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Identification of alternative pathway serum complement activity in the blood of the American alligator (*Alligator mississippiensis*)

Mark E. Merchant[a,*], Cherie M. Roche[a], Damon Thibodeaux[a], Ruth M. Elsey[b]

[a]*Department of Chemistry, McNeese State University, P.O. Box 90455, Lake Charles, Louisiana 70609, USA*
[b]*Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, 5476 Grand Chenier Hwy, Grand Chenier, Louisiana 70643, USA*

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Abstract

Incubation of different dilutions of alligator serum with sheep red blood cells (SRBCs) that had not been sensitized with antibodies resulted in concentration-dependent hemolytic activity. This hemolytic activity was not affected by the presence of ammonium hydroxide and methylamine, known inactivators of the classical complement cascade. However, the hemolytic activities were inhibited by EDTA and salicylaldoxime, indicating that the alternate pathway is primarily responsible for these activities. Immunofixation of electrophoretically-resolved alligator serum proteins with antihuman C3 polyclonal antibodies resulted in detection of a protein antigenically similar to human C3 in alligator serum. SDS-PAGE, followed by Western blot analysis, revealed the presence of two alligator serum proteins with nearly identical molecular weights as human C3α and C3β. SRBC hemolysis and antibacterial activity by alligator serum was significantly reduced in the presence of antihuman C3 antibodies. The hemolytic effect of alligator serum was shown to occur rapidly, with significant activity within 5 min and maximal activity occurring at 15 min. SRBC hemolysis was also temperature-dependent, with reduced activity below 15 °C and above 30 °C. These data suggest that the antibiotic properties of alligator serum are partially due to the presence of a complement-facilitated humoral immune response analogous to that described in mammalian systems.
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1. Introduction

Eukaryotic organisms must continuously defend themselves against infiltration and colonization by microorganisms. The humoral immune response comprises a significant portion of the immune system and acts as an initial defense mechanism against microbial growth shortly after infection occurs. The serum complement system, an important component of the humoral immune response, is composed of 25–30 proteins that can be activated to initiate the inflammatory response, recruit leukocytes to the site of infection, mediate opsinization of particulate foreign materials and kill microorganisms directly by the assembly of a multiprotein membrane attack complex in the outer membrane of microbes (Muller-Eberhard, 1986; Dalmasso et al., 1989). Because of the immunological importance of the serum complement system, a deficiency or mutation in any complement protein is almost always associated with multiple recurring infections (Morgan and Walport, 1991; Pascual and French, 1995).

Complement proteins are expressed and circulated as inactive precursor proteins that can be activated in a very precise and highly coordinated fashion (Campbell et al., 1988). The complement cascade can be initiated by three distinct mechanisms: an antibody-dependent classical pathway, an antibody-independent alternative pathway, and a lectin pathway that results in the modulation of immune function. The serum complement system has been fully characterized in humans as all of the proteins have been purified to homogeneity, their functions in each pathway identified, and their genes isolated (Campbell et al., 1988). Although several studies have reported the presence of

* Corresponding author.
E-mail address: mmerchant@mail.mcneese.edu (M.E. Merchant).

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complement components in a variety of reptiles (Koppenheffer, 1986), the serum complement system is not well characterized in reptilian systems. The results from this study strongly suggest a potent complement system exists in the serum of the American alligator.

2. Materials and methods

2.1. Chemicals and biochemicals

Nutrient broth (cat. # G-3055-50) and nutrient agar (cat. # G-3056-40) were purchased from ISC Bioexpress (Kaysville, UT, USA). Lyophilized ATCC cultures (*Escherichia coli*, ATCC 25922) were purchased from Remel (Lenexa, KS, USA). An ATCC-registered strain of protease derived from *Streptomyces griseus* was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Lyophilized goat antihuman C3 polyclonal antibodies ($F(ab)_2$) were obtained from ICN Biomedicals, Inc. (Irvine, CA, USA, cat. # 55062). Sheep red blood cells were purchased from Rockland Immunologicals (Gilbertsville, PA, USA cat. # R405-0050). All other chemicals were purchased from Sigma-Aldrich.

