Abstract

Serum complement in the serum of two divergent Central African crocodiles, the African dwarf crocodile (*Osteolaemus tetraspis*) and the slender-snouted crocodile (*Mecistops cataphractus*) was assessed using a sheep red blood cell (SRBC) hemolytic assay. The hemolysis for both crocodilian species was serum volume-, time-, and temperature-dependent. Although the serum volume-dependent activities were similar for both species (CH50 = 81 µL for *Osteolaemus tetraspis* and 96 µL for *Mecistops cataphractus*), the kinetic curves show a greater amount cooperativity, and thus more rapid SRBC lysis, for *Osteolaemus tetraspis*. In addition, the hemolytic activities were very similar at 10 – 35°C, but the serum from *Osteolaemus tetraspis* was more active than that of *Mecistops cataphractus* at the temperature extremes tested (5°C and 40°C). The activities for both species were almost completely inhibited by 1 mM EDTA, indicating the dependency on divalent metal ions. However, the EDTA-inhibited hemolysis could be restored by the addition of excess Mg²⁺ and Ca²⁺, but not Ba²⁺, Cu²⁺, or Fe²⁺, which exhibited the specificity for Mg²⁺ or Ca²⁺. These data indicate that these sympatric, but evolutionarily and ecologically divergent crocodile species have similar SRBC hemolysis activities with similar mechanisms, thus reinforcing the idea that serum complement is an ancient, innate immune host defense system.

Keywords: Crocodilian; Innate immunity; Serum complement

Introduction

Serum complement is a key component of innate immunity that can be found in all vertebrates and many ancient invertebrates [1,2]. Although serum complement constitutes a non-specific immune response, it is an important first line of defense upon initial infection [3]. Mutations in mammalian serum complement proteins result in serious clinical conditions [4], and acquired serum complement deficiencies can result in a plethora of disease states [3].

Serum complement can be activated by three distinct mechanisms: 1) the classical mechanism, which relies on the interaction of antibody-antigen complexes, 2) the lectin pathway, which is dependent upon common microbial surface carbohydrate pattern recognition, and 3) the alternative pathway, which is activated by the cleavage of an internal thioester in a specific protein component in response to foreign antigens. All of these three complement activation mechanisms involve a proteolytic cleavage cascade of common components, and eventually culminate in the formation of a multiprotein “membrane attack complex” in the outer envelope of microbes, which compromises the integrity of the membrane, and results in eventual lysis. The complement cascades can be activated by, and attack, a broad spectrum of microbes, and do not result in immunological memory, and thus are considered to be part of the innate immune system [5]. However, several of the proteolytic products that are generated during complement activation have important function in the activation of the acquired immune response. For instance, after complement component C3 is cleaved into C3a and C3b, C3a acts as an anaphylatoxin [6] and C3b functions as a potent opsonin, rendering microbes susceptible to phagocytosis [7]. In addition, C1q and C4a act as chemoattractants for the chemotaxis of dendritic cells and neutrophils, respectively [8,9] thereby recruiting important cellular components to the site of infection. Furthermore, complement protein C4a mediates inflammation [10] by activation of phagocytic cells, stimulation of the release of granule-based enzymes, and generation of oxidants [11].

Previous studies in our laboratory have shown that several crocodilian species exhibit potent and broad-acting serum complement activities [12-15]. This study was conducted to examine the complement activity in two species of sympatric, but morphologically and ecologically divergent crocoddilians in Central Africa.

Materials and Methods

Chemicals and Biochemicals

SRBCs (10% v/v, washed and pooled) were purchased from Rockland Immunochemicals (Rockland, MD, USA). Ethylene diamine tetraacetate (EDTA), calcium chloride, magnesium chloride, barium sulfate, ferrous chloride, cupric chloride and pronase derived from *Streptomyces griseus* were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment of Animals

Crocodiles were captured from the N’Dougoou Lagoon, Bongo River, and Nyanga River areas of the Gamba Complex region in Gabon, Africa. Animals were captured using standard crocodilian capture techniques, including by hand, tongs, locking snare, or research darting [16]. Blood was collected from a total of 22 *Osteolaemus tetraspis* and 15 *Mecistops cataphractus*. 5 mL of whole blood were collected from animals measuring less than 80 cm, and ≥ 10 mL of blood were collected from larger animals, depending on body size and condition. Blood was...
collected from crocodiles via the spinal vein [17,18] using 3.8 cm, 18 ga needles and 10 mL syringes. The whole blood was allowed to clot at ambient temperature, and the serum was separated and stored on ice at the site of capture. All of the activities were approved by McNeese State University and University of Florida Animal Care and Use Committees.

