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Interactions between earthworm hemolysins and sheep red blood cell membranes

Philippe Roch, Calogero Canicatti * and Pierre Valembois

Département de Physiologie des Invertébrés-URA CNRS 1138, Université de Bordeaux I, Institut de Biologie Animale, Talence (France)

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The hemolytic activity exhibited by the coelomic fluid of the Annelid *Eisenia fetida andrei* is mediated by two lipoproteins of mass 40 and 45 kDa, each of them capable of hemolysis. Such an activity is not inhibited by zymosan, inulin or lipopolysaccharide (LPS), nor by hydrazine or methylamine, suggesting that earthworm hemolysins are not related to C3 or C3b complement components. Among the membrane lipids tested (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin and cholesterol) only spingomyelin inhibited hemolysis. The analysis of *E.f. andrei* proteins bound to sphingomyelin microvesicles, as well as to sheep red blood cell (SRBC) membranes, revealed a polymerization of *E.f. andrei* 40 kDa and/or 45 kDa hemolysins. Consequently, sphingomyelin appears a likely candidate for hemolytic complex receptor. Electron microscopy observations suggested that the polymerization causes an open channel through the lipid bilayer. As demonstrated using metal ions, heparin, chondroitin sulfate, poly(L-lysine) and protamine chloride, the mode of action of earthworm hemolytic complex is not analogous to that of C9 or perforine.

Introduction

Pointing out the fact that invertebrate hemolysins and complement have similar target cells, some authors have hypothesized a phylogenetic relationship between both systems [1–3]. In a few cases, the existence of a complement-like activity was deduced from experimental investigations using complement inhibitors [4]. However, concerning the hemolytic system of the echinoderm *Holothuria polii*, zymosan, inulin and LPS, known to link the C3b and consequently activating the complement alternative pathway [5], had no effect. Nucleophils, such as hydrazine and methylamine, were also ineffective [6]. In mammals, these nucleophils dissociate the internal thioester bond of the complement key component C3, leading to a loss of functional activity [7,8]. In the absence of precise molecular data, such as the knowledge of the complete amino-acid sequence of the active molecules, invertebrate lytic activities must be considered to be mediated by original factors not related to the complement system.

In the Annelid Oligochaeta *Eisenia fetida andrei*, the coelomic fluid exhibits a hemolytic activity directed against various mammalian erythrocytes [9,10]. The hemolytic activity is mediated by two lipoproteins of molecular mass 40 and 45 kDa, released by the chloragogue cells [11]. These molecules are also capable of bacteriostatic activity directed against earthworm pathogenic bacteria [12,13] and they are involved in the clotting of coelomic fluid [14]. They have been extensively studied both at biochemical and genetical levels [11,15] but only little is known about their mechanism of action.

The prescot experiments were undertaken to determine the interactions between *E.f. andrei* hemolysins and SRBC membranes. Various molecules and metal ions known to act on the complement system were tested and electron microscope observations of lysed membranes were performed.

* Present address: Dipartimento di Biologia, Universita degli Studi di Lecce, Italy.

Abbreviations: SRBC, sheeped blood cell; LPS, lipopolysaccharide; HF, hemolytic fraction; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HMWP, high-molecular-weight protein; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing.

Correspondence: P. Roch, Département de Physiologie des Invertébrés, Université de Bordeaux, 1-UA CNRS 1138, Institut de Biologie Animale, Avenue des Facultés, 33405 - Talence Cédex, France.

Material and Methods

Earthworms

Oligochaeta, Lumbricidae, *Eisenia fetida andrei*, were washed then submitted to 5 V electric stimulations provoking the extrusion of the coelomic fluid throughout the dorsal pores [10]. The coelomic fluid from 50–100 earthworms was pooled, centrifuged for 5 min at 11 000 $\times g$ then the supernatant submitted to chromatography. Partial purification of hemolysins was achieved by gel filtration on Superose 12 (Pharmacia) as previously described [16,17]. After salt elimination by ultrafiltration on an Amicon PM 10 membrane, the protein content of the hemolytic fraction (referred to as HF) was adjusted to 0.5 mg/ml. All the experiments reported here were performed with HF as activity source in preference to more purified material whose hemolytic activity was partially lost. No change in the protein content nor loss of activity (hemolysis, antibacterial, agglutination) were observed during the storage of HF at $-20\,^{\circ}C$.

