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Characterization of serum complement activity in serum of the Komodo dragon (*Varanus komodoensis*)

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ABSTRACT

Incubation of different volumes of serum from the Komodo dragon (*Varanus komodoensis*) with sheep red blood cells (SRBCs) resulted in volume-dependent hemolysis, as measured spectrophotometrically at 540 nm. The hemolysis occurred rapidly, with almost 90% of the hemolytic activity occurring within 20 min of incubation. A thermal profile showed that Komodo dragon serum exhibited low activity from 5˚C-20˚C, but exerted maximum activity at 35˚C, which was substantially reduced at 40˚C. The maximum activity was observed near optimal temperatures to which Komodo dragons thermoregulate. Mild heat treatment of Komodo dragon serum (56˚C, 30 min) depleted the ability to hemolyze SRBCs. In addition, preincubation of Komodo dragon serum with only 5 mM EDTA or phosphate, both chelators of divalent metal ions, reduced the hemolytic activity sharply. These results indicate that the hemolytic activity was due to the presence of a potent serum complement system. Incubation of Komodo dragon serum with 5 mM EDTA and 15 mM Ca^{2+} or Mg^{2+}, but not Ba^{2+}, Zn^{2+}, or Fe^{2+}, completely restored activity. These results indicate that Komodo dragon serum complement activity requires the presence of Mg^{2+} or Ca^{2+}. This is the first assessment of innate immune activity of a Varanid.

Keywords: Innate Immunity; SRBC Hemolysis Varanid

1. INTRODUCTION

Innate immunity comprises that part of microbial defense which responds in a non-specific manner to infiltration by microbes. Serum complement is one of the first lines of defense against infection, and is made up of approximately 11 different proteins that can be activated by three different mechanisms, the classical (antibody-dependent) pathway [1], the alternative pathway [2], and the lectin-dependent pathway [3]. Once activated, all three mechanisms work in a proteolytic cascade fashion, resulting in the eventual formation of a multi-protein "membrane attack complex" [4] in the outer membrane of the microbe, causing leakage of cellular contents and lysis. Some of the proteolytic complement protein fragments also act to opsonize microbes for phagocytosis [5], serve as attractants for the chemotaxis of macrophages and neutrophils to the site of infection [6], and function as anaphylactic factors [7], causing mast cell degranulation and vascular permeability [8]. Deficiencies in complement proteins have been associated with susceptibility to bacterial infection [9], Leiner's disease [10], fulminant hepatic failure [11], and many other clinical conditions [12]. Serum complement is among the most important components of innate immunity, and has been identified and characterized across a broad spectrum of diverse taxa, including mammals [13] and ancient invertebrates [14,15].

The Komodo dragon (*Varanus komodoensis*) is the world's largest lizard. Its range is restricted to several small islands in the Indonesian archipelago. These animals are carnivorous reptilians, and are known to feed on prey items much larger than themselves. Komodo dragons typically lie at ambush points and bite their prey, delivering a heavy load of infectious bacteria from their saliva [16,17], and then often follow their prey for days, or even weeks, before they succumb to systemic infection. In addition, Komodo dragons have been reported to deliver potent toxins in their bites that prevent coagulation and cause prey items to succumb to systemic shock [18]. Although these animals exhibit aggressive behaviors toward members of their own species during territorial disputes, and often bite each other during feeding frenzies [19], Komodo dragons do not seem to experience the same fate as their prey. This seems to indicate that these animals have developed an immune system

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that allows them to combat potentially high loads of pathogenic bacteria. However, because of their remote location and endangered status, little is known concerning the physiology, biochemistry, and immunology of these apex predators. Recent studies in our laboratory have shown that the serum from Komodo dragons exhibits potent and broad-acting antibacterial properties [20]. This study was undertaken to examine the serum complement system of the Komodo dragon, and is the first characterization of an innate immune component in a Varanid.

2. MATERIALS AND METHODS

Chemicals and biochemicals: Sheep red blood cells (SRBCs) were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Ethylene diamine tetraacetate (EDTA), $CaCl_2$, $MgCl_2$, $BaSO_4$, $FeCl_2$, and $ZnCl_2$ were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Collection of samples: Blood samples were collected from Komodo dragons at the Houston and San Antonio zoos. Blood was drawn from the tail vein, transferred to Vaccutainer™ tubes, and allowed to clot for at least five h before the serum was collected. The amount of blood collected from each individual depended on the size of the animal, and was at the discretion of the attending veterinarian at each institution. Blood was collected from the tail caudal veins three adults (20 - 81.5 kg) and five juveniles (1.5 - 6.2 kg), transferred to Vaccutainer™ tubes, and allowed to clot for at least five hr before serum was collected by centrifugation. The serum was pooled so that average antibacterial values for this species could be generated. The collection of blood from these animals was conducted in accordance with the Animal Care and Use institutional policies of the Houston and San Antonio Zoos.