Serum complement assay

A 100 µL suspension of 2% SRBCs (v/v) in 100 mM Tris-HCl (pH 7.4) was treated with an equal volume of various dilutions of crocodile serum. The SRBCs were incubated for 30 min at ambient temperature, and then centrifuged at 16,000g to pellet intact cells and cellular debris. The resulting (175 µL) supernatant was transferred to a microtiter plate and the optical density (540 nm) was measured in a BioRad Benchmark Plus™ microtiter plate reader.

For assays in which the kinetic parameters of SRBC hemolysis were examined, 1 mL of serum was mixed with 1 mL of 2% SRBCs (v/v). Samples were removed to 10 µL of 1 M EDTA at different time points, and immediately centrifuged (3000g, 5 min, ambient temperature). The resulting supernatants (175 µL) were removed to a microtiter plate, and the absorbance at 540 nm was measured.

To determine the temperature-dependency of SRBC hemolysis for the crocodile serum, samples and 2% SRBCs (v/v) were incubated separately at different temperatures for 10 minutes to reach thermal equilibrium. Equal volumes of SRBC and serum were mixed, and incubated for 30 minutes, followed by centrifugation at ambient temperature (5 min, 3000g). The absorbencies of the supernatants were measured at 540 nm as described above.

The requirements of divalent metal ions on the hemolysis of 1% SRBCs by crocodile serum were determined by including different concentrations of EDTA (0.1 - 5.0 M) in the incubation. The samples were incubated for 30 min at ambient temperature, and then processed as previously described. The effects of Mg²⁺, Ca²⁺, Ba²⁺, Fe²⁺, and Cu²⁺ on the EDTA-inhibited SRBC hemolysis were investigated using similar methodology.

Statistics and controls

All data are displayed as the results of means ± standard deviations of four independent determinations. The hemolysis value for each sample was compared to a positive hemolysis control (1% SRBCs) and represent the results of four independent determinations. The statistical significance between treatment groups was determined by analysis of variance using Duncan’s post-hoc comparisons [19].

Results

Substantial serum volume-dependent hemolytic activity was observed with only 100 µL of serum (per 1 mL reaction volume) from both Mecistops cataphractus (52.0 ± 6.0%) and Osteolaemus tetraspis (61.6 ± 6.01%) serum, and maximum activity (102.4 ± 1.0%) was achieved at only 300 µL of serum for Osteolaemus tetraspis (Figure 1). In contrast, the serum from Mecistops cataphractus exhibited 94.7 ± 0.9% activity at 300 µL and then slowly increased to 99.1 ± 1.2% and 102.4 ± 0.9% at 400 and 500 µL, respectively.

The rate of SRBC hemolysis was measured for Osteolaemus tetraspis and Mecistops cataphractus (Figure 2). Serum from Osteolaemus tetraspis exhibited measurable hemolytic activity (4.3 ± 0.1%, P<0.05) within 2 min of exposure to SRBCs and near-maximum activity (75.5 ± 6.2%) was observed at 15 min. In contrast, Mecistops cataphractus serum did not display measurable hemolytic activity until 10 min exposure and maximum activity was observed at 20 min (75.8 ± 1.1%; Figure 2). Despite the delay in activity of the Mecistops cataphractus serum, the slopes of the lines are very similar (0.0854 for Osteolaemus tetraspis and 0.0833 for Mecistops cataphractus). Despite the differences in the initial rate of reaction, the serum from both species reached the same maximum values, 78.8 ± 0.7% for Osteolaemus tetraspis and 78.6 ± 0.2% for Mecistops cataphractus (P>0.05), at 30 min.

The serum complement activities for both species were the same
at 10 and 15°C (P>0.05). The activity of serum complement for *Osteolaemus tetraspis* was slightly higher at 20-35°C (P<0.05). However, the activity for *Osteolaemus tetraspis* at (Figure 3) the temperature extremes of 5°C (71.8 ± 2.5%) and 40°C (66.7 ± 2.7%) was significantly higher than that for *Mecistops cataphractus* (41.7 ± 4.6% and 31.5 ± 2.8%, respectively; P<0.01).

