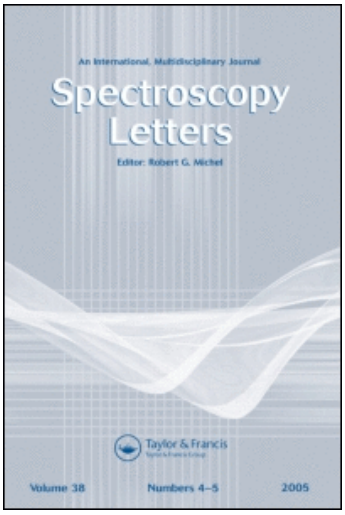

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Rapid and Inexpensive Method for the Spectroscopic Determination of Innate Immune Activity of Crocodilians

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Abstract: We have employed a spectroscopic assay based on the hemolysis of sheep red blood cells (SRBCs) to assess the immune system of the American alligator (*Alligator mississippiensis*). The assay is based on the hemolytic disruption of the SRBCs by the immunological proteins in the crocodilian serum. Incubation of 1% SRBCs (v/v) with alligator serum resulted in hemolysis that was measured at 540 nm in a microtiter plate reader. The hemolysis was concentration-, time-, and temperature-dependent. This assay is rapid, inexpensive, easy to perform, requires small sample volumes, and is useful for evaluating the humoral immune response of crocodilians.

Keywords: Crocodilian, hemoglobin, humoral immunity, innate immunity, serum complement, spectrophotometry

INTRODUCTION

Alligators are territorial animals that often engage in interspecies disputes, particularly during the mating and nesting seasons. The clashes often result in serious injuries, including the loss of entire limbs. However, despite the

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fact that alligators live in marshy environments rich in potentially infectious microbes, these severe injuries heal very rapidly and most often without infection. Recent studies in our laboratory have indicated that serum from the American alligator exhibits potent and broad-acting antibacterial,[1] antiparasitic,[2] and antiviral[3] activities. These activities have been reported to be due to the presence of a potent serum complement system of proteins in the alligator.[4] Further studies have shown that the antibacterial activities are not unique to alligators but are also exhibited by all living crocodilian species.[5]

The serum complement system comprises a significant portion of innate immunity. Serum complement proteins circulate as inactive precursors but become active when exposed to foreign antigens. Activation of this group of proteins results in the formation of a macromolecular complex in the outer membrane of the pathogen. Formation of the protein complex causes a pore to form in the membrane, resulting in the leakage of cellular contents and lysis of the cell. The assay described in this study is based on the complement-mediated lysis of sheep red blood cells (SRBCs). Exposure of SRBCs to alligator serum results in the lysis of these cells. The alligator serum does not distinguish the SRBCs from a pathogenic microbe. Sheep are used as the source of red blood cells because the cell from this species has been shown to elicit a strong immune response from the serum of other animals.[6] The hemolytic activity can easily be measured at 540 nm as the hemoglobin leaks from the cells. This study was conducted to develop a rapid and inexpensive method for the assessment of alligator immune function.

MATERIALS AND METHODS

Treatment of Animals

Alligators were captured at the J. D. Murphree Wildlife Management Area in Port Arthur, Texas. Alligators were captured at night with use of spotlight and cable snare. The alligators were processed rapidly and generally returned to their environment within 5–10 min of capture to minimize stress to the animals.

Collection of Blood Samples

Blood samples were collected from the spinal vein[7,8] of six alligators using $11/2''$ 18-gauge needle and 3-mL syringes. The samples were transferred to Vacutainer™ tubes and allowed to clot for approximately 6 h. The clots were removed by centrifugation at $2500 \times g$ for 15 min (20°C). The resulting serum samples were pooled and used for analyses.

SRBC Hemolysis Assay

One milliliter of alligator serum was incubated with 1 mL of 2% unsensitized SRBCs. The samples were incubated for 20 min, except during the kinetic study, and centrifuged at 2000 × *g* for 2 min. The resulting supernatant was transferred to a 12 × microtiter plate, and the optical density was measured at 540 nm in a Cary 50 UV/visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). For the kinetic hemolysis analysis, 5 mL of 10%, 25%, or 50% alligator serum was added to 5 mL of 2% SRBCs (v/v). Five hundred microliter aliquots were removed at various time points, and the samples were centrifuged at 2500 × *g* to pellet the unlysed cell and the insoluble membrane materials of the lysed cells. Two hundred microliters of the resulting supernatant was removed to a 96-well microtiter plate. The optical density of each sample was measured at 540 nm.

