Hemolysins: Pore-forming proteins in invertebrates

C. Canicatti

Department of Biology, University of Lecce, I-73 100 Lecce (Italy)

Summary. Invertebrates possess lytic molecules which lyse vertebrate erythrocytes. In all the species studied so far, hemolytic activity depends on proteins which possess a wide range of reactivity. It is generally calcium-dependent and heat-labile, although calcium-independent and heat-stable hemolysins have also been detected. The molecules interact with sugars or lipids which could represent the membrane receptors by which circular lesions on target membranes are produced.

On the basis of some analogies with vertebrate lytic molecules it is conceivable that the hemolysins evolved from a common ancestral gene which also led to vertebrate pore-forming proteins.

Key words. Lytic system; hemolysins; pore-forming proteins.

Introduction

Agglutinins, lysins, clotting and antibacterial proteins represent the most important factors in invertebrate humoral immunity. Among these molecules particular attention will be devoted to lysins, biologically active substances characterized by their lytic properties against foreign targets. This activity resembles that of the terminal components of the vertebrate complement system.

Lytic activity has been demonstrated in the body fluids (i.e. hemolymph, coelomic fluid and serum) or tissue extracts of several groups of coelomate invertebrates including annelids, arthropods, molluscs and echinoderms [2, 8, 12, 22, 27, 38, 47, 48, 65, 66, 70, 71]. In addition, tissue extracts of acelomate invertebrates have been demonstrated to be cytolytic [8, 34, 45] (table 1). Since this biological activity was generally demonstrated using vertebrate erythrocytes as experimental target membranes, the resulting lytic effect was referred to as hemolytic. The hemolytic properties of invertebrates depend on molecules of a proteinaceous nature. However, bioactive, non-proteic substances, extracted from a number of invertebrates, have been demonstrated to be hemolytic against erythrocytes. They include echinoderm saponin, a water-soluble, heat-stable mixture of at least six glycosides [44], and cnidaria phosphorylglycerylethers, hemolytic components of the water-soluble extract of the hydroid *Soanderia secunda* [30]. Since the chemical nature, the role and probably the mechanisms of action of these powerful hemolytic agents differ from that of hemolysins, they are not considered as humoral effectors of the invertebrate immune response but as toxins. Proteic toxins that are hemolytic have also been characterized. The toxin of the hydroid *Stoichactis helianthus*, probably derived from the nematocytes, was demonstrated to be strongly hemolytic against erythrocytes derived from a variety of animal species [4].

Table 1. Occurrence of hemolytic activity in metazoa

	Body fluids	Body extracts
Porifera		+
Coelenterata		+ +
Platyhelminthes		n.d.
Sipunculida	+	n.d.
Anellida	+ +	+
Mollusca	+	+
Arthropoda	+	n.d.
Echinodermata	+ + +	+ +
Urochordata	n.d.	n.d.

+ evidenced only in few species; + + evidenced in some species; + + + evidenced in a wide range of species; n.d., not observed.

Generally the hemolytic activity of invertebrate hemolysin is directly effective against a wide range of vertebrate erythrocytes including frog, chicken, pig, horse, calf, sheep, rabbit and human ABO. The reactivity is variable and, as demonstrated in *Holothuria polii*, it depends on the number and type of erythrocytes used [47] and is probably related to the different structures of the target cell membranes (Canicatti, unpublished).

A similar spectrum of hemolysis can be obtained with the body fluids from all the species so far studied. The only exception is the echinoid *Strongylocentrotus droebachiensis*, whose body fluid lyses only rabbit erythrocytes [6].

Gram-negative bacteria are also used to demonstrate the lytic properties of the invertebrate biological fluids [3, 32, 69]. The killing properties do not resemble, at least for the annelid *Glycera dibranchiata* [3], the lysozyme-type enzymes described in a number of other bactericidal systems [23, 24]. They appear to define a new class of antibacterial molecules [3]. However, in the earthworm *Eisenia foetida* the antibacterial spectrum is represented by the same proteins that have hemolytic activity [55, 56, 69]. In this species the hemolytic activity is effective against all cell types which have a complementary receptor structure for it [35].

