Antibacterial activities of serum from the Komodo Dragon (Varanus komodoensis)

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Abstract

Komodo dragons (Varanus komodoensis) are able to feed on large prey items by injecting a dose of toxic bacteria with their bite that, over time, kills the prey by systemic infection. Dragons also suffer bites from other members of their own species during territorial disputes and feeding frenzies. However, they do not suffer the same fate as their prey, suggesting that they have developed a strong immunity to bacterial infections. This study was undertaken to determine the antibacterial activities of serum from the Komodo dragon. Bacterial cultures were treated with different volumes serum from Varanus komodoensis and the growth was monitored by optical density at 430 nm. In addition, the serum was treated with protease, chelators of divalent metal ions, or with mild heat to determine the mechanism of antibacterial activities. Treatment of bacterial cultures with serum from Komodo dragons (Varanus komodoensis) resulted in a volume-dependent decrease in bacterial growth. Cultures of Escherichia coli, Staphylococcus aureus, and Klebsiella oxytoca exhibited moderate-strong growth inhibition by V. komodoensis serum, while cultures of Streptococcus epidermidis, Salmonella typhimurium, Providencia stuartii, and Shigella flexneri were nearly completely obliterated for 24 h by only 10% (v/v) serum. The antibacterial activity of V. komodoensis serum occurred very rapidly, as 18% of E. coli growth was inhibited by a five min exposure to serum. Furthermore, 10- and 20-min incubations of E. coli with serum from V. komodoensis resulted in 43 and 68% inhibition of bacterial growth, respectively. The bacterialic capacity of the serum against E. coli was 2,075,000 bacteria/µL serum, and was inhibited by mild heat treatment, pronase, EDTA, and phosphate, indicating that the antibacterial action is most probably due to the presence of a potent serum complement protein system.

Introduction

The Komodo dragon (Varanus komodoensis) is an endangered species of monitor lizard indigenous to only five islands in the Lesser Sunda region of southeast Indonesia.1 It is the largest lizard in the world, reaching lengths of more than 3 meters.2 These reptiles primarily inhabit the tropical savannahs, and their boundary woodlands, of these islands. They feed as both scavenger and predators,3 eating a broad spectrum of food items.

Komodo dragons kill prey items with a lethal bite that is believed to inject toxic bacteria into the blood stream of these animals.4 5 However, recent studies have shown that Komodo dragon saliva also contains a potent and complex venom, which causes anticoagulation and induction of systemic shock in prey animals.6 After days, or even weeks, depending on the size of the prey and the number of bites, the prey succumbs to the lethal effects of systemic infection or shock. However, despite the fact that Komodo dragons fight and inflict bites on each other,2 these lizards are not known to be affected by septic infections. This would seem to indicate that these animals have developed some type of immune mechanism(s) that protect against potential sepsis due to bites from other Komodo dragons.

Despite the endangered status of the Komodo dragon, practically nothing is known about the immune systems of these threatened reptilians. Knowledge of the immune system will be of interest to both evolutionary biologists and conservationists of these reptilians. The primary goal of this project was to characterize the antibacterial properties of serum from the Komodo dragon.

Materials and Methods

Chemicals and biochemicals

Nutrient broth and low EEO agarose were purchased from Intermountain Scientific (Kaysville, UT). Bacterial cultures (American Type Culture Collection) were purchased from Remel (Lenexa, KS). The following species were used for representatives of Gram-negative species: Escherichia coli (ATCC 25922), Shigella flexneri (ATCC 700930), and Klebsiella oxytoca (ATCC 49131), Salmonella typhimurium (ATCC 14028), Providencia stuartii (33672) and the following Gram-positive species: Staphylococcus aureus (ATCC 6538), and Streptococcus epidermidis (ATCC 19615). Pronase from Strepomyces griseus was purchased from Sigma Chemical Company (St. Louis, MO).

Collection of blood 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 at ambient temperature 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...

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the attending veterinarian. Blood was collected from three adults (20-81.5 kg) and five juveniles (1.5-6.2 kg), and the serum was pooled so that average antibacterial values for this species could be generated. The samples were stored at -20°C until used for the antibacterial assays. The collection of blood from these animals was conducted in accordance with the animal care institutional policies of the Houston and San Antonio Zoos.

**Serum volume-dependent antibacterial assays**

Two hundred µL samples containing various volumes (20-200 µL) of Komodo dragon serum in sterile microtiter plates were inoculated with five µL of bacterial culture from log phase cultures and incubated for 24 h at 37°C. The optical density of each sample was measured at 0, 3, 6 12 and 24 h using a Biorad Benchmark Plus™ microtiter plate reader at 430 nm.