Statistics and Controls

For each SRBC hemolysis assay, a complete lysis positive control was acquired by rapidly syringing a solution of 1% SRBCs through a 1-mL tuberculin syringe in the presence of 1% (v/v) Triton X-100 detergent. This action resulted in 100% lysis of the SRBCs, as inspected by phase contrast microscopy at 400 × magnification, and this sample was used as a comparison

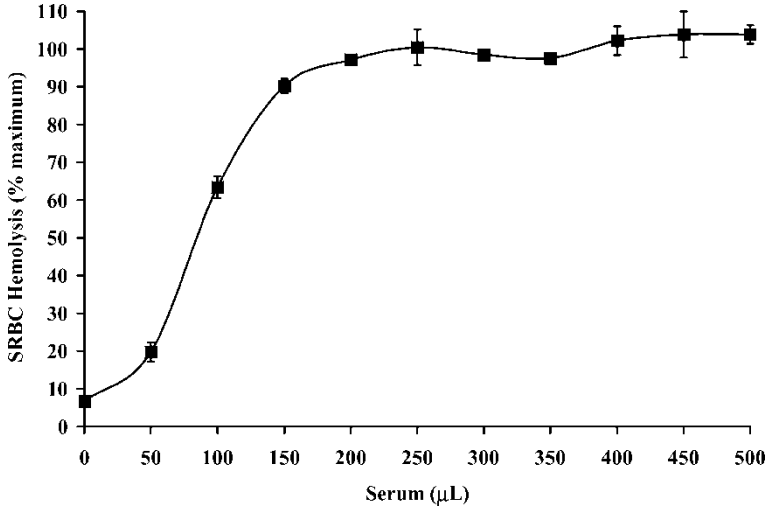

Figure 1. Different concentrations of alligator serum (1 mL total volume) were exposed to SRBCs for 20 min at 25°C. The samples were centrifuged at 2500 × *g* for 1 min, and the optical density (540 nm) of the supernatant was determined using a microtiter plate reader.

for all other samples. Each sample was analyzed in triplicate so that valid statistical results could be obtained. All results presented represent the means standard $+/-$ the deviations. The results obtained from each experiment were subjected to analysis of variance using Scheffe's post hoc comparisons.[9]

RESULTS

Figure 1 displays the concentration-dependent hemolysis of SRBCs by alligator serum. The results are expressed as percent maximum hemolysis, based on comparison with a positive hemolysis control, and represent the means $\pm$ standard deviations for three independent determinations. Incubation of SRBCs with only 5% alligator serum for 20 min at ambient temperature resulted in 13.1% of maximal hemolysis, as compared with the positive control. In addition, incubation of 10%, 15%, and 20% alligator serum with SRBCs resulted in 56.6%, 83.4%, and 90.4% of maximal activity, respectively. Incubation of 1% SRBCs (v/v) with increasing concentrations of alligator serum resulted in a concentration-dependent increase in hemolysis. The 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 extrapolated value for the CH_{50} on Fig. 1 is 0.086 mL. However,

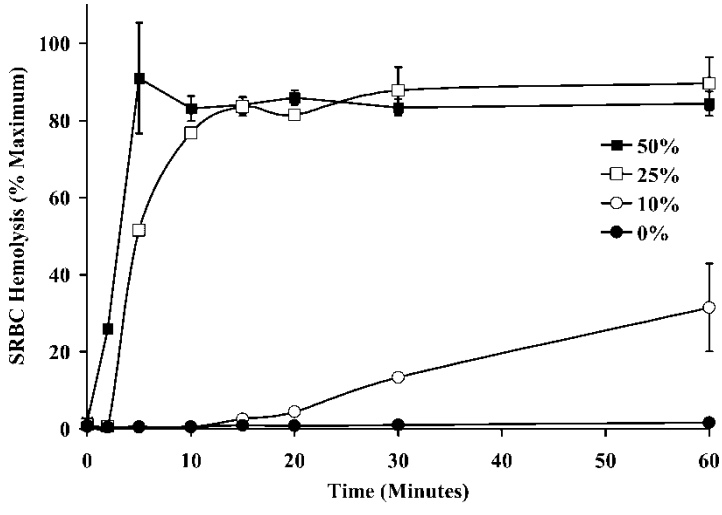

Figure 2. Different concentrations of alligator serum (v/v) were exposed to SRBCs at 25°C for various amounts of time. The samples were centrifuged at 2500 × *g* for 1 min, and the optical density (540 nm) of the supernatant was determined using a microtiter plate reader.

because 25% alligator serum was used, the calculated CH_{50} value was 0.344 mL (4 × 0.086 mL) for alligator serum.

