serum to rupture SRBC membranes. In this case, MH% values of SRBCs with EDTA (14.4 ± 2.41) and heat (17.2 ± 2.17) were compared. Both are considered to be inactivators of the complement system (Morgan 2008). The absorbance recorded indicated that there were significant reductions in the maximum hemolysis of SRBCs when complement-system inhibitors were added ($p < 0.001$; Fig. 1).

The effects of temperature on the hemolysis of SRBCs by broad-snouted caiman serum are shown in figure 2. Broad-snouted caiman serum showed a strong positive correlation of temperature with the MH% up to 35°C ($p < 0.001$, $R^2 = 0.98$). The lowest activity of caiman serum of disrupting the integrity of SRBCs was identified at 5°C (13.48% ± 0.408%). This ability slightly increased with temperature (10°C: 15.27% ± 1.85%; 15°C: 31.43% ± 1.71%, and 20°C: 59.37% ± 1.68%). From 25 to 35°C, hemolysis increased approximately 20% from 47.64% (± 0.17%) to 72.91% (± 1.32%), respectively. Peak hemolysis was measured at 35°C (84.42% ± 1.91%). After

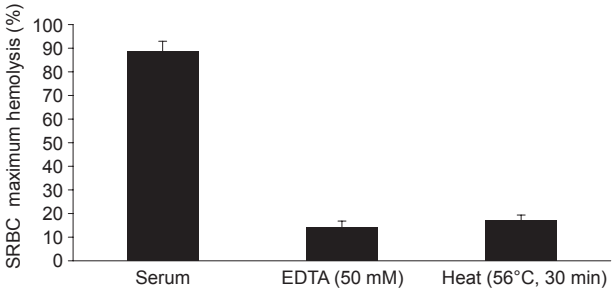

Fig. 1. Effects of treated (50 mM EDTA and heat at 56°C for 30 min) and untreated serum on the hemolysis of sheep red blood cells (SRBCs) by broad-snouted caiman serum. The untreated caiman serum (Serum) demonstrated a greater ability to disrupt SRBCs compared to the 2 treatment groups ($p < 0.001$).

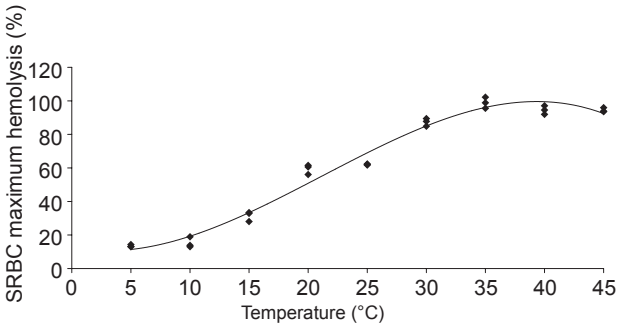

Fig. 2. Caiman serum and 1% (v/v) sheep red blood cells (SRBCs) were incubated at different temperatures (5 to 45°C, at 5°C intervals). Broad-snouted caiman serum showed a positive correlation between temperature and the percentage of maximum hemolysis (MH%): MH% = 12.448 + 1.2973[T°] - 0.235[T°]2 - 0.0037[T°]3; $p < 0.001$, $R^2 = 0.98$. Results are presented as the MH% of 3 determinations.

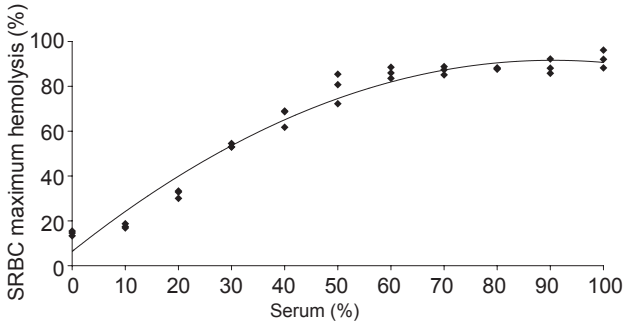

Fig. 3. Incubation of 1% (v/v) sheep red blood cells (SRBCs) with increasing concentrations of caiman serum. Results are presented as the percentage of maximum hemolysis (MH%) of 3 determinations: MH% = 6.3746 + 1.8842[Serum (%)] - 0.0104[Serum (%)]^2; $p < 0.001$, $R^2 = 0.96$.