There was no significant difference in the effects of 0, 0.1, or 0.5 mM EDTA on crocodile serum-mediated SRBC hemolysis for serum from either species (Figure 4; P>0.05). However, the addition of 1 mM and 5 mM EDTA reduced the activity to 9.7 ± 0.5% and 8.8 ± 0.2 % activity, respectively (p<0.01).

The effects of a variety of divalent metal ions on the EDTA-inhibited hemolysis of SRBCs were investigated, and the results are illustrated in Figure 5. Untreated *Osteolaemus tetraspis* and *Mecistops cataphractus* sera samples generated 96.2 ± 3.65 and 94.1 ± 2.1% of maximum activity, respectively. Treatment of the sera samples with 1 mM EDTA produced an approximate 90% decrease in activity for both species. However, treatment with 1 mM EDTA and 5 mM Ca²⁺ or Mg²⁺ produced corresponding SRBC lysis values of 94.3 ± 1.1% and 96.1 ± 2.0% *Osteolaemus tetraspis* serum, and 96.1 ± 1.9% and 94.3 ± 2.7% *Mecistops cataphractus* serum. However, treatment of *Osteolaemus tetraspis* serum with 1 mM EDTA and 5 mM Ca²⁺ or Mg²⁺ resulted in only 7.2 ± 0.4, 9.6 ± 0.8, or 8.9 ± 0.7%. None of these results were significantly different from treatment with 1 mM EDTA alone (P>0.5). Likewise the same treatments for serum from *Mecistops cataphractus* produced 4.3 ± 0.7%, 6.9 ± 0.4%, and 8.6 ± 0.5% of maximum activity, none of which were significantly different from treatment with EDTA alone (P>0.05).

**Discussion**

Unlike acquired immunity, innate immune responses are not specific to a particular pathogen, but instead rely on the recognition of conserved molecular patterns on the surface of microbes [20]. The serum complement system of proteins, considered to be among the most important components of innate immunity, acts to kill microbes in a nonspecific manner. In addition, serum complement proteins work to activate other elements of innate immunity, and also act as critical stimulators of some components of acquired immunity [21].

Some crocodilian species are known to exhibit potent and broad-acting serum complement activities and have been shown to demonstrate other effective innate immune components including serum phospholipase A₂ activity [12,22,23], the production of superoxide [24], and iron withholding [14]. However, at least some components of acquired immunity, including the presence of well developed B- and T-lymphocytes, seem to be less developed than those of mammals [25]. This discrepancy in innate versus acquired immune capacity in crocodilians, and other ancient vertebrates (e.g., teleost fish; [26]), supports it as the basal condition and advanced acquired immunity is a more recently derived feature of younger tetrapod lineages like birds and mammals [27].

Serum from *Osteolaemus tetraspis* and *Mecistops cataphractus*
exhibited potent serum complement activities relative to other crocodilian species. For instance, CH₅ values, the volume of serum required for hemolysis of 50% of 1 mL solution of 1% (v/v) SRBCs, for *Osteolaemus tetraspis* (81 µL) and *Mecistops cataphractus* (96 µL) are much lower than that reported for *A. mississippiensis* (539 µL, [13]), *Caiman latirostris* (386 µL, [15]), *Crocodylus acutus* (329 µL, [28]), and *Crocodylus porosus* and *Crocodylus johnstoni* (473 µL and 451 µL, respectively, [29]). This may be a result of their largely equatorial distribution where hot, stable climates are conducive to continual bacterial growth year round. If we control for potentially confounding effects of shared evolutionary descent by examining this trend at the family level we see this pattern emerging (i.e., Alligatoridae - *A. mississippiensis* vs. *Caiman latirostris* sampled at latitudes of 30° and -26°, respectively; *Crocodylidae* - *Crocodylus porosus, C. johnstoni* and *C. acutus* versus *Osteolaemus* and *Mecistops* sampled at -17°, 23° and -2°, respectively). Sampling of latitudinally-distributed populations within a given species would be necessary before further conclusions can be drawn. Interestingly, it has recently been hypothesized that the *Mecistops* + *Osteolaemus* clade is sister to the true crocodiles [30-32] suggesting that there is, additionally, a phylogenetic underpinning to certain innate immune serum activities. Little work has been done on the phylogenetic basis for convergent or divergent innate immune response between closely related species and it is likely to be informative of these processes in the future.