Hemolytic assays

Hemolysis activity test was carried out in 100 µl of *E.f. andrei* HF mixed with 100 µl ($6 \cdot 10^7$ cells) of SRBCs. The samples were incubated for 10 min at 20 °C then the reaction stopped by adding 1 ml phosphate-buffered saline (Dulbecco's PBS, 0.05 M, pH 7.2) and the samples centrifuged for 10 min at 400 $\times g$. The hemoglobin content of the supernatant was determined by spectrophotometry at 541 nm. The percentage of hemolytic activity was calculated from the following formula: 100 × (absorbance in test − background of absorbance)/ (absorbance in 100% hemolysis − background of absorbance). The background of absorbance consisted of the absorbance of the supernatant of SRBC incubated with PBS alone. Maximum hemolysis (100%) was determined after osmotic lysis of SRBCs in distilled water.

Inhibition assays

HF was incubated for 30 min at 20 °C with various inhibitors prior testing its hemolytic activity. To take into account the dilution due to inhibitor addition, the hemolytic activity of untreated samples was determined in diluted (1 : 2) HF.

(1) Complement inhibitors. 1 ml of HF was incubated with 1 ml of PBS containing 10 or 20 mg/ml of zymosan, inulin or LPS from *Escherichia coli* (Sigma). Reaction mixtures were then centrifuged for 10 min at 11 000 $\times g$ and the supernatant tested. For hydrazine and methylamine (Sigma), 1 ml of different concentrations (6.25, 12.5, 25, 50 and 100 mM) were incubated with 1 ml of HF. They were then extensively dialysed against PBS before the hemolytic activity test.

(2) Phospholipids and cholesterol. Lipid microvesicles were obtained by mechanically stirring 1 mg of commercial pure phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, spingomyelin or cholesterol (Sigma) with 1 ml of PBS. Inhibition assays were performed incubating 0.5 ml of such microvesicle preparations with 0.5 ml of HF. Microvesicles were then pelleted by 5 min centrifugation at 11 000 $\times g$ and the supernatants tested for hemolytic activity.

(3) C9 and perforin inhibitors. C9 and perforin inhibitors were assayed mixing 100 µl of HF with 100 µl of different concentrations (0.6, 1.25, 2.5, 5 and 10 mg/ml) of heparin or chondroitin sulfate A (Sigma) in PBS or with 100 µl of poly(L-lysine) HBr salt or protamine chloride (Sigma) in PBS at concentrations ranging from 0.06 to 1 mg/ml.

(4) Neutralization of lysine and arginine. Neutralization was carried out according to Tschopp and Masson [19]. Briefly, 1 ml of HF was dialysed against 0.2 M sodium borate buffer containing 50 mM EDTA (pH 8.5), then incubated with 2, 4 or 8 mM diketene (Fluka) in 1 ml dialyse buffer. The reaction was stopped by overnight dialysis against 0.2 M carbonate/bicarbonate buffer (pH 8.5).

(5) Metal ion treatment. 1 ml of $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeSO_4$, $CuCl_2$, $CdCl_2$, $HgCl_2$ and $ZnSO_4$ (Merck) solutions at final concentrations of 0.03, 0.06, 0.1, 0.2, 0.5, 1, 2 and 4 mM were incubated with 1 ml of HF.

(6) Pre-incubation with SRBC ghosts. Ghost preparation consisted in washing 10^6, 10^7 and 10^8 SRBCs with large volumes of distilled water until the red color of hemoglobin disappeared. The ghosts were then pelleted by 5 min centrifugation at 11 000 $\times g$, and incubated in HF (v/v). The hemolytic activity of the supernatants was determined after centrifugation at 11 000 $\times g$.

