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Analyzing complement activity in the serum and body homogenates of different fish species, using rabbit and sheep red blood cells

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ABSTRACT

Alternative complement activity was determined in whole body homogenates (WBHs) and serum samples of different fish species, by measuring the amount of sample that induces 50% hemolysis of red blood cells using the ACH50 assay (Alternative Complement pathway Hemolytic activity). Values of ACH50 obtained for serum samples were about two-fold higher when using rabbit red blood cells (RRBC), as compared to sheep red blood cells (SRBC). The increase in ACH50 when using RRBCs for WBH samples was 28, 7 and 4 folds for guppy, molly and zebrafish, respectively. Large variability in complement activity was evident between fish species for both serum and WBHs. Evaluating the effect of freeze-thaw cycles on complement revealed significant reduction in complement activity in all tested samples. Loss of activity following three freeze-thaw cycles amounted to 48–59% when serum was tested and over 95% loss in activity for WBH. To our knowledge, this is the first study where fish WBHs were used for assaying complement activity. Our results support the suitability of this method in evaluating complement activity in small fish species or larvae, where blood cannot be obtained, as long as samples can be tested upon first thawing.

1. Introduction

The complement system plays an important role in host innate immune defense. It comprises of large number of plasma proteins that work together to achieve lysis of pathogens. The structural and functional diversity of complement proteins in teleost fish have resulted in extensive innate immune recognition capabilities (Oriol Sunyer et al., 1998). Complement activity has been extensively used as an indicator of innate humoral immune response in various fish species. The three different activation pathways: classical, alternative and lectin-dependent, have been well studied in fish (Forsgren and Quie, 1974; Montero et al., 1998).

Complement proteins in fish exhibit high degree of structural and functional conservation along with isotype diversity of components as a result of gene duplications (Boshra et al., 2006; Nakao et al., 2006; Nakao et al., 2003). Data obtained from studies on the complement system in several fish species have demonstrated the presence of almost all homologues to mammalian complement proteins (Nakao et al., 2011). The alternative pathway comprises of C3 and factors B and D which can be activated by bacterial lipopolysaccharide and β-glucans, leading to formation of C3-convertase. In normal physiological conditions at non-activated state, C3 is suppressed by regulatory proteins, factors H and I, and suppression is released by the various complement activators (Nakao et al., 2003).

Analysis of the alternative complement activity is usually achieved by measuring the ability of serum samples to lyse red blood cells, also known as the ACH50 assay (Alternative Complement pathway Hemolytic activity), in which the volume of serum that provides 50% lysis of sheep or rabbit red blood cells is calculated. These assays have been widely used to measure complement activity, mostly in blood plasma and serum.

Precursor zymogens of complement proteins are distributed in body tissues and synthesized at multiple sites including hepatocytes and cell surfaces (Morgan and Gasque, 1997; Nesargikar et al., 2012). Thus measuring complement activity in tissues and organs should be possible and could be an alternative for employing this analysis in cases where collection of blood is impractical.

This manuscript addresses several topics in complement analysis.

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(1) The source of red blood cells (RBCs): RBCs from sheep and rabbit have been used interchangeably for complement analysis in fish (Chiu et al., 2008; Matsuyama et al., 1988; Pionnier et al., 2013; van den Berg et al., 1993). In this study we carry out a comparative analysis using RBCs from these two animals. (2) Examining the application of complement analysis to small-sized fish, from which blood cannot be drawn (*i.e.* small ornamental species and fish larvae). (3) Analyzing the effects of freeze and thaw on complement activity in serum and WBHs.

2. Materials and methods

2.1. Fish

Adult guppies (*Poecilia reticulate* Peters; males only), mollies (*Poecilia sphenops*) and zebrafish (*Danio rerio*) were obtained from commercial aquaculture farms in the Arava Valley, Israel. The fish were maintained in 100-L plastic tanks, at 25 ± 1.5 °C with light:dark cycle of 12:12 h, and were fed at 2% of their body weight once a day with a commercial fish feed (Ocean Nutrition, San Diego, USA). Water quality parameters were monitored once or twice a week. Serum samples were obtained from barramundi (*Lates calcarifer)*, catfish (*Clarias gariepinus*) and rabbitfish (*Siganus rivulatus*), maintained at experimental aquaculture facilities (Ben-Gurion University and the National Center for Mariculture, Eilat, Israel).

2.2. Preparation of whole body homogenates and sampling of blood

Whole body homogenates (WBH) were prepared from guppies, mollies and zebrafish. Anaesthetized fish (clove oil, 200 ppm) were weighed and dissected to remove the gut, gall bladder and tail fin; the remaining body was then frozen at −80 °C. Frozen fish were ground using a mortar and pestle filled with liquid nitrogen. Liquid nitrogen was constantly added, and the frozen powder carefully transferred to 2 ml tubes. Immediately after homogenization, cold PBS (0.05 M, pH 6.2) was added (2 ml per gram of fish weight), and samples were centrifuged for 30 min at 16000 × *g* at 4 °C. The supernatant was carefully removed with a glass Pasteur pipette, avoiding the overlying lipid layer, and stored in aliquots at −80 °C.

