ELSEVIER

Comparative Biochemistry and Physiology, Part B 141 (2005) 289 – 293

CBP

www.elsevier.com/locate/cbpb

Role of divalent metal ions in serum complement activity of the American alligator (*Alligator mississippiensis*)

Mark E. Merchant[a,*], Brannon Verret[a], Ruth M. Elsey[b]

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

Received 20 December 2004; received in revised form 30 March 2005; accepted 30 March 2005
Available online 26 May 2005

Abstract

Treatment of alligator serum with different concentrations of EDTA resulted in a concentration-dependent inhibition of serum-mediated sheep red blood cell (SRBC) hemolysis. This inhibition of serum-dependent hemolysis was observed for other chelators of divalent metal ions, such as phosphate and citrate. Treatment of alligator serum with 5 mM EDTA completely inhibited SRBC hemolysis, which could be totally restored by the addition of 5 mM Ca^{2+} or Mg^{2+}, but not Cu^{2+} or Ba^{2+}. These data indicate a specific need for Ca^{2+} and/or Mg^{2+} in the serum-mediated hemolysis of SRBCs. Kinetic analyses revealed that the addition of 30 mM EDTA 1 min after incubation of SRBCs with serum resulted in only 30% inhibition of hemolytic activity. However, addition of EDTA as early as 3 min post-incubation resulted in complete SRBC hemolysis. Pretreatment of serum with EDTA inhibited the hemolytic activity, but the activity could be restored in a time-dependent manner by the addition of Ca^{2+}or Mg^{2+}. These data indicate that, as in human serum, the need for divalent metal ions occurs early in the alligator serum complement cascade.

Keywords: Alligator; Calcium; Complement; Magnesium; Mississippiensis; Serum

1. Introduction

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 to 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).

Recent studies in our laboratory have shown that serum from the American alligator exhibits potent and broad-acting antibacterial (Merchant et al., 2003), amoebacidal (Merchant et al., 2004), and antiviral (Merchant et al., 2005a) activities. Further studies have shown that these activities are likely due to an active serum complement system in the blood of alligators (Merchant et al., 2005b). This study was conducted to investigate the role and kinetic requirements of divalent metal ions in alligator serum complement function.

2. Materials and methods

2.1. Chemicals and biochemicals

Ethylene diamine tetraacetic acid (EDTA), orthophosphate, calcium chloride, barium chloride, and cupric chloride were purchased from Sigma-Aldrich Chemical

* Corresponding author. Tel.: +1 337 475 5773; fax: +1 337 475 5950.
E-mail address: mmerchant@mcneese.edu (M.E. Merchant).

1096-4959/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2005.03.006

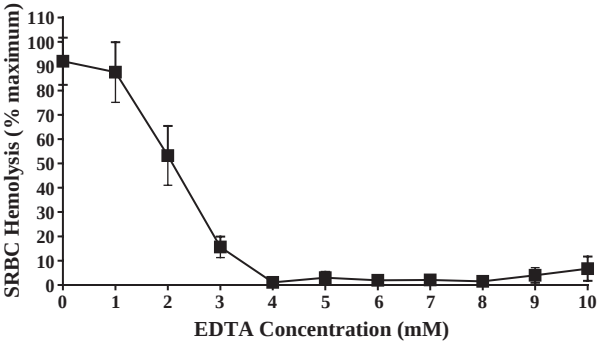

Fig. 1. Concentration-dependent inhibition of serum-mediated SRBC hemolysis by EDTA. Alligator serum was pretreated with different concentrations of EDTA. The effects of the EDTA on SRBC hemolysis are displayed. The results are expressed as the percent of activity of a positive lysis control, and represent the means ± standard deviations of four independent determinations.

Company (St. Louis, MO, USA). Sheep red blood cells (SRBCs) were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA).

2.2. Treatment of animals

Wild alligators were captured and then housed at the Rockefeller State Wildlife Refuge in Grand Chenier, Louisiana, USA. Adult alligators were typically captured at night with use of a cable snare. 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 4.57 m long × 1.83 m wide. Several small alligators (up to 91 cm in length) were housed in a single tank.

The environment in the tanks consisted of 50% dry bottom and 50% water of approximate 12.5 cm depth. The temperature was maintained at approximately 30–31 °C. The alligators were fed formulated dry pellets four–five times per week and the tanks were cleaned five times per week. Blood samples were drawn from the spinal vein (Olsen et al., 1975, Zippel et al., 2003) using a 3.81-cm, 18-gauge needle and a 20-mL or 60-mL syringe and transferred to either serum or plasma (50 mM EDTA) vacutainer tubes.

