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ARTICLE *in* MOLECULAR IMMUNOLOGY · AUGUST 1993

Impact Factor: 2.97 · DOI: 10.1016/0161-5890(93)90017-6 · Source: PubMed

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Molecular Immunology, Vol. 30, No. 10, pp. 929–934, 1993
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INVOLVEMENT OF α_2-MACROGLOBULIN AND C-REACTIVE PROTEIN IN A COMPLEMENT-LIKE HEMOLYTIC SYSTEM IN THE ARTHROPOD, *LIMULUS POLYPHEMUS*

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(*First received* 7 *July* 1992; *accepted in revised form* 22 *December* 1992)

Abstract—Homologues of two plasma proteins of vertebrates, α_2-macroglobulin and C-reactive protein, participate in a hemolytic system of the ancient arthropod, *Limulus polyphemus*. C-reactive protein, which can under the appropriate circumstances activate the classical pathway of the mammalian complement system, is an essential element of the hemolytic system of *Limulus*. The selective removal of C-reactive protein from the plasma with phosphorylethanolamine–agarose inactivated hemolysis. Addition of affinity-purified C-reactive protein to inactive plasma restored activity. Exposure of plasma to phosphorylethanolamine in solution potentiated hemolysis. α_2-Macroglobulin is a member of the same protein family as the complement protein C3 and both require an intact thiol ester for activity. Treatment of *Limulus* plasma with methylamine under conditions that inactivate thiol-ester-containing proteins reduced the hemolytic activity of some plasma preparations. Addition of purified *Limulus* α_2-macroglobulin to the methylamine-treated plasma restored hemolytic activity. However, α_2-macroglobulin is not necessary for hemolysis since the hemolytic activity of some pooled plasma preparations was insensitive to methylamine treatment under conditions that inactivated α_2-macroglobulin. Purified C-reactive protein was hemolytic in the absence of α_2-macroglobulin. These observations suggest that the proteins in *Limulus* plasma that participate in hemolysis represent the components of an ancient invertebrate defense system with distant evolutionarily affinities to the vertebrate complement system.

INTRODUCTION

One of the important immune defense strategies employed by animals is to kill invading pathogenic organisms by inducing their cytolysis in the blood. In higher vertebrates, cytolysis is mediated by the complement system with its associated regulators and receptors (Law and Reid, 1988). The key factor in the mammalian complement system is the protein C3, which binds to the surfaces of target cells, marking them for destruction by cytolysis and phagocytosis. Binding involves the covalent bonding of a reactive internal thiol ester of the C3 molecule with hydroxyl and amino residues at the surface of the target particle (Law and Levine, 1977). C3 is a member of the α_2-macroglobulin family of proteins, based on peptide sequence homology and the presence of the reactive thiol ester (Tack, 1983). A recently-emergent topic in the complement field is the evolution of this complex defense system (Dodds and Day, 1992; Farries and Atkinson, 1991). Lower vertebrates show many but not all of the elements of the mammalian system (Dodds and Day, 1992). Invertebrates also have plasma- or hemocyte-based cytolytic systems (Bertheussen, 1983; Canicatti and Cuilla, 1987, 1988; Cenini, 1983; Komano and Natori, 1985; Phipps *et al.*, 1989; Tucakková *et al.*, 1986), but none had been convincingly demonstrated to be related to the vertebrate complement system until Enghild *et al.* (1990) observed that the form of α_2-macroglobulin found in the blood of the American horseshoe crab *Limulus polyphemus* (Armstrong and Quigley, 1987; Armstrong *et al.*, 1985; Quigley and Armstrong, 1983, 1985; Quigley *et al.*, 1991) is a component of the plasma-based cytolytic system of that animal. Based on the molecular similarity between α_2-macroglobulin and C3 and the involvement of the thiol ester in the activities of both molecules, these results are consistent with the possibility that, in *Limulus*, α_2-macroglobulin serves a function like that of C3 in vertebrates.