Serum volume-dependent SRBC hemolysis: The functionality of the Komodo dragon serum complement system of proteins was examined using a SRBC lysis assay modified from the method of Mayer [21], and previously described for crocodilians [22]. To measure the volume dependence of SRBC activity on Komodo dragon serum, different volumes of serum (0 - 15 µL) were diluted to 150 µL total volume with a 0.9% saline solution and then 150 µL of 2% SRBCs was added to the solution. The samples were allowed to incubate for 60 min, followed by centrifugation at 16,000 × g for 5 min at ambient temperature. The optical densities of the resulting supernatants were measured using the Bio-Rad Benchmark Plus™ microplate spectrophotometer at 540 nm.

Kinetic analysis of SRBC hemolysis: For the determination of the hemolytic kinetic profile, a solution composed of 250 µL of Komodo dragon serum, 5.15 mL of a 0.9% saline solution, and 5.40 mL of a 1% SRBC was prepared. In quadruplicate, 290 µL of solution was dispensed into separate vials. At different time intervals (0 - 120 min), the samples were centrifuged and transferred to microtiter plates to measure optical density (540 nm) as described above.

Temperature-dependent SRBC hemolysis: Komodo dragon-mediated serum complement SRBC hemolysis activity was also assayed at different temperatures. Aliquots of Komodo dragon serum (5 µL) and 145 µL of saline were incubated at different temperatures (5˚C - 40˚C in increments of 5˚C) for 10 min. The reaction was initiated by the addition of 150 µL of 2% SRBCs, and allowed to continue for 30 min. The samples were then centrifuged (16,000 × g) and transferred to microtiter plates to measure optical density (540 nm) as described above.

Effects of divalent metal ions and heat on Komodo dragon complement: To examine the effects chelators of divalent metal ions on the Komodo dragon complement protein system, 100 µL of serum solution with SRBC and saline solution was spiked with either 1 µL of 500 mM EDTA or 500 mM Na_3PO_4. The resulting serum samples (5 µL), which contained 5 mM EDTA or Na_3PO_4, were incubated (30 min) with 145 µL of saline and 150 µL of 1% SRBCs. Another aliquot of serum (100 µL) was heated to 56˚C for 30 min. The serum was allowed to cool to ambient temperature, and then 5 µL was incubated (30 min) with 145 µL of saline and 150 µL of 1% SRBCs. These samples were centrifuged at 16,000 × g for 5 min, and the optical density of each sample was measured using the Bio-Rad Benchmark Plus™ microplate spectrophotometer at 540 nm.

To determine the specific requirement of Komodo dragon serum complement activity for divalent metal ions, serum was treated with 5 mM EDTA in the absence, and in the presence, of 15 µM $CaCl_2$, $MgCl_2$, $ZnCl_2$, $BaSO_4$, and $FeCl_2$ (**Table 1**). The samples (150 µL) were then incubated with 150 µL of 2% SRBCs for 30 min at ambient temperature. The samples were centrifuged, and the optical densities of the supernatants were measured at 540 nm as described above.

Statistics and controls: All results presented in this study represent the means ± standard deviations for four independent determinations. The results are expressed as the % maximum of a positive control for SRBC hemolysis, which was generated by treating SRBCs with 0.1% Triton-X detergent (v/v), and passing the cells repeatedly through a TB syringe until all of the cells had been disrupted (10 passes), as determined by examination under 400× magnification. The statistical comparisons between groups were conducted using analyses of variance with Duncan's post-hoc comparisons, and $p < 0.05$ was chosen as the standard for statistical significance.

Table 1. Effects of heat treatment and EDTA on hemolytic activity of serum from the Komodo dragon. Komodo dragon serum was treated with EDTA, or subjected to mild heat treatment, and then exposed to 1% SRBCs for 30 min at ambient temperature. The results are expressed as the percentage of maximum lysis, and represent the means ± standard deviation for four determinations.