2.3. SRBC hemolysis assay

Five hundred microliters of alligator serum was added to 500 μL of 2% SRBCs (v/v) in a 2-mL microcentrifuge tube. Except in the kinetic study, during which the incubation time varied, the samples were incubated at room temperature (RT) for 30 min. The samples were centrifuged at 2500×*g* (5 min, RT) in a microcentrifuge to pellet intact cells and debris from lysed cells. The optical density of the supernatants was quantified using a Varian Cary 50 spectrophotometer (525 nm) and a microprobe accessory designed for the measurement of optical density in samples of small volume. For experiments in which EDTA, Ca^{2+} and/or Mg^{2+} were included, 20 μL of each was added at 10× the final concentration. The final concentration of SRBCs was kept at 1% and the reaction volume was maintained at 2 mL.

2.4. Statistics and controls

All experiments were performed in triplicate to enable valid statistical evaluation of the results. The results of each assay are expressed as the percent of maximum lysis by comparison to the positive control value obtained by full hemolysis of SRBCs by physical disruption in a hyposaline solution. All results represent the means ± standard deviations. The results were subjected to analysis of variants using Sheffe's post-hoc comparisons.

3. Results

The inhibition of alligator serum complement activity is shown in Fig. 1. Pretreatment of alligator serum with 1 mM EDTA, prior to incubation with SRBCs, did not result in a statistically significant reduction of SRBC hemolysis ($p < 0.05$). However, pretreatment of the serum with 2 mM, 3 mM, or 4 mM EDTA resulted in reductions to 53%, 15%, and 1% of the total hemolytic activity, respectively. Treatment of the alligator serum with concentrations of EDTA higher than 4 mM resulted in full inhibition of SRBC hemolysis.

The effects of divalent metal ion chelators on the hemolysis of SRBCs by alligator serum are depicted in Fig. 2. Incubation of SRBCs with untreated alligator serum resulted in 90% of maximum hemolysis, while treatment of serum with 50 mM EDTA produced only 9% of maximum hemolysis. Treatment of serum with 50 mM phosphate caused the hemolytic activity to drop to 22%,

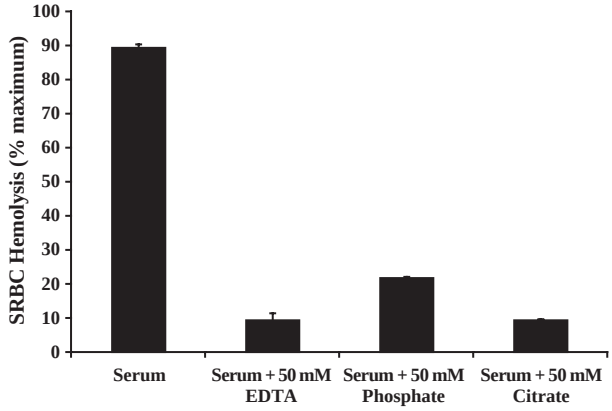

Fig. 2. Effects of divalent metal ion chelators on the hemolysis of SRBCs by alligator serum. Alligator serum was incubated with 30 mM EDTA, citrate or inorganic phosphate. The results are expressed as the percent of activity of a positive lysis control, and represent the means ± standard deviations of four independent determinations.

while treatment of the alligator serum with 50 mM citrate resulted in a decrease in hemolysis to 10% of the maximum activity. These values were obtained by comparing each treatment group to SRBCs incubated with the same volume of normal saline.

The ability of excess Ca^{2+} or Mg^{2+} to overcome the inhibitory effects of EDTA is illustrated in Fig. 3. Pretreatment of alligator serum with 4 mM EDTA followed by exposure to SRBCs resulted in a nearly complete inhibition of hemolytic activity, when compared to control SRBCs, which were completely hemolysed by syringing in a hyposaline solution. However, cotreatment of the serum with 4 mM EDTA and 1 mM, 2 mM, or 5 mM Mg^{2+} restored the hemolytic activity to 31%, 63%, or 87% of maximal activity, respectively. Cotreatment of the serum with 4 mM EDTA and 5–20 mM Mg^{2+} did not result in further increase in hemolytic efficiency ($p>0.05$). The results obtained for Ca^{2+} restoration of EDTA-inhibited hemolysis were nearly identical to those obtained for Mg^{2+}. Cotreatment of serum with 4 mM EDTA and 1 mM, 2 mM, or 5 mM Ca^{2+} restored the hemolytic activity to 31%, 73%, 87% of maximal activity, respectively. Cotreatment of alligator serum with SRBCs in the presence of 4 mM EDTA was not affected by the addition of Ba^{2+}(1–100 mM) or Cu^{2+} (1–100 mM).