(7) Interaction with sphingomyelin and with SRBCs. Aliquots of diluted (1 : 5) HF were labeled with 1 mCi ^{125}I (Amersham) according to MacConahey and Dixon [20] then extensively dialysed against PBS. 0.5 ml of ^{125}I-labeled HF (^{125}I-HF) was incubated with 0.5 ml of 1 mg/ml sphingomyelin microvesicles or with 0.5 ml of $6 \cdot 10^8$ SRBC/ml. The mixtures were centrifuged for 5 min at 11 000 $\times g$ and the pellets were washed three times in 50 mM EDTA. They were then dissolved in 100 µl electrophoresis sample buffer containing 2% SDS and 5% β-mercaptoethanol, boiled for 5 min and analyzed in 15% SDS-PAGE.

SDS-PAGE analysis

Electrophoretic analysis was carried out under denaturing conditions as previously described [16]. Gels of 0.75 mm thickness were silver stained according to Merill et al. [21] with slight modifications. With radioactive samples, the gels were dried and exposed to Kodak X-Omat film for 48 h at $-70\,^{\circ}C$.

Immunoblot analysis

Western blotting of SDS-PAGE was performed with the semi-dry Nova-Blot LKB apparatus on Hybond-C membrane (Amersham). 1% gelatin in the presence of 0.3% Tween 20 was used as a blot-quenching agent. Detection occurred with 1/400 rabbit antiserum prepared against *E.f. andrei* hemolysins. The specificity of such an antiserum has been controlled by both Western labeling and immunoprecipitation of only the 40 and 45 kDa hemolysins among coelomic fluid, HF and in vitro total mRNA translation. Rabbit-Ig binding was revealed with 1/500 peroxidase-labeled goat Fab anti-rabbit Ig (Biosys) using H_2O_2-diaminobenzidine (Serva) as substrate.

TEM observations

SRBC membranes were prepared by incubating a pellet of 40 µl of packed SRBCs with 0.5 ml of HF at 30°C for 5 min. After centrifugation for 3 min at 11000 × g, a drop of suspension taken at the pellet-supernatant interface was layered on collodion-carbon-coated grids previously dipped in 0.1% poly(L-lysine) solution. The excess of liquid was removed after 5 min and the grids were treated for negative staining by 2 min floating upside down on 2% disodium phosphotungstate (pH 7.0). Observations were made at 80 kV on a Philips 201 electron microscope. Magnifications were calibrated with catalase crystals (Agar Aids) of 8.75 and 6.85 nm lattice spacing.

Results

Effect of complement inhibitors. Concentrations of 10 or 20 mg/ml of zymosan particles, inulin or LPS did not decrease the *E. f. andrei* hemolytic activity. Similar absence of inhibition was obtained with 100 mM of both hydrazine (hemolytic activity of 69.4 ± 3.6%) and methylamine (62.2 ± 2.3%), compared to 69.4 ± 3.6% obtained in untreated HF. The lower concentrations of 50, 25, 12.5 and 6.25 were also inactive (data not shown).

Effect of cholesterol and some membrane phospholipids. Fig. 1 shows that only spingomyelin induced a potent inhibition (75%), while cholesterol and the other phospholipids tested failed to inhibit hemolysis. Such inhibition could be the result of hemolysins trapped on sphingomyelin microvesicles representing artificial membrane. SDS-PAGE analysis of proteins bound to sphingomyelin microvesicles revealed the presence of two proteins of molecular mass 40 and 45 kDa (Fig. 2). The presence of a new high-molecular-weight protein (HMWP), penetrating the 7% top gel but not the 15% separating gel and totally absent from coelomic fluid or HF, was also noted. A similar pattern was observed when ^{125}I-HF was used. In Western blots, the two proteins of molecular mass 40 and 45 kDa and the HMWP were labeled by the specific anti-hemolysins antiserum (Fig. 2).

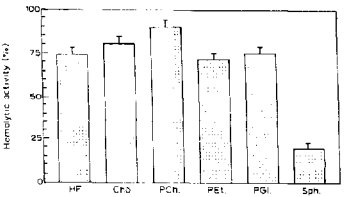

Fig. 1. Effect of cholesterol and various membrane phospholipids on the hemolysis of SRBC induced by *E.f. andrei* hemolytic fractions. Abbreviations: HF, hemolysis induced by untreated hemolytic fraction; Cho, cholesterol; PCh, phosphatidylcholine; PEt, phosphatidylethanolamine; PGl, phosphatidylglycerol; Sph, sphingomyelin. Bars represent S.E. on arithmetic mean values from triplicates.