We have confirmed these observations, but suggest that the involvement of α_2-macroglobulin in the hemolytic system of *Limulus* may differ in significant respects from that of C3 in the mammalian complement system. In addition, we have demonstrated that C-reactive protein is an essential participant in the hemolytic system of *Limulus*. C-reactive protein that has been activated by appropriate ligands (e.g. C-polysaccharide or polyanions) has been shown to activate the complement system in mammals (Miyazawa and Inoue, 1990; Volanakis, 1982; Volankis and Kaplan, 1974). Its involvement in the cytolytic system of *Limulus* strengthens the notion that the cytolytic system of this invertebrate shows evolutionary affinities with the vertebrate complement system.

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Abbreviations: PEG, polyethylene glycol-8000.

MATERIALS AND METHODS

Plasma was obtained from freshly-collected adult *Limulus* under conditions that avoided exocytosis of the blood cells, as described previously (Armstrong, 1985b). Animals were released into the ocean unharmed after bleeding. Hemocyanin was removed by ultracentrifugation at 120,000 *g* for 16 hr or by precipitation with 3% polyethylene glycol-8000 (PEG) with centrifugation at 30,000 *g*, 0.5 hr. C-reactive protein was removed from the plasma by exposure to phosphorylethanolamine–agarose (0.1 volumes of resin per volume of plasma) (Sigma Chemical Co., St. Louis, MO). C-reactive protein was purified by passage of plasma depleted in hemocyanin by treatment with 3% PEG over a column of Sepharose 4B (0.2 volumes of resin per volume of plasma) equilibrated with Ca-saline (0.9% NaCl, 10 mM $CaCl_2$, 20 mM Tris, pH 7.3). The Sepharose-passaged plasma was then exposed to phosphorylethanolamine–agarose (0.03 volumes of resin per volume of plasma) with stirring (3–5 hr, room temp). Exposure to the resin in bulk suspension was utilized because C-reactive protein precipitated onto the resin bed when plasma was passed over phosphorylethanolamine–agarose packed in a column. The phosphorylethanolamine–agarose resin was then transferred to a column and washed with several column volumes of Ca-saline, then with Ca-saline with 1 M NaCl, and was eluted with 0.1 M citrate buffer, pH 6.7. The C-reactive protein in the eluate was precipitated with 10% PEG and the precipitate redissolved in Ca-saline. When analyzed by SDS–polyacrylamide gel electrophoresis the C-reactive protein was free of other plasma components and showed either a close-space dimer or a single broad band at about 24 kDa (Fig. 2, lane 6). The extinction coefficient of *Limulus* C-reactive protein was 13.9, as determined by amino acid analysis using norleucine to correct for loss during hydrolysis. α_2-Macroglobulin was purified from *Limulus* plasma as described previously (Armstrong *et al.*, 1991; Sottrup-Jensen *et al.*, 1990) and yielded a preparation with a single protein band in SDS–polyacrylamide gel electrophoresis (reducing conditions) at 185 kDa (Fig. 2, lane 5). Purification of both proteins was conducted at room temperature. Protein concentrations were determined by the optical absorption at 280 nm, using extinction coefficients (1%, 1 cm) of 13.9 for C-reactive protein and 11.7 for *Limulus* α_2-macroglobulin (Enghild *et al.*, 1990).

The hemolytic activity of the plasma was determined in duplicate or triplicate samples using sheep red blood cells (Gee, 1983; Kabat and Mayer, 1961; Sim, 1981). Unactivated sheep erythrocytes in Alsevers solution were obtained from Cappel, West Chester, PA (reference number 55875) and Becton Dickinson and Company, Cockeysville, MD (reference number 12388). The reaction mixtures contained 3×10^7 washed sheep red cells in a final volume of 800 μl. The buffer system was DGVB (71 mM NaCl,0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose, 0.1% gelatin, 2.5 mM sodium barbital, pH 7.3). The samples were incubated at 22–23°C for 4 hr, and the reaction was terminated by adding 2 ml of ice cold phosphate-buffered saline containing 5 mM ethylenediaminetetraacetic acid, followed by centrifugation to remove the red cells. The extent of hemolysis was determined by monitoring released hemoglobin in the supernatant by the optical absorbance at 412 nm and was compared to full hemolysis produced by hypotonic lysis of the red cells.