**Kinetic antibacterial assay**

Five hundred µL of Komodo dragon serum were inoculated with 20 µL of an E. coli culture in log phase growth. At various time points, 50 µL of a dilution of each sample were spread onto the surface of nutrient broth agar plates to determine the CFUs for each sample. The samples were typically plated at five different dilutions (1:100-1:106) in sterile isotonic saline to obtain plates with a quantity of colonies such to provide a reasonable estimate of bacterial density (50-400 CFUs per plate). To determine possible mechanisms of antibacterial action, pooled serum from Komodo dragons was incubated with one unit/mL pronase (isolated from Streptomyces griseus), 5 mM EDTA (pH 8.0), or 5 mM phosphate for 30 min at ambient temperature. In addition, four aliquots of serum were incubated at 56°C for 30 min. These serum samples (500 µL) were all incubated with 20 µL of an E. coli culture in log phase growth. The samples were incubated for 15 min at ambient temperature, diluted serially (1:100 -1:106) in sterile saline, and 50 µL of each sample were plated on nutrient agar to determine the CFUs for each sample. The serum inhibited the growth of E. coli by generating chemotactic factors and producing cytokines that initiate the development of acquired immune responses.

**Statistics and controls**

All assays were conducted in quadruplicate, and the results are expressed as the means ± standard deviations for four independent replicates. The statistical comparisons between groups were conducted using analyses of variance and Duncan’s post-hoc comparisons and P<0.05 was chosen as the standard probability of statistical significance.

**Results**

The results in Figure 1 show that serum from V. komodoensis inhibits the growth of Gram negative bacteria. The serum inhibited growth of E. coli in a volume-dependent manner (Figure 1A). At 24 hrs, 10% V. komodoensis serum (v/v) produced a 44% decrease in E. coli growth, while 25, 50, 75, and 100% serum caused 58, 75, 93, and 98% decreases in growth, respectively. By way of comparison, incubation of 10% V. komodoensis serum with Klebsiella oxytoca cultures resulted in 34% growth inhibition at 24 hrs, while 25% serum caused a 65% decrease in bacterial density (Figure 1B). All serum volumes at, or above, 50% completely inhibited the growth of K. oxytoca cultures at 24 hrs. Serum from V. komodoensis (10% volume) inhibited Shigella flexneri growth by 95% at 24 hrs (Figure 1D), and exhibited similar results for cultures of Salmonella typhimurium (Figure 1E). Cultures of Providencia stuarti growth were completely obliterated (100% inhibition) by only 10% serum (Figure 1C).

Figure 2 illustrates the effects of serum from V. komodoensis on the growth of Gram positive bacteria. Serum (10% volume) from V. komodoensis inhibited the growth of Staphylococcus aureus by 64% at 24 hrs, while 25, 50, 75, and 100% serum resulted in 68, 84, 93, and 98% reduction in growth, respectively (Figure 2A). In contrast, 10% serum from V. komodoensis inhibited the growth of Streptococcus epidermitis cultures by 49% (Figure 2B).

The bacterial-killing capacity of serum from V. komodoensis for E. coli is displayed in Figure 3. A 20 µL sample of positive control culture of E. coli in log phase growth was found to have 16.4±0.4 million viable bacteria. In contrast, the addition of 25% (v/v) of serum from V. komodoensis to the bacterial growth medium caused a significant decrease (P<0.05) to 5.2±2.1 million CFU. Further increases in serum to 50% resulted in decreased bacterial viability (1.3±0.2 million CFU). Because only 20 µL of the bacterial culture were used for colony determination, we calculated that one µL of serum from V. komodoensis has the capacity to kill approximately 2.24 million E. coli bacteria.

Figure 4 shows the results of a kinetic analysis of the ability of serum from V. komodoensis to inhibit the growth of E. coli bacterial cultures. The antibacterial activity occurred quickly, as 18% of the bacteria (2.95 million) were killed within 5 min of exposure to serum from V. komodoensis. Further incubation resulted in 43% (7.05 million) of the bacteria killed at 10 min of exposure, and the antibacterial action was maximal at 20 min as 68% (11.2 million) of the E. coli were killed.

The data in Table 1 exhibits the results of a mechanistic study, showing the susceptibility of the antibacterial effects to mild heat treatment and chelators of divalent metal ions. Treatment of E. coli cultures with serum from the Komodo dragon results in 96.4% of the bacteria killed. However, mild heat treatment (56°C, 30 min) of the serum resulted in only 4.0% antibacterial activity. Likewise, treatment of the serum with 5 mM EDTA or 5 mM phosphate resulted in only 6.3% or 8.7% antibacterial activity, respectively.

**Discussion**

Eukaryotic organisms must continuously defend themselves against infiltration and colonization by microorganisms. Host defense occurs via complex mechanisms and can be divided into two distinct, but interrelated, types of responses: acquired immunity and innate immunity. Acquired immunity requires prior exposure to a specific antigen before a full immunological assault can be established by the host organism. In addition, the acquired immune response is complex and can often take several days to become fully activated. The innate immune response comprises a significant portion of the immune system and acts as an initial defense mechanism against microbial growth shortly after infection occurs. These innate defense responses are activated shortly after exposure and act to slow or stop an infection in the initial stages so that the acquired immune response can be initiated. Also, the innate immune system functions in the activation of the acquired immune system by generating chemotactic factors and producing cytokines that initiate the development and maturation of specific T-cell and B-cell populations. Anecdotal evidence suggests that Komodo dragons are resistant to microbial invasion.