SRBC Treatment	% Maximum Activity
None	0.0%
Komodo dragon serum	97.3 ± 2.9%
Serum, 56˚C, 30 min	6.3 ± 0.1%
Serum + 5 mM EDTA	4.2 ± 0.2%
Serum + 5 mM EDTA + 15 mM Ca^{2+}	93.8 ± 3.4%
Serum + 5 mM EDTA + 15 mM Mg^{2+}	94.7 ± 2.6%
Serum + 5 mM EDTA + 15 mM Zn^{2+}	6.7 ± 0.9%
Serum + 5 mM EDTA + 15 mM Ba^{2+}	7.8 ± 1.1%
Serum + 5 mM EDTA + 15 mM Fe^{2+}	3.7 ± 0.4%

3. RESULTS

Exposure of different dilutions of serum from *Varanus komodoensis* to SRBCs resulted in volume-dependent hemolysis (**Figure 1**). Treatment of a 500 µL suspension of 1% SRBCs (v/v) with only 3 µL of serum resulted in measureable ($p < 0.5$) hemolysis, as 5.5% ± 0.9% of the SRBCs were hemolyzed. Significant hemolysis of SRBCs occurs with infusion of a small quantity of Komodo dragon serum. Increased volumes of 5 and 7 µL of serum produced 60.0% ± 6.5% and 93.6% ± 2.6% hemolysis, respectively. However, a further increase to 10 µL of serum produced 99.3% ± 0.6% hemolysis, which indicated that a near-maximal response could be obtained by the use of only 7 µL of serum.

Figure 2 shows the kinetic response of the hemolytic activity of serum from *Varanus komodoensis*, at two different volumes, toward SRBCs. At the lower volume (3 mL), an increase in SRBC hemolysis was not observed until 30 min of incubation with *Varanus komodoensis* serum. The activity increased to only 15.9% ± 0.3% of maximum activity at 60 min. However, the activity increased sharply to 91.2% ± 0.8% at 120 min. In contrast, the higher serum volume (7 mL) produced a sharp increase (26.0% ± 2.7%) at only 5 min of incubation with 1% SRBCs. The activity increased rapidly, in a linear fashion, to 88.5% ± 2.8% at 20 min. The linear relation of the increase ($y = 0.0464x - 0.011$) exhibited a correlation coefficient of 0.985.

Incubation of 1% SRBCs with serum from *Varanus komodoensis* at different temperartures (5˚C - 40˚C) resulted in temperature-dependent hemolysis (**Figure 3**). Hemolysis at 5˚C - 20˚C was low (7.4% - 9.5% of maximum), and was not statistically different between these

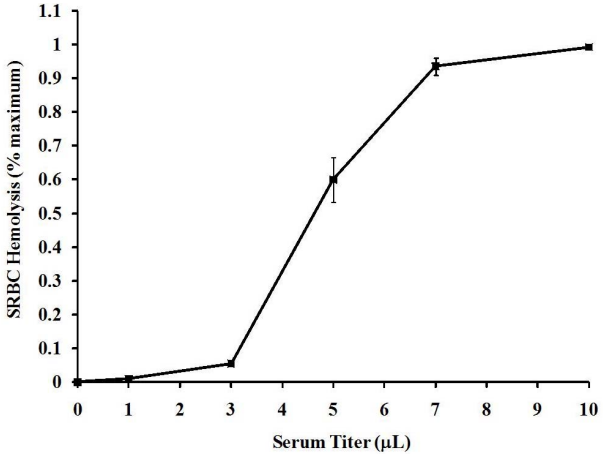

Figure 1. Titer-dependent hemolysis of SRBCs by Komodo dragon serum. Incubation of 1% (v/v) SRBCs with different volumes of Komodo dragon serum for 30 min resulted in a volume-dependent hemolysis, as measured spectrophotometrically as described in the Materials and Methods. The results are expressed as % of maximum hemolysis, relative to a positive control, and represent the means ± standard deviations, and represent the results of four independent determinations.