RESULTS

Hemocyanin-depleted *Limulus* plasma showed a dose-dependent hemolytic activity (Fig. 1). The red cells were not sensitized by pretreatment with anti-erythrocyte antisera for these experiments. Hemolysis was Ca^{2+}-dependent, since EDTA and Mg^{2+}–EGTA abolished

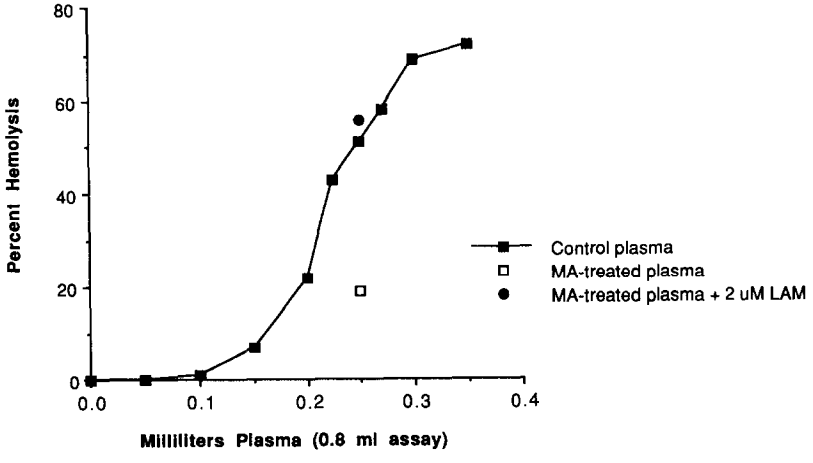

Fig. 1. Hemolytic activity of *Limulus* plasma that was depleted in hemocyanin by ultracentrifugation (120,000 *g*, 16 hr). Untreated plasma (■). Methylamine-treated plasma (□) shows reduced hemolytic activity, which can be restored by the addition of 2 μM purified *Limulus* α_2-macroglobulin (●). For reasons that are not understood, preparations that contain plasma from several horseshoe crabs retain hemolytic activity even after the α_2-macroglobulin has been inactivated by methylamine (data not shown) whereas single-animal plasma preparations, as shown in figure, are sensitive to methylamine.

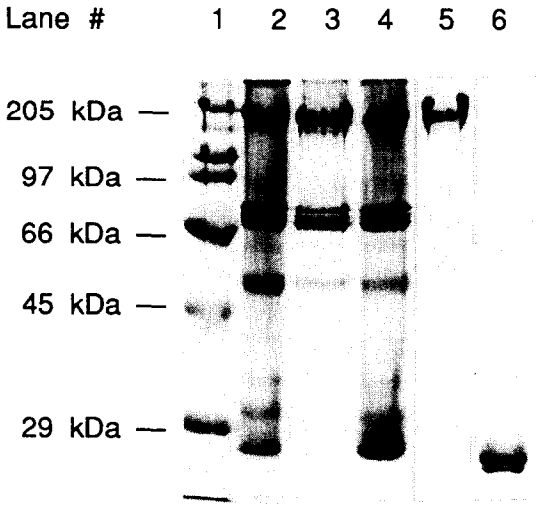

Fig. 2. SDS–polyacrylamide gel electrophoresis (reducing conditions: samples treated with 5%β-mercaptoethanol) of 3% PEG supernatant of *Limulus* plasma (lane 2 = untreated plasma; lane 3 = plasma exposed to phosphorylethanolamine–agarose; lane 4 = plasma exposed to plain agarose) and purified *Limulus* α_2-macroglobulin (lane 5) and *Limulus* C-reactive protein (lane 6). The molecular weight standards are contained in lane 1. Lane 2 contained 11.7 μl of *Limulus* plasma, lane 5 contained 5.8 μg of α_2-macroglobulin, and lane 6 contained 4 μg of C-reactive protein.