Effect of SRBC ghosts. Pre-incubating HF with 10^6 SRBC ghosts did not modify the hemolytic activity (75.5 ± 2.1%). In contrast, the ghosts from 10^7 and 10^8 SRBCs markedly decreased the hemolytic activity to 18.6 ± 2.1% and 5.2 ± 2.1%, respectively (Fig. 3).

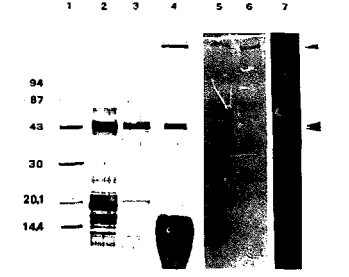

Fig. 2. SDS-PAGE analysis of HF proteins interacting with SRBC membranes and sphingomyelin microvesicles. Lane 1, Kit of molecular weight standards (14400: lalalbumin; 20100: trypsin inhibitor; 30000: carbonic anhydrase; 43000: ovalbumin; 67000: albumin; 94000: phosphorylase *b*) Lane 2, Total coelomic fluid protein content. Lane 3, HF obtained by FPLC gel filtration of total coelomic fluid. Lanes 4 and 5, HF proteins bound to sphingomyelin microvesicles. Lanes 6 and 7, HF proteins bound to SRBC membranes. Proteins were revealed by silver staining (1–4) or autoradiography using ^{125}I-HF proteins [7], and on Western blots incubated with specific anti-hemolysins antiserum revealed with the peroxidase-DAB system [5,6]. Note the presence of the two hemolysins of molecular mass 40 and 45 kDa (arrows) and the new HMWP (top arrow) on 4–7. The 70 kDa band in 5 and 6 represents β-mercaptoethanol artefact frequently found in a track even without a sample.

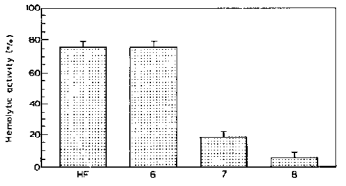

Fig. 3. Hemolytic activity of *E.f. andrei* HF (HF) compared to the hemolytic activity of HF previously incubated with the ghosts from 10^6 (6), 10^7 (7) and 10^8 (8) SRBCs. Symbols defined as in Fig. 1.

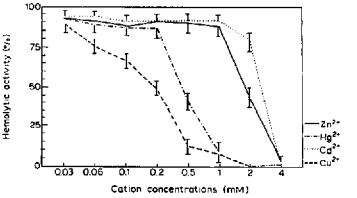

Fig. 4. Hemolytic activity of *E.f. andrei* HF in the presence of various concentrations of $CuSO_4$, $HgCl_2$, $ZnSO_4$ and $CdCl_2$. Symbols defined as in Fig. 1.

To check whether the ghost inhibition was the result of hemolysin binding on SRBC, an analysis in SDS-PAGE was conducted on SRBC membranes. To exclude the SRBC proteins we used ^{125}I-HF. As shown in Fig. 2, a strong HMWP band which failed to penetrate the 15% gel was detected. Using Western immunoblotting, this HMWP band was labeled by the specific anti-hemolysins antiserum (Fig. 2).

Effects of metal ions. As shown in Table I, 4 mM of Cu^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+} totally suppressed the hemolysis. The 4 mM of Fe^{2+} ions produced a noticeable decrease (about 35%) of the hemolytic activity. In contrast, Ca^{2+}, Mg^{2+} and Mn^{2+} were inactive whatever the concentration tested (from 0.03 to 4 mM). Among the inhibitory metal ions, an inhibition of about 50% was observed in the presence of 0.2 mM Cu^{2+} or 0.5 mM Hg^{2+} (Fig. 4). At 1 mM, Zn^{2+} and Cd^{2+} were not inhibitors. However, the metal ions' inhibitory effect was due to protein precipitation, visually observed when adding Cu^{2+}, Hg^{2+}, Cd^{2+} or Zn^{2+} solutions. The precipitates, collected by $11\,000 \times g$ centrifugation, resuspended in distilled water and submitted to SDS-PAGE analysis, revealed the same protein pattern as control HF.