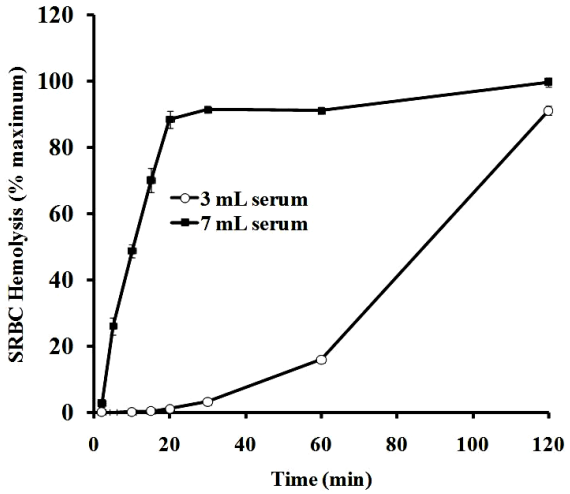

Figure 2. Kinetic analysis of SRBC hemolysis by Komodo dragon serum. Incubation of 1% SRBCs (v/v) with Komodo dragon serum, diluted in normal saline, for different amounts of time resulted in a time-dependent hemolysis. The results are expressed as the % of maximum hemolysis, relative to a positive control, and represent the means ± standard deviations, and represent the results of four independent determinations.

temperatures ($p > 0.05$). The hemolytic activity increased ($p < 0.05$) at 25˚C (28.7% ± 2.8%), 30˚C (53.3% ± 2.3%), and 35˚C (68.7% ± 5.1%). However, the activity at 40˚C resulted in decreased ($p < 0.05$) activity, to 52.0% ± 2.6% of maximum activity.

Table 1 illustrates the effects of mild heat treatment and chelators of divalent metal cations on the hemolysis of SRBCS by Komodo dragon serum. Incubation of Komodo dragon serum at 56˚C for 30 min decreased its

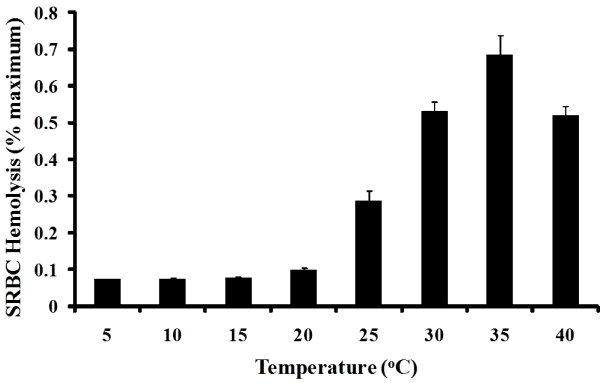

Figure 3. Effects of temperature on hemolysis of SRBCs by Komodo dragon serum. Incubation of 1% SRBCs (v/v) with 0.5% Komodo dragon serum, diluted with normal saline, for 30 min resulted in a temperature-dependent hemolysis. The results are expressed as % of maximum hemolysis, relative to a positive control, and represent the results of four independent determinations.

ability to hemolyze SRBCs to only 6.3% ± 2.8% of maximal activity. Likewise, pretreatment of Komodo dragon serum with 5 mM EDTA resulted in only 4.2% ± 0.2% hemolytic activity. However, incubation of serum with 5 mM EDTA and 15 mM Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, or Fe^{2+} resulted in 93.8% ± 3.4%, 94.7% ± 2.6%, 7.8% ± 1.1%, 6.7% ± 0.9%, or 3.7% ± 0.4% of maximal hemolytic activity, respectively.

4. DISCUSSION

The components of the innate immune systems of eukaryotic organisms constantly survey their surroundings, primarily through molecular pattern recognition, distinguishing self from non-self tissues, and respond to invasion of microbes in a nonspecific manner. The response is rapid, and acts as a first line of defense against infection. In higher vertebrates, such as mammals, both the innate and acquired immune components are well-developed [23]. However, the immune systems of reptilians are believed to have a less developed acquired immune system [24], but the innate immunity of some, particularly crocodilians, have been shown to have a highly developed innate immunity [25-29]. In addition, it has been suggested that more ancient poikilothermic vertebrates, such as teleost fish [30-32] and crocodilians [33], have followed the evolutionary path to develop a more efficient innate immune system, while mammals have developed more effective adaptive immunity. Previous studies have demonstrated that serum complement activity in several different crocodilian species is far more potent than that of other phyla [29,33-35]. The results presented in this study show that the complement activity in the serum of the Komodo dragon is much more active than that of crocodilians.

The SRBC hemolysis assay has been used as a clinical tool to assess human complement activity for many years [36]. In addition, this assay has been modified to assess crocodilian complement activity [37]. The fact that the SRBC hemolysis by Komodo dragon serum is extremely heat-labile, sensitive to proteases (data not shown), and requires either Mg^{2+} or Ca^{2+} are all indications that this activity is due to the presence of serum complement. The alternate serum complement pathway is activated, in the absence of antibody:antigen interaction (classical pathway) or protein:carbohydrate interaction (lectin pathway), by the detection of non-self molecular pattern recognition, which results in the formation of a membrane attack complex (MAC) in the outer membrane of microbes. However, in the presence of SRBCs, the MAC causes rapid hemolysis, which provides an easy way to measure complement activity *in vitro* by the measurement of hemoglobin spectrophotometrically at 540 nm [37].