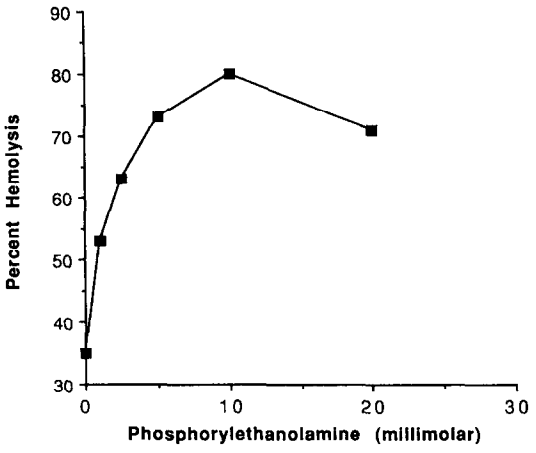

Fig. 3. Phosphorylethanolamine activates the hemolytic activity of *Limulus* plasma. A 1 M solution of phosphorylethanolamine buffered to pH 6.8 with 1 M Tris base was added to an 0.8 ml reaction mixture to give the indicated final concentration of phosphorylethanolamine and incubated for 5 min with plasma before red blood cells were added. All samples contained 100 μl of *Limulus* plasma that had been cleared of hemocyanin by ultracentrifugation. An equivalent amount of 1 M Tris, pH 7.5 failed to activate plasma. Phosphorylethanolamine applied in the absence of plasma failed to elicit hemolysis.

hemolysis (data not shown). The three most abundant proteins of the hemocyanin-depleted fraction of plasma were α_2-macroglobulin (Fig. 2, lane 2, 185 kDa) (Quigley and Armstrong, 1985), hemocyanin (Fig. 2, lane 2, 70 kDa), and C-reactive protein (Fig. 2, lane 2, 24 kDa) (Nguyen *et al.*, 1986; Robey and Liu, 1981). α_2-Macroglobulin is present in *Limulus* plasma at 1–2 mg/ml (Enghild *et al.*, 1990) and C-reactive protein at 1-5 mg/ml (Nguyen *et al.*, 1986). An involvement of α_2-macroglobulin in hemolysis was demonstrated by the reduction in the hemolytic titer of plasma following treatment with methylamine (Fig. 1). The conditions of treatment eliminated greater than 95% of the activity of α_2-macroglobulin, as assayed by the ability of α_2-macroglobulin to bind trypsin (Armstrong *et al.*, 1985). Hemolytic activity was restored by the addition of purified *Limulus* α_2-macroglobulin (Fig. 1; Fig. 2, lane 5). Methylamine-treated *Limulus* α_2-macroglobulin was unable to restore the hemolytic activity of methylamine-treated plasma (Table 1). These results confirm observations reported previously (Enghild *et al.*, 1990). Interestingly, although preparations containing plasma from single horseshoe crabs (e.g. unpooled samples) were sensitive to methylamine treatment, two pooled plasma preparations that contained, respectively, plasma from 21 and nine horseshoe crabs retained hemolytic activity even after the α_2-macroglobulin has been inactivated by methylamine (data not shown).

The *Limulus* homologue of C-reactive protein appears to play an essential role in hemolysis. Treatment of plasma with the C-reactive protein reactant, phosphorylethanolamine, resulted in a dose-dependent elevation of hemolytic activity (Fig. 3). Removal of C-reactive protein with phosphorylethanolamine–agarose (Fig. 2, lane 3) significantly reduced activity (Fig. 4). Activity was restored to the phosphorylethanolamine–agarose-treated plasma by the addition of purified C-reactive protein (Fig. 4; Fig. 2, lane 6).