The effects of different combinations of Ca^{2+} and Mg^{2+} on the alligator serum-mediated lysis of SRBCs are depicted in Fig. 4. Alligator serum pretreated with 5 mM EDTA and 10 mM $CaCl_2$ was titrated with $MgCl_2$. The addition of 1 mM $MgCl_2$ significantly elevated serum complement activity by approximately 11% ($p<0.01$). Addition of higher concentrations of $MgCl_2$ did not further change the complement activity ($p>0.05$). The titration of $MgCl_2$-spiked serum with $CaCl_2$ displayed almost identical effects. The addition of 1 mM $CaCl_2$ caused a 9% increase in hemolytic activity and addition of concentrations of $MgCl_2$ above 1 mM did not further increase the hemolytic activity of the alligator serum ($p>0.05$).

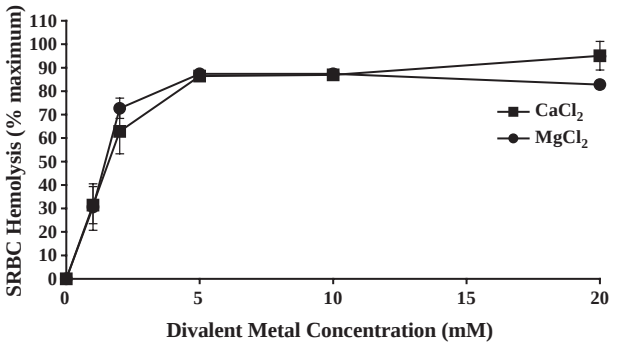

Fig. 3. Optimal concentration of Mg^{2+} and Ca^{2+} in alligator serum for the hemolysis of SRBCs. Alligator serum was incubated with 5 mM EDTA and then titrated with different concentrations of Mg^{2+} or Ca^{2+}. The results are expressed as the percent of activity of a positive lysis control, and represent the means ± standard deviations of four independent determinations.

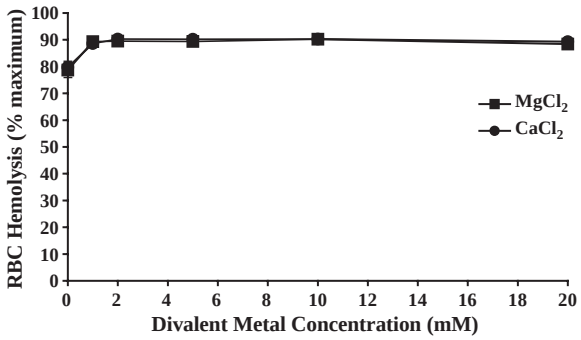

Fig. 4. Effects of different combinations of Ca^{2+} and Mg^{2+} on the alligator serum-mediated lysis of SRBCs. Alligator serum was treated with 5 mM EDTA and either 10 mM Ca^{2+} or Mg^{2+}. The serum treated with Ca^{2+} was titrated with different concentrations of Mg^{2+}, and vice versa, to determine the combined effect of these metals on SRBC hemolysis. The results are expressed as the percent of activity of a positive lysis control, and represent the means ± standard deviations of four independent determinations.

The dynamics of SRBC hemolysis by alligator serum is shown in Fig. 5. Treatment of alligator serum with 30 mM EDTA prior to, and at different times after, incubation with 1% SRBCs resulted in time-dependent effects on hemolytic activity. Pretreatment of the serum with 30 mM EDTA resulted in strong inhibition ($p<0.01$, not significantly different from negative controls) of SRBC hemolysis at all time points. However, treatment of serum that had been incubated with SRBCs for 1 min resulted in only 10% of maximal hemolytic activity, compared to positive lysis controls. Addition of 30 mM EDTA to alligator serum 2 min after the addition of SRBCs resulted in 90% of the maximal SRBC hemolysis activity, while 3 min post-SRBC EDTA incubation resulted in only 100% of hemolysis.

