

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

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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 salicylaldehyde, 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 C3 $\alpha$  and C3 $\beta$ . 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 opsonization of particulate foreign materials and kill microorganisms directly by the assembly of a multiprotein membrane attack complex in the outer mem-

brane of microbes (Muller-Eberhard, 1986; Dalmaso 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

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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)<sub>2</sub>) 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 anti-goat 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 µL of veronal buffer (without Mg<sup>2+</sup> or Ca<sup>2+</sup>) and 700 µL of undiluted alligator serum. The samples were centrifuged at 1500 ×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 salicylaldehyde, or 60 mM EDTA.

For samples tested for the effects of antihuman C3 antibodies, the sample was increased to a total volume of 1500  $\mu\text{L}$  using saline, or a C3 antibody solution. The samples were incubated for 30 min at room temperature, centrifuged at 1500  $\times g$  for 3 min, and the optical density measured at 525 nm using a Varian Cary 50 UV/Vis spectrophotometer.

### 2.8. Von Krogh transformation analysis

Different concentrations of alligator serum were incubated with 1% SRBCs ( $v/v$ ) in vitro in a 1 mL reaction at room temperature for 30 min. The samples were centrifuged at 1500  $\times g$  for 3 min and the optical density measured at 525 nm using a Varian Cary 50 UV/Vis spectrophotometer.

### 2.9. Statistics and controls

All experiments were performed in quadruplicate to obtain valid statistical evaluation of the results. Colony-forming units per milliliter (CFUs/mL) were calculated by multiplying the number of colonies counted by the dilution factor and then by ten (due to the fact that only 50  $\mu\text{L}$ , or 10% of the sample, were plated on each dish). To obtain a value for 100% lysis while conducting the SRBC hemolytic assays, a 1% solution of SRBCs in 1% Triton X-100 ( $v/v$ ) was syringed 5 times with a 1 mL TB syringe. All results presented represent the means  $\pm$  standard deviations.

## 3. Results

Sheep red blood cell (SRBC) hemolytic assays have been used for years to assess the functionality of serum complement in the clinical laboratory (Mayer, 1967). The data listed in Table 1 display the ability of alligator serum to disrupt SRBCs in vitro. Incubation of 1% SRBCs ( $v/v$ ) with alligator serum for 30 min at room temperature resulted in a  $94 \pm 4\%$  increase (relative to veronal buffer controls) in optical density at 525 nm. Pretreatment of alligator serum samples for 30 min at 56  $^{\circ}\text{C}$ , prior to incubation with

Table 1  
Effects of heat treatment and protease treatment on the hemolysis of SRBCs by alligator serum

Alligator serum	Optical density (525 nm, % maximum)
Untreated	$94 \pm 4$
Heat-treated	$14 \pm 2$
Protease-treated	$8 \pm 1$

Undiluted alligator serum samples were heat-treated or protease-treated and then incubated with 1% SRBCs for 30 min at room temperature. Samples were centrifuged at 1500  $\times g$  and the optical densities of the supernatants were determined at 525 nm. The results are expressed as the maximum of the positive sample control sample lysed with a TB syringe in the presence of 1% Triton X-100 detergent. The results represent means  $\pm$  standard deviations for four determinations.

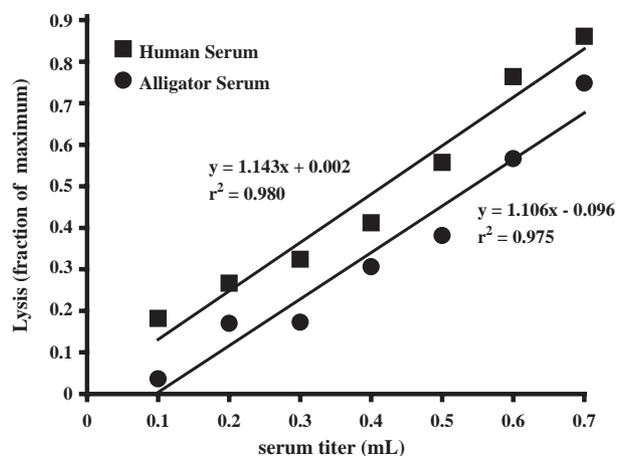


Fig. 1. Krogh plot: Concentration-dependent lysis of SRBCs by alligator and human serum. Serum samples were incubated with 1% SRBCs in a 1.0 mL reaction for 30 min at ambient temperature. The optical density of each sample was determined at 525 nm. The results are expressed as the percentage maximum lysis and represent the means  $\pm$  standard deviations for four determinations.