Figure 2 illustrates the kinetics of the hemolysis of SRBCs by alligator serum. The results are expressed as percent maximum hemolysis, based on comparison with a positive hemolysis control, and represent the means ± standard deviations for three independent determinations. Incubation of SRBCs with 50% alligator serum (v/v) resulted in 26.0 ± 1.3% of maximal hemolysis within 2 min, and 91.0 ± 14.4% of maximal hemolysis within 5 min. The hemolysis of SRBCs by 25% serum was slower, with 51.5 ± 0.2% of maximum hemolysis after 5 min, and 76.8 ± 1.3% of maximum hemolysis 10 min. In contrast, incubation with 10% serum resulted in only 31.5% of maximal hemolysis after an incubation of 60 min. Incubation of SRBCs with isotonic saline resulted in only 1.6 ± 0.1% of maximal hemolysis after 60 min.

Figure 3 shows the effects of temperature on the hemolysis of SRBCs by alligator serum. The results are expressed as percent maximum hemolysis, based on comparison with a positive hemolysis control, and represent the means ± standard deviations for three independent determinations. Incubation of 25% serum with SRBCs at 5°C or 10°C did not result in substantial hemolytic activity after 20 min. However, this activity increased to 3.5% ± 0.5%, 47.2% ± 4.4%, and 88.7% ± 2.8% for 15°C, 20°C, and 25°C, respectively. The hemolytic effects were not statistically different from 100% at 30°C, 35°C, or 40°C ($p > 0.05$).

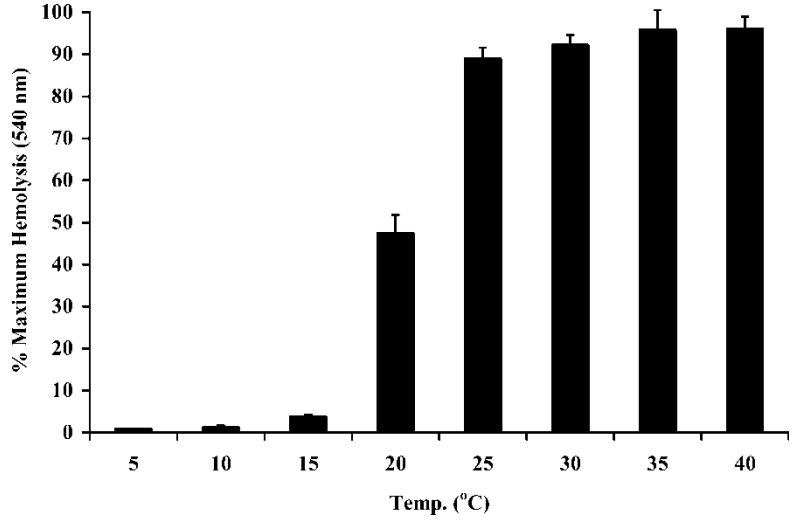

Figure 3. Alligator serum (25%, v/v) was exposed to SRBCs at different temperatures for 20 min. The samples were centrifuged at 2500 × *g* for 1 min, and the optical density (540 nm) of the supernatant was determined using a microtiter plate reader.

DISCUSSION

The recent results from our laboratory have shown that crocodilians have evolved powerful and broad-acting immune systems. These activities are currently being investigated as a potential source of novel antibiotic drugs, and thus much research attention will be focused on the immune systems of these ancient reptiles. The assay described in this study can be used to assess the immune systems of crocodilians for drug development. In addition, veterinary scientists can use this assay to assess the health of captive crocodilian specimens in zoological parks. Also, because environmental pollutants often affect the immune systems of critical indicator species, this assay may be useful for the assessment of the biological effects of environmental pollutants. This assay can be performed using a standard spectrophotometer or miniaturized and conducted using a microtiter plate reader. The assay requires a small sample size. Because the samples are diluted to 25% with saline prior to testing, and only 200 μL of diluted serum are required for each analysis, only 500 μL of whole blood is required from each alligator for analysis in triplicate. This assay can serve as an inexpensive and rapid method for the assessment of crocodilian humoral immunity.

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