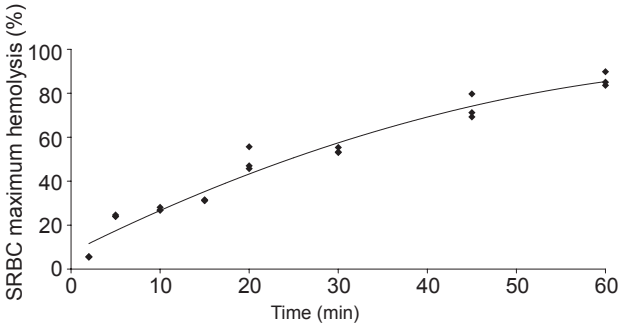

Fig. 4. Incubation of 25 ml of serum with 25 ml of 1% sheep red blood cells (SRBCs) (v/v) performed to evaluate the in vitro kinetics of SRBC hemolysis caused by caiman serum. Aliquots were removed at different time points. Results are presented as the percentage of maximum hemolysis (MH%) of 3 determinations: MH% = 7.6047 + 2.0274[min] - 0.0122[min]^2; $p < 0.001$, $R^2 = 0.96$.

that, a slight reduction of hemolysis was observed at 40 and 45°C (80.08% ± 1.47% and 80.0% ± 0.74%, respectively).

Hemolysis of SRBCs by broad-snouted caiman serum was concentration dependent, as shown in figure 3 ($p < 0.001$). The ability of broad-snouted caiman serum to disrupt SRBC membranes showed a positive relationship with an increase in the serum concentration describing a logistic model. Incubation of 10 ml of SRBCs without caiman serum at room temperature resulted in a maximal hemolysis of 13.2% ± 0.54%. Hemolysis of SRBCs with 20%, 30%, and 40% caiman serum exhibited activities of 17.64% ± 1.02%, 39.01% ± 0.51%, and 51.97% ± 2.36%, respectively. Consequently, caiman serum-mediated SRBC hemolysis increased until it reached the highest value at 100% (77.64% ± 2.29%). Specifically the hemolysis percentages at 50%, 60%, 70%, 80%, and 90% caiman serum were 64.95% ± 4.72%, 71.50% ± 1.43%, 72.59% ± 1.07%, 73.43% ± 0.16%, and 74.19% ± 1.86%, respectively. The CH_{50} value was calculated to be 386 μL for caiman serum.

The results of the MH% found after exposing SRBCs to broad-snouted caiman serum at different time points are displayed in figure 4. The capacity of *C. latirostri*s serum to damage SRBCs depended of the length of exposure ($p < 0.001$, $R^2 = 0.96$). Two minutes of exposure showed maximal hemolysis of 5.54% ± 0.05%, and this ability slowly rose from 5 (24.19% ± 0.22%) to 30 min (10 min: 27.25% ± 0.43%; 15 min: 31.28% ± 0.12%; 20 min: 49.45% ± 3.12%; and 30 min: 53.90% ± 0.72%). Hemolysis continued to increase at 45 min (73.37% ± 3.21%) and 60 min, at which time the maximum response (86.15% ± 1.88%) occurred.

DISCUSSION

Crocodilians are exposed daily to potential pathogens as consequences of injuries caused by social disputes, population density, and others factors. They are constantly threatened by microorganisms, but they rarely show signs of infection (Manolis et al. 1991, Madsen 1993, Madsen et al. 1998). These animals have evolved and diversified in response to many factors, mainly the environment in which the organisms live. The serum complement systems of fish and other poikilothermic vertebrates are more diverse than those of higher vertebrates, so a broader range of antigens can be recognized (Sunyer et al.

1998). These findings may be related to the high resistance of crocodilians to infections (Merchant et al. 2005b).

In recent years, there has been increasing emphasis on phylogenetic studies of immune competence. Many researchers reported the presence of complement-system serum in reptiles (Koppenheffer 1987, Sunyer et al. 1998). Mastellos et al. (2004) described the complement system as a phylogenetically conserved arm of innate immunity. Carey et al. (1999) included the complement system as a part of the innate defense mechanisms in urodele and anuran amphibians together with antimicrobial peptides, natural killer cells, and phagocytic cells.

Various antimicrobial activities of crocodilian tissues were described (Shaharabany et al. 1999, Merchant et al. 2003 2004 2005a, Merchant and Britton 2006, Siroski et al. 2009) and were attributed to an effective complement system. However, to our knowledge, the detection and characterization of the broad-snouted caiman's complement system has not been investigated until now.