SRBCs, reduced the  $A_{525}$  by 80%. In addition, pretreatment of a 100  $\mu\text{L}$  sample of alligator serum with 20  $\mu\text{L}$  of a protease derived from *S. griseus* reduced the serum's ability to disrupt the integrity of the SRBCs. The protease treatment inhibited hemolysis by alligator serum by 96%. The inability of heat-treatment to completely obliterate the hemolytic activity of alligator serum may indicate the presence of cationic peptides or other heat-stable molecules.

The data depicted in Fig. 1 represents a modified Krogh transformation for the concentration-dependent disruption of SRBCs by alligator serum (Fike, 1997). Incubation of 1% SRBCs ( $v/v$ ) with increasing concentrations of alligator serum resulted in a concentration-dependent increase in hemolysis. The data were subjected to linear regression analyses. The slope of the line was 1.106. The  $\text{CH}_{50}$  value derived from this graph (Fig. 1) represents the concentration of serum required to produce 50% of the maximum hemolysis. This value is used clinically to determine the relative activity of a serum sample. The calculated  $\text{CH}_{50}$  value was  $0.539 \pm 0.043$  mL for alligator serum.

Table 2 shows the effects of specific serum complement inhibitors on the hemolysis of SRBCs by alligator serum in vitro. The treatment of alligator serum with 20 mM ammonium hydroxide or 20 mM methylamine did not affect the hemolytic activity of the serum. These compounds are known to interfere with the activity of the C4 protein and inhibit classical complement activity (Gordon et al., 1926; Gorski and Howard, 1980; Blom et al., 2003). The presence of these compounds, in the absence of serum, did not affect the integrity of the SRBCs. However, the presence of 20 mM salicylaldehyde or 30 mM EDTA resulted in only 23% and 2% of the total hemolytic activity, respectively. Salicylaldehyde has been shown to inhibit the alternate complement pathway (Austen and Brocklhurst, 1961). In addition, EDTA inhibits the alternate pathway by seques-

Table 2  
Effects of specific serum complement inhibitors on the hemolysis of SRBCs by alligator serum

	Hemolytic activity (% maximum)
Alligator serum	100±3
Alligator serum+20 mM ammonium hydroxide	94±6*
Alligator serum+20 mM methylamine	98±5*
Alligator serum+20 mM salicylaldoxime	24±4
Alligator serum+30 mM EDTA	3±1

Undiluted alligator serum samples were incubated with or without saline and ammonium hydroxide, methylamine, salicylaldoxime, or EDTA for 30 min at room temperature. The serum samples were then incubated with 1% SRBCs for 30 min. Samples were centrifuged at 1500 ×g and the optical densities of the supernatants were determined at 525 nm. The results are expressed as the maximum of the positive sample control sample lysed with a TB syringe in the presence of 1% Triton X-100 detergent. The results represent means±standard deviations for four determinations. \* =not statistically different from treatment with alligator serum alone ( $p>0.05$ ).

tering  $Mg^{2+}$  ions that are required for the interaction of C3b with factor B (Gotze et al., 1977).

The native agarose gel electrophoretic analysis of human and alligator serum revealed remarkably similar protein patterns (Fig. 2A). The albumin band in the alligator serum constituted only 25% of the total serum protein, as determined by densitometry, compared to approximately 50–60% for human serum (data not shown). The albumin band migrated slightly more anodal than that of the human serum. Immunofixation of the human C3 protein showed a single band just cathodal to the application point on the gel (Fig. 2A, lane 2). The same antibodies (goat antihuman polyclonal C3) detected a single band in alligator serum (Fig. 2A, lane 4) that migrated slightly more anodal than the human C3. The appearance of only one band indicates the specificity of human C3 antibodies for the alligator C3 protein. SDS-PAGE followed by Western blot analysis revealed the presence of two polypeptides similar in molecular weight to the human C3 $\alpha$  and C3 $\beta$  proteins. The interaction of the

Table 3  
Effects of polyclonal C3 antibodies on the hemolysis of SRBCs by alligator serum

	Optical density (525 nm, % maximum)
Alligator serum	93±3
Alligator serum+saline	91±2*
Alligator serum+C3 Abs	48±4