2.2. Treatment of animals

Alligators were captured and housed at the Rockefeller State Wildlife Refuge in Grand Chenier, Louisiana, USA. Numerous juvenile alligators, which were hatched in captivity from eggs collected in the wild, were maintained at Rockefeller Refuge in fiberglass-lined concrete tanks approximately 4.36 m long × 2.18 m wide. Several small alligators (up to 1 m in length) were housed in a single tank. Adult alligators were typically captured at night with the use of a cable snare.

The environment in the tanks consisted of 50% dry bottom and 50% water of approximately 15 cm depth. The temperature was maintained at approximately 30–31 °C. The alligators were fed formulated dry pellets 4–5 times per week and the tanks were cleaned five times per week. Blood samples were drawn from the spinal vein using a 3.81 cm 18 gauge needle and a 10 mL syringe (Olsen et al., 1977; Zippel et al., 2003) and transferred to serum vacutainer tubes.

2.3. Bacterial cultures

Bacteria were maintained on nutrient agar slants at 4 °C. The day before the experiment, a 4 mL nutrient broth liquid culture was inoculated from the slant with a sterile cotton swab. The bacteria were allowed to incubate at 37 °C overnight to obtain log-phase culture. Serial dilutions of the log-phase cultures were plated on nutrient broth agar in 100 mm Petri dishes to determine the colony-forming units (CFUs) in each culture.

2.4. Antibacterial assay

E. coli cultures in log growth phase were treated with alligator serum in the presence or absence of antihuman C3 antibodies. The samples were incubated at 37 °C for 1 h and the CFUs for each culture were determined by the solid medium bacterial growth assay as described below. To determine the CFUs in each sample, 50 μL of several serial dilutions of each sample was spread onto the surface of nutrient broth agar plates to determine the CFUs for each sample. Samples were typically plated at three different dilutions to obtain plates with a quantity of colonies such to provide a reasonable estimate of bacterial density (50–400 CFUs/plate).

2.5. Immunofixation assay

Detection of C3 complement protein in human and alligator serum was achieved by immunofixation using a SPIFE Combo protein analyzer (Helena Laboratories, Beaumont, Texas). The analysis was performed according to the manufacturer's instructions, with the exception that goat polyclonal antihuman C3 antibodies were used for immunofixation. The human serum was analyzed at a 1 : 3 dilution while the alligator serum was analyzed undiluted.

2.6. SDS-PAGE and Western blot analysis

Alligator (35 μg) and human (5 μg) serum proteins were resolved on 8.5% polyacrylamide gels for approximately 5 h at 75 V. The proteins were transferred to a PVDF membrane at 100 V for 3 h (5 °C) in Tris glycine buffer (pH 8.6) containing 20% MeOH. The membrane was blocked using NAP™ blocker (BioRad) as per manufacturer's instructions. The blot was probed using goat polyclonal antihuman C3 antibodies, followed by rabbit antigoat secondary antibodies conjugated to horseradish peroxidase. Color development was achieved using Opti-4CN HRP color development kit (BioRad).

2.7. Sheep red blood cell (SRBC) hemolytic assay

The functionality of the alligator serum complement system of proteins was investigated by a SRBC lysis assay modified from the method of Mayer (1967). Three hundred microliters of 1% SRBCs were mixed with 300 mL of veronal buffer (without Mg^{2+} or Ca^{2+}) and 700 μL of undiluted alligator serum. The samples were centrifuged at 1500 $\times g$ and the optical density of the supernatant was measured at 525 nm using a Varian Cary 50 UV/Vis spectrophotometer. To examine the effects of different specific inhibitors of the complement protein system, The serum veronal buffer was spiked with 20 mM ammonium hydroxide, 20 mM methylamine, 20 mM salicylaldoxime, or 60 mM EDTA.

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