Biochemical and physiological processes in ectothermic vertebrates are closely tied to environmental temperatures and this relationship may serve as the basis for an alternate explanation for the serum complement potency seen in *Osteolaemus* and *Mecistops*. Not only do both species show more potent serum complement activity than that seen in other crocodilian species, but they exhibit a much higher range of temperature-dependent activity as well [13,29,15,28], another factor possibly explained by the phylogenetic sister relationship of *Mecistops* and *Osteolaemus* compared to other crocodilians. Even though these species were sampled near the equator, an area which experiences more stable annual temperature cycles, *Osteolaemus* and *Mecistops* both prefer heavily forested wetlands [33,34] that provide little opportunity for basking, perhaps necessitating more potent innate immunity. For these same reasons, ecological differences between these two species are likely driving differences observed in the serum temperature-dependent activity range between them; the serum from *Osteolaemus tetraspis* exhibits a higher range of activity than that of *Mecistops cataphractus* (Figure 3). *Osteolaemus* spends the day in subterranean burrows exposing it to much lower temperatures with less capacity for thermoregulation than *Mecistops*.

While the direct relationship between temperature-dependent serum activity has not been assessed in *vivo*, assuming they are the same it seems unlikely that internal body temperatures in these crocodilians would regularly reach as high as 40°C, considering that most crocodilians thermoregulate to 31-32°C [35]. However, a febrile response to infection has been previously shown in the American alligator with internal body temperatures rising as high as 37.5°C [M. Merchant, unpublished observations] [36]. Therefore, it is conceivable that, during a febrile response, *Osteolaemus tetraspis* could potentially elevate internal temperatures to near 40°C, particularly considering the tropical climate of the Gabonese forests in which this crocodilian resides relative to the temperate climate of alligators. At the lower end of temperature-dependent serum activity, the internal body temperature of a crocodilian at the equator would certainly never be below 10°C and, therefore, it is unlikely that the high complement activity of *Osteolaemus. tetraspis* at 5°C is physiologically relevant and may be an artifact of increased thermodynamic stability and increased protein interactions at these temperature extremes.

The data displayed in Figure 2 show the prototypical sigmoidal kinetic curves that are indicative of positive cooperativity in enzyme systems [37]. This cooperative enzyme action of serum complement enzymes has been reported in other vertebrates [38]. Although the sera from both species catalyze the hemolysis of SRBCs to the same extent (maximum activity), the activity of the *Osteolaemus tetraspis* serum occurs much more rapidly. This is probably due to a higher degree of cooperativity between the complement enzymes in the serum of *Osteolaemus tetraspis* relative to that of *Mecistops cataphractus*, which may also be a result of the aforementioned ecological and behavior differences related to thermoregulation between these two species.

Both *Osteolaemus tetraspis* and *Mecistops cataphractus* serum complement exhibit high sensitivity to EDTA (Figure 4). Divalent metal ions, specifically Ca²⁺ and Mg²⁺, are known to be important for serum complement activity in mammals [39], birds [40], reptilians [13], amphibians [38], and fish [26]. Since EDTA efficiently binds divalent metal ions and sequesters them away from complement proteins, it is a potent inhibitor of serum complement activity at low concentrations [39]. The threshold for inhibition of serum complement activity in both *Osteolaemus tetraspis* and *Mecistops cataphractus* was between 0.5 and 1.0 mM EDTA (Figure 4); however, the inhibitory effects of EDTA can be overcome by the addition of excess Mg²⁺ or Ca²⁺ (Figure 5). Further, EDTA-mediated inhibition of complement activity is not affected by other Periodic Group 1 metals (Ba²⁺) or transition divalent metal ions (Fe²⁺ and Cu²⁺). This specificity has also been reported in other crocodilians [13,29]. In contrast, human serum complement requires both Ca²⁺ or Mg²⁺ for functionality [40].

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**References**


32. Shirley MH, KA Viet, AN Carr, JD Austin. In Review. Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proceedings of the Royal Society B: Biology


42. Shirley MH, KA Viet, AN Carr, JD Austin. In Review. Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proceedings of the Royal Society B: Biology


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