Hemolytic assays were traditionally used to assess the functional activities of the complement system (Kirschfink and Mollnes 2003). The incubation of broad-snouted caiman serum with 1% SRBCs resulted in hemolytic activity determined by an increase in the optical density at 540 nm due to the release of hemoglobin by disrupted erythrocytes. Müller-Eberhard (1969) and Mollnes et al. (1988) demonstrated that EDTA is a potent chelator of divalent metal ions, which blocks activation of the complement system *in vitro* via its calcium- and magnesium-binding properties. These were shown to be essential components of the *A. mississippiensis* complement system activation cascade (Merchant et al. 2005c). Moreover, heating to 56°C for 30 min (a process commonly called decomplementing; Morgan 2008) led to a significant drop in the hemolytic activity of the serum. The complement system was first described in the 1890s as a heat-labile protein in serum that "complemented" the killing of bacteria by heat-stable antibodies (Fujita et al. 2004). This is consistent with the idea that hemolytic activity is not due to antimicrobial peptides because they are considered to be heat stable (Diamond et al. 1991). Therefore, an antibody interference study was not conducted. Observations of similar findings in normal human (Morgan 2008), American alligator (Merchant et al. 2005b), and freshwater and saltwater crocodile serum (Merchant and Britton 2006) strongly suggest definite physiological significance of the serum complement system in broad-snouted caiman serum in terms of protection against various pathogens. The hemolytic activity of *C. latirostri*s serum toward SRBCs was characterized in terms of dependence on the concentration, temperature, and kinetics.

Ectothermic vertebrates are suitable models for studying the influence of temperature on a variety of physiological functions (Pxytycz and Zkowicz 1994). These data support previous studies which indicated that environmental temperature plays a fundamental role in ectothermic vertebrate homeostasis, including antibody formation and the immune response (Klesius 1990). In addition, crocodilians prefer to maintain their body temperatures within a narrow range of 28-33°C by using thermogradients in their natural environment, e.g., sunshine/shade and warm surfaces/cold deep waters (Huchzermeyer 2002). One of the important physiologic activities influenced by environmental temperatures is the serum complement system. As we show in figure 2, the hemolysis of SRBCs by broad-snouted caiman serum *in vitro* depends on the temperature at which it is incubated. The highest hemolytic activity occurred between 30 and 40°C. Since the activity was measured at 5°C intervals, it is difficult to pinpoint a specific temperature for the maximum, but this peak could be near the temperature range caimans prefer during thermoregulation for normal physiological processes (Bassetti 2002). In other species of crocodilians studied, the peak serum complement activity was observed at 30°C in the freshwater crocodile, 25°C in the saltwater crocodile (Merchant and Britton 2006), and 35°C in the American alligator (Merchant et al. 2006). The American alligator and caiman belong to the same family (Alligatoridae) with crocodiles in another family (Crocodylidae). These differences may be attributed to adaptive preferences (optimal temperature range) of each crocodilian family.

The complement system was implicated in the pathogenesis of several disorders. Frequently, hemolytic assays are used in clinical laboratories to evaluate the complement system and identify human patients with complement deficiencies. The relationship of the hemolytic activity of *C. latirostri*s toward non-sensitized SRBCs was generated on the basis of the data obtained from simultaneous serum titrations carried out with 11 different serum concentrations. The slope of the curve and absorbance values coincided with other crocodilian species investigated (Merchant et al.

2005b, Merchant and Britton 2006). As mentioned above, the CH_{50} is an index used to measure the functionality of the complement system, and the value determined in this study was 386 μL. Other CH_{50} values obtained in similar studies were 539 μL for the American alligator (Merchant et al. 2005b), 451 μL for the freshwater crocodile, and 473 μL for the saltwater crocodile (Merchant and Britton 2006).

The incubation of broad-snouted caiman serum with SRBCs showed a time-dependent reaction (Fig. 4). When caiman serum was exposed to SRBCs, hemolysis steadily occurred, with measurable activity at 2 min which increased to a maximum of 86% SRBC hemolysis at 60 min. The kinetics of SRBC lysis by broad-snouted caiman serum is similar to the *in vitro* actions of antibacterial effects detected in alligator serum (Merchant et al. 2003) and the human complement against *Escherichia coli* previously reported (Wright and Levine 1981). Also, the kinetics data shown are very similar to those found in other crocodilians studied (Merchant et al. 2005b, Merchant and Britton 2006). These observations can be considered a rapid response of the immunological properties of caiman serum to avoid any challenge from microbial infection.

The ability of crocodilians to avoid infection depends in part on the mechanisms of their innate immunity. There is a tendency to emphasize the roles of humoral and/or cellular immunological systems against infection. However, it is clear that those systems are not triggered rapidly enough to provide immediate protection of organisms against exposure to pathogens (Hancock and Scott 2000). The serum complement system in some ectothermic species is present in multiple forms, and researchers hypothesized that this complement diversity is used by the species as a strategy to expand its innate immune recognition capabilities (Sunyer et al. 1998). From our results, we could not distinguish if the hemolytic activities were due to classical or alternative pathways.

This study indicated that the mechanisms of serum complement activities for diverse crocodilian species studied are similar. The foregoing data highlight the significant contribution of the complement system to immunological activities of this species. Crocodilians represent an extremely successful group of organisms that have changed little for millions of years. The serum-complement system could be a very important innate immune component in the resistance to attack by micro-organisms, and could be one of the reasons for their longevity.

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