Undiluted alligator serum samples were incubated with or without saline and polyclonal goat antihuman C3 antibodies for 30 min at room temperature. The serum samples were then incubated with 1% SRBCs for 30 min. Samples were centrifuged at 1500 ×g and the optical densities of the supernatants were determined at 525 nm. The results are expressed as the maximum of the positive sample control sample lysed with a TB syringe in the presence of 1% Triton X-100 detergent. The results represent means±standard deviations for four determinations. \* =not statistically different from treatment with alligator serum alone ( $p>0.05$ ).

alligator protein with the human C3 antibodies, the similar migration of the native protein band, and the similarity of the molecular masses of the human and alligator C3  $\alpha$  and  $\beta$  components lead us to believe that this protein represents a crocodylian complement component analogous to the mammalian C3 protein.

The data listed in Table 3 illustrate the effects of antihuman C3 antibodies on the ability of alligator serum to disrupt SRBCs in vitro. Incubation of alligator serum with 1% SRBCs ( $v/v$ ) resulted in 93% of the maximum hemolytic activity, relative to positive hemolysis controls. The serum-mediated hemolysis of SRBCs was decreased to 48% by preincubation with antihuman C3 polyclonal antibodies. The results from this experiment highlight the functional effects of the proposed alligator serum C3 protein.

The data in Table 4 illustrate the effects of antihuman polyclonal C3 antibodies on the antibacterial properties of alligator serum. Inoculation of 0.5 mL of serum with  $10^4$  CFU *E. coli* resulted in only 11±6% survival. However, pretreatment of the serum with the antihuman C3 antibodies resulted

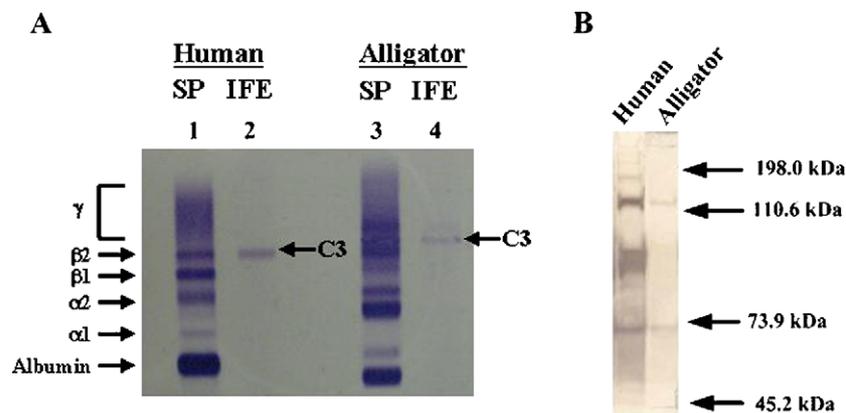


Fig. 2. Detection of serum complement C3 protein in human and alligator serum by immunofixation and Western blot analysis. A. Human (diluted 1:3 with saline) and alligator (undiluted) serum samples were resolved on a 1.2% native agarose gel and the total protein was precipitated using 5% acetic acid (lanes 1 and 3). Protein was visualized using acid violet Coomassie-type stain. The cathodic C3 protein band was detected by immunofixation (lanes 2 and 4) using goat antihuman C3 antibodies. B. Denatured serum samples (SDS) were resolved on an 8.5% SDS-PAGE gel, transferred to a PVDF membrane, and probed using goat antihuman C3 primary antibodies.

Table 4

Effects of polyclonal C3 antibodies on antibacterial properties of alligator serum

	Bacterial survival (% maximum)
Alligator serum	11±6
Alligator serum+saline	9±4*
Alligator serum+C3 Abs	73±9
Alligator serum+transferrin Abs	8±4*
Alligator serum+ $\alpha_1$ -macroglobulin Abs	14±4*
Alligator serum+ $\alpha_1$ -antitrypsin Abs	12±3*

Undiluted alligator serum samples were incubated with or without saline and polyclonal goat antihuman C3 antibodies for 30 min at room temperature. The serum samples were then inoculated with  $10^4$  CFU of *E. coli*. The results are expressed as percentage maximum of the positive control of  $10^4$  CFUs inoculated into nutrient broth bacterial growth medium. These results represent the means±standard deviations for four determinations. \* = not statistically different from treatment with alligator serum alone ( $p > 0.05$ ).

in substantial (73±9%) increase in bacterial survival. In addition, pretreatment of the serum with goat antihuman antibodies directed toward transferrin,  $\alpha_1$ -macroglobulin, or  $\alpha_1$ -antitrypsin resulted in 8±4%, 14±4%, and 12±3%, respectively. None of these treatments exhibited a significant effect on the antibacterial effects of the alligator serum.