TABLE I

Effect of 4 mM metal ions on hemolysis

Values presented are arithmetic means from triplicates.

Metal ions	Hemolytic activity (%)
Untreated HF	89.1
Ca^{2+}	89.9
Mg^{2+}	91.8
Mn^{2+}	80.8
Fe^{2+}	53.4
Cu^{2+}	0
Hg^{2+}	0
Cd^{2+}	0
Zn^{2+}	0

Effect of glycosaminoglycans and positively charged peptides. Heparin and chondroitin sulfate A were used to investigate possible inhibitory effect of sulfated glycosaminoglycans on the hemolytic activity. As shown in Table II, these two substances, even at high concentration (10 mg/ml), did not modify the hemolytic activity ($71.3 \pm 2.1\%$ for heparin and $72.2 \pm 2.1\%$ for chondroitin sulfate A compared to $76.0 \pm 2.1\%$ for untreated

TABLE II

Effect of glycosaminoglycans and positively charged peptides

Values presented are arithmetic means from triplicates.

Glycosaminoglycan (mg/ml)	Hemolytic activity (%)
Untreated HF	76.0
Heparin	
0.6	73.2
1.25	75.3
2.5	73.9
5	70.3
10	71.3
Chondroitin sulfate A	
0.6	73.1
1.25	74.6
2.5	76.4
5	73.6
10	72.2
Protamine	
0.06	74.8
0.12	73.1
0.25	74.2
0.5	73.6
1	74.4
Poly(L-lysine)	
0.06	71.8
0.12	72.3
0.25	68.7
0.5	70.3
1	70.7

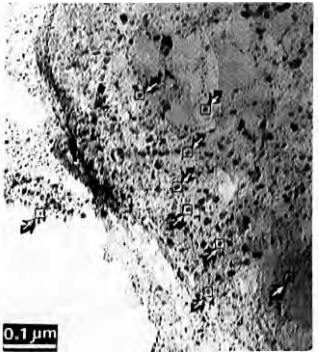

Fig. 5. Ultramicroscopical aspect of SRBC membranes treated with HF. Fragments of ghosts were taken at the bottom-supernatant interface of a centrifuged suspension of SRBC incubated with HF. They were layered on a collodion carbon-coated grid and negatively stained with 2% sodium phosphotungstate. The surface of the ghosts appeared as closely dotted with round deposits of phosphotungstate of diameter 5–20 nm. Most of these deposits were surrounded by a clear ring (arrows).

HF). Similarly, the addition of highly positively charged peptides, protamine and poly(L-lysine), failed to induce inhibitory effect on the hemolysis even at concentrations of 1 mg/ml.

Effect of diketene. In order to control whether lysine or arginine could represent amino acids of functional importance in hemolysins, the HF was treated with diketene, known to specifically neutralize the positive charges of such groups. None of the concentrations used, which ranged from 2 to 8 mM, modified the hemolysis (data not shown).

TEM observations of lysed SRBC. As shown in Fig. 5, lesions of the erythrocyte membrane apepared as more-or-less regular round-shaped deposits of phosphotungstate with diameters of 5–10 nm. Exceptionally, larger deposits (more than 20 nm) of irregular shape could he observed. Round deposits of 10 nm could be seen edged by a clear ring of 1.5 ± 0.5 nm. The lesions were generally very numerous with more than 10000 per square micrometer.