Purified C-reactive protein was hemolytic in the absence of other components (Fig. 5). The hemolytic activity of purified C-reactive protein was maximal at an

Table 1. Role of α_2-macroglobulin in hemolysis

Treatment of plasma[a] (μl)	α_2-Macroglobulin (μM)	Treatment of α_2-macroglobulin	Hemolysis (%)
Untreated	0.0	—	60
Methylamine[b]	0.0	—	39
Methylamine	2.0	Untreated	61
Methylamine	2.0	Methylamine[b]	38

[a]All samples contained 250 μl of unpooled *Limulus* plasma in a total assay of 800 μl.

[b]Samples were treated for 20 hr with 0.2 M methylamine, pH 8.0, 25°C.

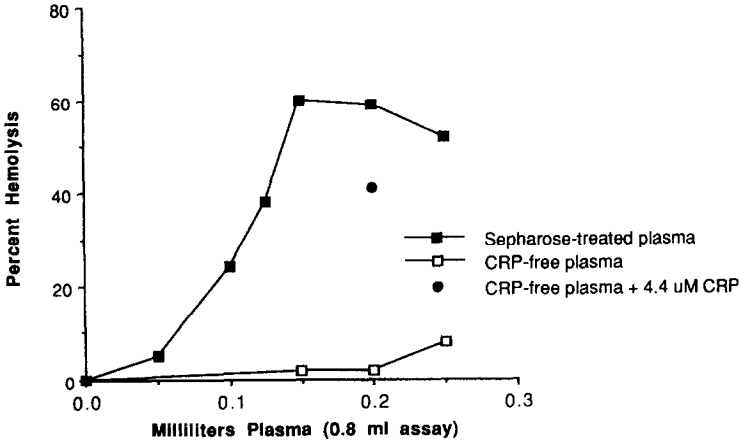

Fig. 4. Removal of C-reactive protein from ultracentrifuged *Limulus* plasma by passage over phosphorylethanolamine–Sepharose reduces its hemolytic activity. Ultracentrifuged plasma passed over unconjugated Sepharose 4B (■) was strongly hemolytic whereas phosphorylethanolamine–Sepharose-treated plasma (□) showed reduced activity. Phosphorylethanolamine–agarose removes C-reactive protein (Fig. 2, lane 3). Addition of 4.4 μM C-reactive protein (●) restored activity.

ionic strength of 0.15–0.2 M and was reduced at lower ionic strength (data not shown). The hemolytic activity of purified C-reactive protein was unaffected by treatment with methylamine (Table 2).

DISCUSSION

Limulus has long been known to possess a plasma-based ability to lyse foreign cells (Day *et al.*, 1970; Enghild *et al.*, 1990; Noguchi, 1903). In mammals, the principal plasma-based cytolytic pathway is mediated by complement, which consists of 20 or more plasma proteins plus another 40 or more receptor and regulatory proteins (Kinoshita, 1991; Law and Reid, 1988). The possibility that invertebrates possess isolated elements of the vertebrate complement system has been suggested previously (Bertheussen, 1982, 1983; Day *et al.*, 1970, 1972), but the demonstration that α_2-macroglobulin and C-reactive protein participate in hemolysis in *Limulus* is the first direct identification of an involvement of components related to elements of the vertebrate complement system in an invertebrate cytolytic system.