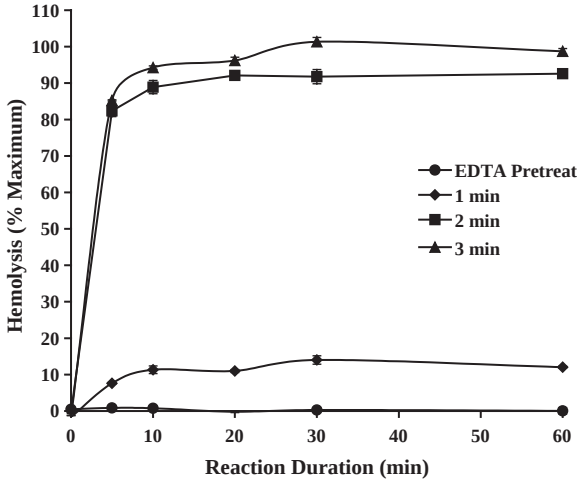

Fig. 5. Kinetics of the effects of EDTA on the hemolysis of SRBCs by alligator serum. Alligator serum was mixed with SRBCs, and 30 mM EDTA was added at different time points after the addition of SRBCs. The results are expressed as the percent of activity of a positive lysis control, and represent the means ± standard deviations of four independent determinations.

4. Discussion

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. While higher eukaryotes have evolved complex adaptive immune systems, Sunyer et al. (1997) have theorized that more ancient vertebrates, such as teleost fishes, have evolved diverse innate immune systems. Sunyer has revealed that bony fish have evolved five different isoforms of C3. This allows fish to respond immunologically to a broad spectrum of stimuli. This broad-acting innate immunity is similar to that which was described for alligator innate immunity by our laboratory (Merchant et al., 2003, 2004, 2005a). Previous studies in our laboratory have provided evidence for the existence of a functional crocodilian serum complement system in the American alligator (Merchant et al., 2005b).

We have employed the sheep red blood cell (SRBC) hemolysis assay to probe the role of divalent metal ions in alligator serum complement function. The hemolysis of the SRBCs results in the release of hemoglobin from the cells. The concentration of released hemoglobin can be quantified spectrophotometrically by measuring absorbance at 525 nm.

Early reports by Cernovodeanu and Henri (1906) indicated that Ca^{2+} and Mg^{2+} were required for human serum complement function. Later reports by Levine and coworkers (1953a) indicated that complement-mediated hemolysis of SRBCs is inhibited by ethylene diamine tetraacetic acid (EDTA). These studies also indicated that the guinea pig serum complement system was affected profoundly by treatment of serum with EDTA, prior to incubation with SRBCs. More modern studies have shown the need for these divalent cations in innate host defense in a variety of models (Des Prez et al., 1975; Romanella et al., 1997; Watford et al., 2000). The data displayed in Fig. 1 show that EDTA inhibits alligator serum-mediated hemolysis of SRBCs in a concentration-dependent manner. The pretreatment of serum with 4 mM EDTA (final concentration) completely inhibits the complement-mediated SRBC hemolysis. This phenomenon is also observed following pretreatment with other chelators of divalent metal ions such as inorganic phosphate and citrate (Fig. 2). In addition, the supplement of a molar excess of Ca^{2+} or Mg^{2+} restores the EDTA-inhibited hemolytic activities of alligator serum in a concentration-dependent manner (Fig. 3). These results, in contrast to the findings of Levine et al. (1953b) that demonstrate the requirement for human serum of both Ca^{2+} and Mg^{2+} for complement activity, indicate that either Ca^{2+} or Mg^{2+} alone can restore alligator complement activity. The addition of Ca^{2+} and Mg^{2+} together, at various concentrations, did not increase the hemolytic properties of alligator serum substantially more than either cation alone at equivalent total-cation concentrations (Fig. 4). Although alligator serum seems to lack the complement specificity for Ca^{2+} and Mg^{2+}, there is not a complete lack of specificity of alligator complement for divalent metal ions, as the addition of Ba^{2+} or Cu^{2+} did not restore the hemolytic effects of alligator serum (data not shown).