The data depicted in Fig. 3 demonstrate the time-dependent in vitro hemolytic effects of alligator serum. Incubation of alligator serum with SRBCs resulted in a time-dependent increase in optical density at 525 nm. Significant hemolysis (32±6% of maximum,  $p < 0.01$ ) was observed at 5 min. The activity was increased to 68±8% at 10 min and was maximal at 15 min.

Incubation of alligator serum with SRBCs at different temperatures resulted in temperature-dependent hemolysis (Fig. 4). The hemolytic effects were not statistically different from 100% at 15, 20, 25, and 30 °C ( $p > 0.05$ ). However, a temperature-dependent decrease in activity was

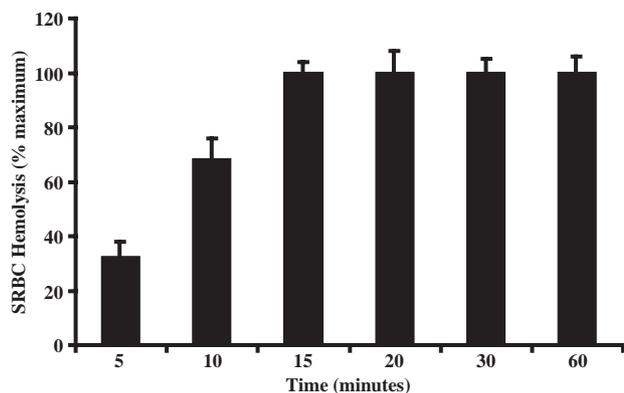


Fig. 3. In vitro kinetic analysis of SRBC hemolysis by alligator serum. Alligator serum (1:2 final dilution) was incubated with 1% unsensitized SRBCs (v/v) at ambient temperature and aliquots were removed at various time points. The aliquots were immediately centrifuged (1500 ×g, 5 min) and the optical densities of the supernatants were measured at 525 nm. The results are expressed as the percentage maximum lysis and represent the means±standard deviations for four determinations.

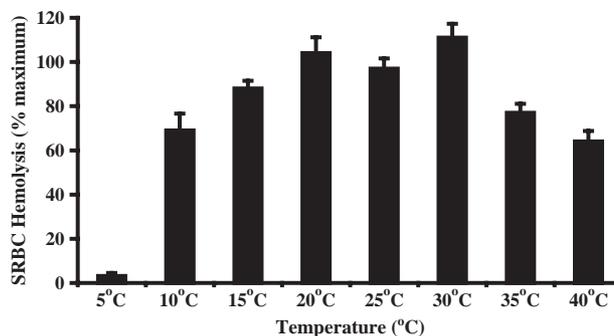


Fig. 4. Temperature-dependent hemolysis of SRBCs by alligator serum. Alligator serum (1:2 final dilution) was incubated with 1% unsensitized SRBCs (v/v) at the indicated temperatures. The optical density of each sample was determined at 525 nm. The results are expressed as the percentage maximum lysis and represent the means±standard deviations for four determinations.

observed at temperatures below 15 °C. The hemolytic activity was decreased by 31% at 10 °C ( $p < 0.01$ ) and 96.8% at 5 °C ( $p < 0.01$ ). In addition, the activity of SRBC hemolysis was decreased at temperatures higher than 30 °C. The serum-dependent hemolytic activity was decreased by 23% at 35 °C and by 46% at 40 °C.

#### 4. Discussion

Several investigators have reported the presence of an active serum complement system in a variety of reptilian species (Koppenheffer, 1987; Sunyer and Lambris, 1998; Sunyer et al., 1998). For instance, Kuo et al. (2000) described the complement-mediated killing of the Lyme disease spirochete (*Borrelia burgdorferi*) in the western fence lizard. Koppenheffer (1986) has demonstrated the presence of both classical and alternative pathways in turtle serum. Other studies have focused on the complement activities in snake serum (Kawaguchi et al., 1978; Dias da Silva et al., 1984). The most extensive characterizations of reptilian complement have been in the cobra (Vogel and Muller-Eberhard, 1985a,b; Fritzing et al., 1992). Shahrabany et al. (1999) described the antibacterial activity of crocodile (*Crocodilus niloticus*) serum. However, to our knowledge, a description of crocodilian complement has not been published to date.