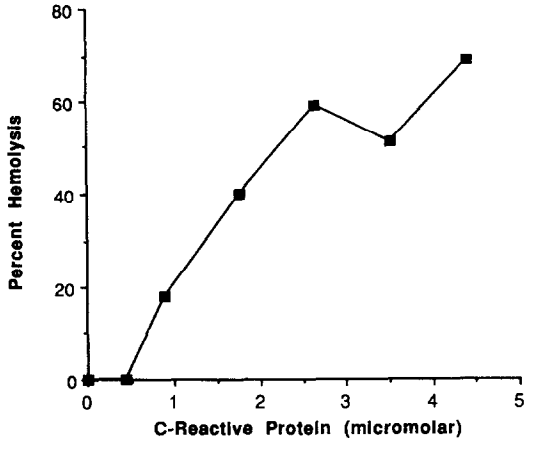

Fig. 5. Hemolytic activity of purified *Limulus* C-reactive protein. Hemolysis was conducted in the presence of 0.19 M NaCl, 0.01 M $CaCl_2$ and in the absence of other plasma components. The only added protein was purified *Limulus* C-reactive protein.

However, important differences between complement-mediated hemolysis and hemolysis in *Limulus* are readily apparent. In the complement system, C3 is absolutely required, whereas in *Limulus* the C3 homologue, α_2-macroglobulin, is dispensable. While inactivation of α_2-macroglobulin reduced the hemolytic activity of unpooled plasma samples, for reasons that are not clear, the hemolytic activity of two independent pooled plasma samples was unaffected by methylamine treatments that inactivated the α_2-macroglobulin present. α_2-Macroglobulin has been reported to be the only methylamine-binding protein in the plasma of *Limulus* (Dodds, personal communication; Enghild *et al.*, 1990), and the hemolytic activity of methylamine-inactivated plasma was restored by addition of purified *Limulus* α_2-macroglobulin (Fig. 1), so an involvement of α_2-macroglobulin is indicated. However, our results suggest that the role is not the obligatory role that is exercised by C3 in the mammalian complement system.

C-reactive protein is an activator of the complement system of mammals, but not a direct participant in the

Table 2. The hemolytic activity of C-reactive protein[a] is not affected by methylamine

C-reactive protein (μM)	Treatment of C-reactive protein	Hemolysis (%)
0.0	—	2
2.0	None	39
2.0	Methylamine[b]	36

[a]Conditions of reaction were the same as for Fig. 5.
[b]Samples were treated for 20 hr with 0.2 M methylamine, pH 8.0, 25°C.

process of complement-mediated cytolysis (Gewurz, 1982; Jiang *et al.*, 1991; Kushner, 1989; Kushner *et al.*, 1992; Miyazawa and Inoue, 1990; Volanakis, 1982). In *Limulus*, C-reactive protein appears to play a more central role in cytolysis.

Phosphorylethanolamine, a ligand that specifically binds C-reactive protein, potentiated the hemolytic activity of plasma (Fig. 3), presumably by activating C-reactive protein. Removal of C-reactive protein from plasma profoundly depressed its hemolytic activity (Fig. 4). Purified *Limulus* C-reactive protein could effect hemolysis in the absence of other plasma proteins (Fig. 5).

In this and most other investigations of plasma-based cytolytic defense systems, the sheep red cell is used as a convenient foreign cell for the assay of cytolysis. The principal physiological function of the complement system in mammals and the cytolytic system in *Limulus* is presumably to destroy bacteria and other invading pathogens, not sheep red cells. *Limulus* blood has been reported to have bactericidal activity [reviewed in Armstrong (1985a, 1991)]. It will be important to learn whether the plasma-based cytolytic system of *Limulus* described in the present communication does indeed contribute to this bacteriolytic activity. In addition to a direct cytolytic activity, the mammalian complement system has opsonic activity that destroys foreign cells by promoting their phagocytosis by monocytes and macrophages. It will also be interesting to learn whether the plasma-based hemolytic system functions similarly to enhance the phagocytic activity of the *Limulus* blood cell (Armstrong and Levin, 1979).

Acknowledgements—This research was supported by Grant No. GM35185 from the National Institutes of Health. We thank Dr Berhane Ghebrehiwet for a critical reading of the manuscript.

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