Blood was withdrawn from the caudal vein of 24 h-starved, anaesthetized barramundi, catfish and rabbitfish. Samples were centrifuged at 2000 × *g* for 15 min at 4 °C, serum was collected and stored in aliquots at −80 °C until further use. Serum and WBH samples were thawed on ice prior to analysis, and due attention was given to sample storage in cold conditions in order to preserve the complement activity.

2.3. Analyses of complement activity (ACH50 assay)

Complement activity was analysed in WBH and serum samples using both sheep and rabbit red blood cells (abbreviated as SRBC and RRBC, respectively) following the method of Sunyer and Tort (Oriol Sunyer and Tort, 1995) with some modifications. For negative control, five samples of guppy WBH and rabbitfish serum were heat inactivated at 56 °C for 30 min. Individual 100 µl aliquots of serially diluted serum/WBH in working buffer (gelatin veronal buffer, EGTA Mg^{2+} GVB; dilutions ranging from 1:16 to 1:1024) were mixed with 30 µl of red blood cells (4×10^8 cells ml^{-1} and 2.5×10^8 cell ml^{-1} for analyses using serum and WBHs, respectively). For 100% lysis, double distilled water (DDW) was used, and for blank, red blood cells were similarly mixed with the working buffer. The reaction mixtures were incubated for 2 h at 24 °C with gentle shaking. The hemolytic reaction was stopped by adding 1 ml of stop solution (working buffer containing 10 mM EDTA). The mixtures were centrifuged at 1600 × *g* for 10 min at 4 °C. Lysis of red blood cells in the reaction was visually evaluated by the absence/presence and evident change in size of the RBC pellet in the tube. Finally 200 µl of the reaction supernatant was transferred to a 96 well plate and the optical density (OD) was measured at 414 nm using a Tecan Sunrise™ microplate reader (Salzburg, Austria). The reciprocal of the sample dilution inducing 50% lysis of RBCs was designated as the ACH50 titer (Oriol Sunyer and Tort, 1995). Results are presented as units per ml. The visual observation of the pellet prior to OD reading was only used as a conformation for the quantitative analysis that followed.

To evaluate the effect of freeze and thaw cycles on complement activity, an often necessary routine in experimental analyses, samples were frozen and thawed multiple times, followed by complement activity analysis. All samples were stored in a −80 °C freezer, and thawing was done in cold conditions, on ice. The percentage loss in activity upon every cycle of freeze-thaw was calculated, based on the value obtained for the same sample at time 0 (before freeze-thawing was applied).

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was carried out followed by a Tukey post hoc test to compare ACH50 levels between fish species. T-test was used to compare ACH50 levels when using RRBCs and SRBCs within fish species, and to compare reduction in activity following freeze-thawing to the time 0 values of the same species. Differences were considered significant at $p < 0.05$. All statistical tests were carried out with Sigma Stat (Systat Software Inc., San Jose, CA, USA).

3. Results and discussion

Substantial amount of complement activity was recorded in WBH of the different fish species. Hemolytic activity was confirmed by visual observation of the red blood cell pellet, including presence of intact RBCs, absence (suggesting complete lysis) or shrinkage of the pellet (suggesting partial lysis of RBCs; these observations are not shown). The assay was also verified by use of appropriate heat inactivated control samples, in which no RBCs lysis was observed. To rule out lysis by other proteases or by the classical and lectin complement pathways, the buffer used in this assay is supplemented with 0.01 M EGTA which chelates Ca^{2+}, thus preventing the activation of the classical complement pathway, and activation of Ca^{2+}-dependent proteases, that are known to be associated with membranes (Kirschfink and Mollnes, 2003; Mellgren, 1987). The added Mg^{2+} aids in the activation of the analysed alternative pathway (Forsgren and Quie, 1974; Okroj et al., 2012).

Comparing RBCs from sheep and rabbit revealed that higher ACH50 levels were obtained when RRBCs were used (Fig. 1). For serum samples, the levels were about two fold higher for RRBCs compared to SRBCs. These results are in line with earlier studies examining complement activity in the serum of other fish species, including tench (*Tinca tinca*), cod (*Gadus morhua*) and sea bream (*Sparus aurata*) (Magnadóttir, 2000; Oriol Sunyer and Tort, 1995; Pionnier et al., 2013).

When analyzing WBH with rabbit and sheep RBCs, differences in ACH50 levels were even greater than for serum samples. In molly, guppy and zebrafish the levels were 7, 28 and 4-fold higher, respectively, for RRBCs as compared to SRBCs (Fig. 1).

Sheep and rabbit RBCs differ in their membrane composition, as sheep blood cells have a layer of sialic acid that interferes with the lysis by the complement's membrane attack complex (Fearon, 1978; Okroj et al., 2012; Oriol Sunyer et al., 1998). In addition, glycosyl phosphatidylinositol proteins are anchored in SRBC's membrane, including CD59, and DAF (decay acceleration factor) that play a regulatory role in protection from autologous complement attack (Okroj et al., 2012; van den Berg et al., 1993). These factors, which are absent from RRBCs, can explain the reduced lysis of SRBCs.

Large variability in complement activity was evident between fish species, as it was significantly higher in the serum of rabbitfish and barramundi compared to catfish. Variability in complement activity was also evident between WBHs of different fish species, and even among batches of same fish species (*i.e.* guppy 1 and 2). Molly and