The data listed in Table 1 illustrate the ability of alligator serum to hemolyse SRBCs in vitro. The ability of human serum to hemolyse antibody-sensitized SRBCs is well documented and is used clinically to detect deficiencies in innate complement defense. This assay was used to assess the complement-like activities of alligator serum. Incubation of alligator serum with 1% unsensitized SRBCs resulted in strong hemolytic activity, as assayed by an increase in optical density at 525 nm. Human complement contains heat-labile proteins that are thermally inactivated when incubated at 56 °C for 30 min. The incubation of alligator serum at classical complement inactivation conditions (56 °C, 30 min) disabled the hemolytic capacity of the samples.

The fact that the hemolytic activity of alligator serum is also compromised under these conditions (Table 1) rules out the possibility that this activity is due to heat-stable cationic peptides and provides supporting evidence that these activities are due to the presence of a crocodilian complement immune system component (Diamond et al., 1991; Moore et al., 1991). The sensitivity of the hemolytic activity of the alligator serum to protease treatment indicates that the activity is due to a protein(s) and not a small organic molecule. Previous studies in our laboratory have indicated that the SRBC hemolytic properties of alligator serum and human serum are the same (data not shown). The ability of alligator serum to lyse SRBCs that have not been sensitized with antibodies marks a fundamental difference in the complement-like activities of alligator and human sera. The hemolytic activity of the alligator serum was not enhanced by the addition of goat anti-SRBC or rabbit anti-SRBC (data not shown). It is well established that human serum will only lyse SRBCs that have been sensitized by interaction with antibodies, thus initiating the classical pathway of complement activation. However, this does not seem to be the case with alligator serum as strong hemolytic activity is observed with serum from animals that have not been challenged with SRBCs. In addition, activity from alligators that were sensitized with SRBCs *in vivo* resulted in the same hemolytic activities, thus indicating that the alligator serum may exhibit alternative complement activity toward SRBCs *in vitro*. This theory is further strengthened by the fact that pretreatment of alligator serum with EDTA prevents SRBC lysis (Fig. 2, Merchant et al., 2005a,b). It is known that, for human serum, only the alternative pathway of complement activation is inhibited by EDTA (Vogt et al., 1977). These results are consistent with previous reports from our laboratory that alligator serum exhibits a broad spectrum of antibacterial (Merchant et al., 2003), antiviral (Merchant et al., 2005a,b), and amoebicidal (Merchant et al., 2004) activities. The serum has been shown to be effective in killing 23 species of bacteria, 3 viruses, and 8 amoeba species tested thus far. It seems unlikely that the alligators would have had natural antibodies against all of these organisms, particularly since many of the microbes are not environmental types but are opportunistic human pathogens that grow optimally at 37 °C. These data provide further evidence for an alternative complement mechanism of antibacterial activity in alligator serum. However, this evidence does not rule out the possibility that the alligator may also have an active classical serum complement pathway.

Fig. 1 displays the ability of alligator serum to hemolyse SRBCs in a concentration-dependent manner. The Krogh plot has been used for years in the clinical laboratory to determine the relative activity of serum complement in human patients (Fike, 1997). The  $CH_{50}$  values represent the volume of blood needed to produce 50% lysis of SRBCs *in vitro*. The  $CH_{50}$  value for pooled alligator serum (0.539 mL) indicates strong hemolytic activity.

The results from early studies revealed that ammonium ions and primary amines bind to a specific activated glutamyl residue in the  $\alpha$  subunit of C4, resulting in glutamylamine conjugates that inactivated the classical complement pathway (von Zabern et al., 1982). The results listed in Table 2 show that these compounds do not affect the activity of alligator serum with respect to SRBC hemolysis. Furthermore, treatment of the serum with 20 mM salicylaldehyde resulted in only 24% of the total hemolytic activity. Salicylaldehyde has been shown to inhibit alternate complement cascade (Austen and Brocklhurst, 1961). The fact that EDTA can prevent alligator serum-mediated SRBC hemolysis but Mg-EDTA does not indicates that the activity is due to the alternate complement pathway. These results suggest a common molecular mechanism for the activation of the classical and alternate pathways of the serum complement system in human and alligator serum.