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**Table 1. Effects of heat treatment, protease treatment, or metal chelators on the antibacterial activity of serum from V. komodoensis against E. coli.** The E. coli survival was determined by manual colony counts. The results represent the means of four independent determinations.

| Treatment          | CFU   | Antibacterial activity |
|--------------------|-------|------------------------|
| None               | 12.7×10⁶ | -                      |
| Komodo dragon serum| 0.46×10⁶ | 96.4%                  |
| Serum, 56°C, 30 min| 12.2×10⁴ | 4.0%                   |
| Serum + pronase    | 12.4×10⁴ | 2.4%                   |
| Serum + 5 mM EDTA  | 11.8×10⁴ | 6.3%                   |
| Serum + 5 mM phosphate | 11.5×10⁴ | 8.7%                   |

CFU, colony-forming units.
infections. These animals often sustain serious injuries, including open wounds, due to interspecies fighting in territorial disputes. Despite the propensity of dragons to resist microbial infection, the mechanisms of immunity are not well-characterized.

Montgomery et al. isolated many bacterial species from both captive and wild Komodo dragons, some of which were extremely toxic to mice, particularly *Pasteurella multocida*, supporting the idea that bite wounds inflicted by Komodo dragons may induce systemic septicemia in their prey. However, it would be reasonable to expect that Komodo dragons would have developed innate immune mechanisms to combat possible infection by the bacteria in their own saliva. In addition, antibodies against *P. multocida* were found in the plasma of the Komodo dragons, supporting the idea that their immune systems are equipped to deal with the heavy bacteria load present in their saliva. Antibacterial activities of other reptilian species have been assessed, particularly those of several crocodilian species. Merchant et al. showed that the bactericidal capacity of serum from the American alligator (*Alligator mississippiensis*) toward *E. coli* was...
54,000 bacteria/µL. Other reptilians have been shown to exhibit potent antibacterial activities, particularly crocodilian species. Merchant et al.10 showed that serum from the American alligator (Alligator mississippiensis), when challenged with cultures of E. coli as low as 10³/mL, could not kill enough bacteria to make a statistical difference in the colony counts, relative to untreated controls cultures. The capacity of alligator serum to kill E. coli was approximately 54,000 bacteria/µL. However, the results shown in Figure 3 show that serum from V. komodoensis has substantially higher activity than that of Alligator mississippiensis. The serum from V. komodoensis showed an approximate 40-fold increase in the capacity to kill E. coli, relative to that of Alligator mississippiensis. The extreme antibacterial properties described in this study could be a result of the evolution of these ancient vertebrates under the conditions of territorial aggression, feeding disputes, challenges for mating rights, etc. Bull et al.11 reported that 70% of the feeding activities of V. komodoensis on large prey items occurred with multiple adult animals in close proximity. The broad spectrum of oral bacteria isolated from this species and the high bacterial load of their saliva,4,11 would certainly be an issue if bite injuries occurred during territorial battles. Therefore, it is not surprising that these animals could have evolved strong innate immunity defenses to deal with the potential for serious infection due to their aggressive lifestyles. It must be noted that the animals used in this study were captive animals, and thus the immune system could potentially be altered by chronic stress of captivity. However, chronic stress typically acts as an immunosuppressive factor in vertebrates,12-17 and thus the antibacterial activity of the serum from wild V. komodoensis could potentially be underestimated in this study.

The antibacterial effects of Komodo dragon serum is most likely due to an efficient serum complement innate immune-defense. The fact that mild heat treatment (30 min. at 56°C) almost completely obliterated the observed activities is an indication that serum complement activity is responsible for the antibacterial activities. It has been well-established that serum complement proteins are extremely vulnerable to mild heat treatment, as some of the key proteins components are thermally unstable.18 In addition, the antibacterial activity was sensitive to pronase, indicating that at least one of the components required for the observed activity is proteinaceous in nature. This idea is reinforced by the dependence of the antibacterial activities on divalent metal ions (Table 1). It has long been known that divalent metals (magnesium and calcium) are required for mammalian serum complement activity.19,20 More recent studies have confirmed the requirement for calcium and magnesium for reptilian serum complement function.21 It is not surprising that V. komodoensis would have serum complement proteins, as most vertebrate, as well as many ancient invertebrates, exhibit complement activity.22 However, the strength with which these proteins kill bacteria is staggering. Before this study, crocodilians were considered to have among the most potent serum complement defense systems.10 However, the results in this study show that the potency of Komodo dragon serum complement is much higher than that of crocodilians.23 This study represents the first report on innate immune activity of V. komodoensis.

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