The relation of Komodo dragon serum volume with hemolytic activity showed a typical sigmoidal curve, with a CH_{50} of approximately 9 µL/1000 µL incubation (**Figure 1**). These data show the potent capacity with which the Komodo dragon serum disrupts the integrity of SRBCs. By way of comparison, the CH_{50} for the American alligator (*Alligator mississippiensis*) was found to be 539 µL [33], or 60 times higher than for the Komodo dragon. These data support the fact that Komodo dragon serum exhibits potent antibacterial activities [20], and might provide a mechanism for these observed activities.

The kinetic curve, depicted in **Figure 2**, shows that different volumes of Komodo dragon serum exhibit different rates of SRBC hemolysis. The shape of the curve with high Komodo dragon serum volume is very similar to that of human complement [38], and also to the several crocodilian species such as the American alligator (*Alligator mississippiensis*) [33], American crocodile (*Crocodylus acutus*) [27], and the differences in the shapes of the kinetic curves strongly suggest that the serum complement enzymes exhibit positive cooperativity. At the lower concentrations, the cooperativity is not observed due to reduced contact between the enzymes, and thus the onset of activity occurs much later. Both of the kinetic curves arrive at the same maximum activities, given enough time, due to the higher degree of cooperativity in samples with the higher plasma protein concentrations.

Because reptilians are ectothermic vertebrates, their physiology and biochemistry depends, to a large extent, on the temperature of their surroundings. Komodo dragons are poikilotherms, whose temperatures fluctuate with their environments. Because the Komodo dragons live in a tropical area, they seek shade in an attempt to maintain stability of body temperature. It is clear that Komodo dragon serum exerted its maximum hemolytic activity at

35˚C (**Figure 3**), very near the 37˚C - 38˚C preferred temperature range to which Komodo dragons thermoregulate [35]. These results are consistent with those obtained for the American alligator [33] and the saltwater (*Crocodylus porosus*) and freshwater (*Crocodylus johnstoni*) crocodiles in Australia [34], and the American crocodile (*Crocodylus acutus*) [27], and the broad-snouted caiman (*Caiman latirostris*) [29].

The results shown in **Table 1** reveal similar characteristics of Komodo dragon serum with the ability of crocodilian serum to hemolyze SRBCs [34,41]. Mammalian serum complement activity is known to be thermally unstable [12], and the results displayed in **Table 1** provide supporting evidence that Komodo dragon serum complement exhibits the same thermal lability. Mild heat treatment of Komodo dragon serum at 56˚C for 30 min, classical serum complement thermal inactivation conditions [39], resulted in a substantial decrease in activity. In addition, mammalian serum complement function is known to require both Mg^{2+} and Ca^{2+} [38], while crocodilian complement function requires either Mg^{2+} or Ca^{2+} [41]. The results from the present study (**Table 1**) shows that Komodo dragon serum complement, like that of crocodilians, requires the presence of either Mg^{2+} or Ca^{2+}. Treatment of Komodo dragon serum with only 5 mM EDTA produced a large decrease in hemolytic activity. However, this activity can be restored by the inclusion of 15 mM Mg^{2+} or Ca^{2+}, indicating the requirement for only one of these metals. In addition, the results clearly illustrate that not all divalent metal ions will restore EDTA-depleted Komodo dragon serum complement. The addition of 15 mM Ba^{2+}, Zn^{2+}, or Fe^{2+} ions resulted in complement activity which was not statistically different ($p > 0.05$) than treatment with EDTA alone. The inabilities of these divalent cations to restore EDTA-depleted Komodo dragon serum complement activity demonstrate the specificity of the complement proteins for Mg^{2+} and Ca^{2+}. This same specificity has been noted in several crocodileian species [27,34,41].

The results presented in this study show that the serum from the Komodo dragon exhibits potent and rapid serum complement activity. The activity occurs in a volume-, time-, and temperature-dependent manner, and requires either Mg^{2+} or Ca^{2+} for activity. These activities are consistent with an animal that lives an aggressive lifestyle and harbors potentially infectious microbes in their saliva for the purpose of feeding. This study represents the first investigation of innate immunity of a Varanid lizard.

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