Discussion

The hemolytic system of *E.f. andrei* is composed of two lipoproteins of molecular mass 40 and 45 kDa, one of which is present in several isoforms [16]. The two lipoproteins are capable of lysing cell membranes, as revealed by SRBC overlaying analytical IEF gel [10]. We demonstrated here that hemolysis is not inhibited by zymosan, inulin or LPS, known to trap the C3b complement component [5]. In *E.F. andrei*, also as in other invertebrates [6], hemolytic activity is not affected by nucleophilic molecules, such as hydrazine and methylamine which open the thioester bond of C3 [22,23]. Consequently, earthworm hemolytic activity is not mediated by a molecule similar to the C3 or C3b complement component. In contrast to numerous enzymes, the hemolytic molecules present a kinetic curve of SRBC hemolysis which does not change between 4 and 45°C [11,18]. Also, as previously demonstrated [16], 100 mM of EDTA or EGTA did not modify the hemolytic activity.

The hemolytic activity involves the binding of hemolysin molecules onto target cell membranes. Such binding has been reported as being partially inhibited by acetylated or methylated sugars [16]. We demonstrated here that phospholipids, and particularly spingomyelin, represent interacting membrane components trapping the hemolysin molecules. It is noticeable that in other invertebrates, sphingomyelin has also been reported as being a potent hemolysis inhibitor [6]. SDS-PAGE analysis of the protein content associated with spingomyelin microvesicles revealed a new HMWP present after incubation with HF. From its immunolabeling by specific anti-hemolysins antiserum, it may be inferred that the HMWP is a polymer composed of the 40 and/or 45 kDa hemolysins. Similar HMWP has been evidenced in SDS-PAGE of SRBC membranes lysed by ^{125}I-HF. Also in this case, the HMWP has been detected in Western blotting by the anti-hemolysins antiserum confirming that (i) polymerization of hemolytic molecules occurs on the target cell membrane during the hemolytic process, and (ii) membrane sphingomyelin could be the receptor site of hemolytic complexes.

It seems that artificial membranes constituted of spingomyelin were less efficient than SRBC in inducing the polymerization of hemolytic molecules. Free 40 and 45 kDa hemolysins were detectable on sphingomyelin microvesicles when they were apparently all transformed into HMWP at the SRBC membrane level. Identical results were obtained in the Echinoderm *Holothuria polii* where the protein pattern associated with lysed membranes [24] or with sphingomyelin microvesicles (unpublished results) also contained a new HMWP.

Polymerization of *E.f. andrei* 40 and 45 kDa proteins has previously been reported as giving rise to clot fibers [14]. In that case, before being organized in fibrils, the polymeric structures appeared as spheric units of 4 nm in diameter. The HMWP detected after interaction with spingomyelin or SRBC is likely to be different. In contrast to the HMWP, the clot-forming polymeric structures can be dissociated by SDS. It may be hypoth-

esized that, following the medium conditions, the 40 and 45 kDa proteins polymerize leading either to a dissociable structure (clot fibers) or to a more stable system (HMWP). Such a hypothesis agrees with electron microscopy observations reported here. Lesions appearing in negative staining as round deposits of phosphotungstate edged by a clear ring suggest a channel formation through the SRBC lipid bilayer, resulting from a protein oligomerization. Very similar pictures have been observed with the *H. polii* hemolysins [25].

The most exhaustively studied hemolytic system is complement. It has been demonstrated that C9 polymerizes spontaneously or after metal ion treatment into a tubular complex resembling the membrane attack complex [26]. Zn^{2+} ions accelerated such C9 extramembrane polymerization, leading to a decreased lytic activity. To check whether a similar effect was operating in *E.f. andrei*, we tested a variety of metal ions evidencing effective inhibition of hemolysis, particularly with Cu^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+}. Meanwhile, SDS-PAGE analysis demonstrated that such inhibition was not specific but due to the precipitation of all the HF proteins without any evidence of polymerization.

Another important difference between *E.f. andrei* hemolysins and complement was evidenced using various C9 inhibitors. Negatively charged glycosaminoglycans, positively charged peptides, protamine and poly(L-lysine), and diketene-neutralizing lysine and arginine residues, inhibit the lytic activity of late complement complex and perforin [19]. As demonstrated in this report, none of these molecules can produce hemolysis inhibition, suggesting that the interaction between *E.f. andrei* hemolysins and target cell membranes occurs by a pathway different from that of C9 and perforin.

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