Levine et al. (1953a) showed that treatment of guinea pig serum with EDTA after SRBC challenge reduced the effectiveness of the EDTA to inhibit hemolysis. Later studies showed that some of the early steps in the serum complement cascade required the presence of divalent cations (Levine et al., 1953b). The results from this study indicated that the need for Ca^{2+}occurred prior to that of Mg^{2+}in the complement cascade. The results displayed in Fig. 5 indicate that the requirement for divalent metal ions occurs very early in the alligator complement cascade. The addition of 30 mM EDTA 3 min after the initial incubation of alligator serum with SRBCs failed to inhibit the hemolytic effects of the alligator serum. Likewise, the addition of EDTA 2 min after incubation exhibited minimal effects. However, the addition of EDTA 1 min after SRBC exhibited a strong inhibition of hemolytic activity. These data indicate that the kinetics of alligator complement at 25 °C is much faster than that of human complement at 37 °C.

The results from this study indicate that alligator serum, like human and guinea pig serum, requires divalent metal ions for complement activity. The data indicate that, for *A. mississippiensis*, the ability to distinguish between Mg^{2+} and Ca^{2+} during complement-mediated hemolytic activity may not be as refined as that for higher eukaryotes. In addition, alligator serum requires these metals much earlier in the kinetic curve than mammalian systems.

Acknowledgements

The authors wish to thank Mr. Phillip “Scooter” Trosclair, III, and Mr. Dwayne LeJeune, of the Louisiana Department of Wildlife and Fisheries, for their assistance with catching and handling wild alligators.

References

Cernovodeanu, P., Henri, V., 1906. C. R. Soc. Biol. 60, 571.

Dalmasso, A.P., Falk, R.J., Raij, L., 1989. The pathobiology of the terminal complement complexes. Complement Inflamm. 6, 36–48.

Des Prez, R.M., Bryan, C.S., Hawiger, J., Colley, D.G., 1975. Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetraacetic acid and $MgCl_2$-ethylene glycol tetraacetic acid. Infect. Immun. 11, 1235–1243.

Levine, L., Cowan, K.M., Osler, A.G., Mayer, M.M., 1953a. The role of calcium and magnesium ions in complement fixation and immune hemolysis. I: uptake of complement nitrogen by specific precipitates and its inhibition by ethylenediaminetetraacetate. J. Immunol. 71, 359–366.

Levine, L., Osler, A.G., Mayer, M.M., 1953b. The role of calcium and magnesium ions in complement fixation and immune hemolysis: III. The respective roles of calcium and magnesium ions in immune hemolysis. J. Immunol. 71, 374–379.

Merchant, M., Roche', C., Elsey, R.M., Prudhomme, J., 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). Comp. Biochem. Physiol., B 136, 505–513.

Merchant, M., Thibodeaux, D., Loubser, K., Elsey, R.M., 2004. Amoebacidal effects of serum from the American alligator (*Alligator mississippiensis*). J. Parasitol. 90, 1480–1483.

Merchant, M., Pallansch, M., Paulman, R., Wells, J., Nalca, A., Ptak, R., 2005a. Antiviral activity of serum from the American alligator (*Alligator mississippiensis*). Antivir. Res. 66, 35–38.

Merchant, M., Roche', C., Thibodeaux, D., Elsey, R., 2005b. Identification of alternative serum complement activity in the blood of the American alligator (*Alligator mississippiensis*). Comp. Biochem. Physiol., B.

Morgan, B.P., Walport, M.J., 1991. Complement deficiency and disease. Immunol. Today 12, 301–306.

Muller-Eberhard, H.J., 1986. The membrane attack complex of complement. Annu. Rev. Immunol. 4, 503–528.

Olsen, G.A., Hessler, J.R., Faith, R.E., 1975. Technics for blood collection and intravascular infusion of reptiles. Lab. Anim. Sci. 25, 783–786.

Pascual, M., French, L.E., 1995. Complement in human disease: looking towards the 21st century. Immunol. Today 16, 58.

Romanella, M., Aminian, A., Adam, W.R., Pearse, M., d'Apice, A., 1997. Involvement of both the classical and alternative pathways in an ex vivo model of xenograft rejection. Transplantation 63, 1021–1025.

Sunyer, J.O., Tort, L., Lambris, J.D., 1997. Structural C3 diversity in fish: characterization of five forms of C3 in the diploid fish *Sparus aurata*. J. Immunol. 158, 2813–2821.

Watford, W.T., Ghio, A.J., Wright, J.R., 2000. Complement-mediated host defense in the lung. Am. J. Physiol., Lung Cell. Mol. Physiol. 279, L790–L798.

Zippel, K.C., Lillywhite, H.B., Mladnich, C.R., 2003. Anatomy of the crocodilian spinal vein. J. Morphol. 258, 327–335.