Complement protein C3 plays a pivotal role in the activation of the serum complement activation by both the classical and alternative pathways. C3 is a 195 kDa protein present at approximately 1.3 mg/mL in human serum, making this protein the most abundant of the complement factors. The data depicted in Fig. 2 reveal the presence of a protein in alligator serum with antigenic character similar to that of the human C3 complement protein. The densitometric analysis of the C3 bands (data not shown) in Fig. 2A revealed a 2.3-fold decrease in intensity of the C3 band in alligator serum despite a two-fold increase in sample load on the gel (relative to the volume of human serum loaded). Although the interactions are relatively weak, the fact that antibodies directed toward human C3 protein bind to alligator C3 complement shows the similar surface antigenic character of human and alligator proteins. These findings contrast those reported by Eggersten et al. (1983) that showed no cross-reactivity of human C3 antisera to cobra C3 complement protein. In addition, the same approximate cathodal migration on the native agarose gel reveals a similar charge/mass ratio for the alligator and human C3 proteins. Fig. 2B exhibits the almost identical molecular masses of the alligator and human C3  $\alpha$  (115 kDa) and  $\beta$  (68 kDa) peptide components. The presence of the C3 protein in both humans and alligators exhibits the immunological importance of this protein in host defense.

Table 3 shows the effects of antihuman C3 antibodies on the hemolysis of SRBCs by alligator serum. The addition of C3 antibodies neutralized the ability of the serum complement system from both species to hemolyse the cells. These data show that the C3-like protein in alligator serum is necessary for SRBC lysis. The results indicate that the proposed alligator C3 protein has a role in complement function.

Table 4 shows the effects of antihuman C3 antibodies on the antibacterial properties of alligator serum. The potent antibacterial properties of alligator serum have been documented in our laboratory (Merchant et al., 2003). The bacterial survival in the presence of alligator serum was

increased from 11% to 73% by the addition of C3 antibodies. The fact that this activity was not completely eliminated was probably due to the relatively weak interaction of the antibodies directed to human C3 with the proposed crocodilian C3 protein. These data indicate that the C3-like protein in alligator serum has a functional role in the antibacterial properties of alligator serum. Further studies showed that goat antihuman antibodies (100 µg/mL) directed toward other human serum proteins such as transferrin,  $\alpha_1$ -macroglobulin, and  $\alpha_1$ -antitrypsin did not exhibit any effect on the antibacterial activities of alligator serum (Table 4). These data demonstrate the specificity of the effects of the antihuman C3 antibodies on the antibacterial properties of the alligator serum. In addition, the data reveal that the effects of the anti-C3 antibodies were not due to a nonspecific response of the alligator serum to goat IgG.

The kinetics of SRBC lysis by alligator serum are remarkably similar to the kinetics of the antibacterial effects of alligator (Merchant et al., 2003) and human serum (Wright and Levine, 1981) previously described. The hemolysis of SRBCs by alligator serum occurs rapidly, with substantial activity observed as early as 5 min (Fig. 3). In addition, the response is maximal at 15 min. These kinetic properties should allow alligators to provide a strong immunological response to microbial challenge, in a nonspecific manner, almost immediately after infection occurs.

Since alligators are poikilothermic, vertebrates whose body temperatures exhibit seasonal and diurnal variation, we investigated the effects of temperature on SRBC hemolysis by alligator serum. The hemolysis of SRBCs by alligator serum was compromised at temperatures below 15 °C (Fig. 4). This may indicate that alligators are immunocompromised during the cold winter months when body temperatures can fall as low as 5 °C (Brisbin et al., 1982). In addition, alligator complement activity was decreased in a temperature-dependent manner above 30 °C. SRBC hemolysis was decreased 23% at 35 °C and by 36% at 40 °C. However, this result might not be physiologically relevant due to the fact that internal temperatures of alligators have not been reported higher than 35 °C in wild, free-ranging alligators (Seebacher et al., 2003).

To our knowledge, the results from this study represent the first report of serum complement activity in a crocodilian species. The complement system is shown to exhibit similar functional and molecular properties of mammalian complement. We believe that the complement system may be, in part, responsible for the antibacterial properties of alligator serum.

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