Effect of cations on the lytic activity

The hemolytic activity of the body fluids so far studied strongly depends on cations. Calcium ions were demonstrated to be the only ions enhancing hemolytic activity [6, 12, 59, 67]. In some cases magnesium can substitute for calcium. No significant differences can be observed in the hemolytic potency of the coelomic fluid of the polychaete *Spirographis spallanzanii* [48] when $CaCl_2$ is replaced by $MgCl_2$ in the reaction medium. In *H. polii* zinc ions seem

to be able to mimic the calcium effect on hemolytic reactions. Low concentrations of $ZnCl_2$ (from 0.25 to 0.5 mM) produce a noticeable increase in the degree of hemolysis in the coelomic fluid, whereas higher concentrations (from 1 to 4 mM) are inhibitors. No other metal ion tested (Cn^{2+}, Hg^{2+}, Cd^{2+}) is as effective in mediating an increase of hemolytic efficiency, at least for *H. polii* coelomic fluid[17]. The chelating agents EDTA or EGTA reduce hemolytic responses in all the body fluids so far studied. A calcium-independent hemolytic activity was found in immune *H. polii* coelomic fluid. After injection of formalinized sheep erythrocytes into the coelomic cavity, a residual activity (40%) was registered in EDTA-dialyzed coelomic fluid, indicating the induction of a calcium-independent hemolytic component[10]. Both calcium-dependent and calcium-independent components were present in the lysate of coelomocytes[11]. Calcium- and magnesium-independent hemolytic activity has recently been demonstrated in both coelomic fluid and coelomocyte lysate of the sea star *Marthasterias glacialis*[20].

In a more advanced lytic system, that of vertebrate complement, the presence of divalent cations from the first transition period of the periodic table (i.e. Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}), or of Cd^{2+} or Tb^{3+}, effectively mediates the formation of activable C_1, which can start the classical complement pathway[74]. The presence of Mg^{2+} permits the binding of modified C_3 to the B-factor and the activation of the alternative complement pathway[43]. In invertebrates, it is not yet known whether cations act as stabilizing agents of the structure of the lytic molecules, or whether they act as mediators for the interaction between hemolysin and target. However, a third hypothesis can be advanced; cations may act as accelerators (or mediators) of hemolysin polymerization during membrane damage to the target cells, as is the case for the ninth component of complement (C_9)[61] or for perforin[52,53]. C_9 does in fact polymerize to a tubular complex, either spontaneously or in a process dependent on metal ions. The presence of Cn^{2+}, Zn^{2+} or Cd^{2+} accelerates C_9 polymerization (poly C_9)[61]. Also, in the presence of calcium ions[39], perforin produces a transmembrane complex which produces lesions similar to those of complement[28].

Physico-chemical and kinetic properties

The hemolytic system of invertebrates presents several physico-chemical properties, depending on the nature of the lytic components. In table 2 some of the features of the most-studied species are summarized. Temperatures ranging from 25 °C to 37 °C are optimal to produce the highest degree of hemolysis, probably because these temperatures contribute to the stability of the lytic components. At lower temperatures, 5 °C and 15 °C, hemolysins generally do not display their highest hemolytic potency[12,20,47,59]. Temperatures exceeding 50 °C generally completely abrogate the hemolytic activity of the body fluids, which indicates that thermo-lability is one of the common characteristics of hemolysins. However, temperature-labile hemolysins are not the only lytic components present in the coelomic fluids. As demonstrated by Canicatti and Parrinello[10], a heat-stable fraction is detectable in the coelomic fluid from *H. polii* fSRBC-injected animals. Moreover, both heat-labile and heat-stable fractions are present in the lysate of circulating coelomocytes[11]. Also in the hemocyte lysate from the asian clam *Corbicula fluminea* the potent hemolytic activity is not completely inactivated at 56 °C, though it is almost fully abrogated at 100 °C[72]. The hemolysin of the sea star *Marthasterias glacialis* was completely thermo-stable[20]. Thermo-lability is not the only common characteristic of hemolysins: the wide range of pH stability and the concentration-dependent time course are also common to almost all the hemolysins studied. Moreover, in many cases, the slope of the dose-response curve is similar. When the degree of hemolysis of a constant number of erythrocytes is plotted against a scalar dilution of the coelomic fluid, the resulting dose-response curve has a sigmoidal shape, indicating that the components have a synergic lytic effect. However, considering the curve shape, it cannot be excluded that the mediation of lysis involves the formation of an aggregate of a single molecular species[19].

Table 2. Salient physico-chemical features of some invertebrate hemolysins

Species	Temperature inactivation	pH stability	Cations dependence
L. terrestris	>50 °C	n.d.	no
E. foetida	56 °C	n.d.	no
S. spallanzanii	56 °C	n.d.	n.d.
T. cancriformis	60 °C	6–10	Ca^{++}-Mg^{++}
M. edulis	56 °C	>3–>12.8	Ca^{++}-Mg^{++}
C. fluminea	100 °C	n.d.	n.d.
H. polii	56 °C	5–9	Ca^{++}
S. droebachiensis	37 °C	4–8	Ca^{++}
P. depressus	56 °C	4–9	Ca^{++}
H. pulcherrimus	56 °C	4–9	Ca^{++}
A. crassispina	56 °C	4–9	Ca^{++}
P. lividus	56 °C	4–9	Ca^{++}
M. glacialis	100 °C	4–9	no

Isolation of the lytic components

Only a few reports exist on the isolation and characterization of hemolytic factors in invertebrates. This is probably due to difficulties in maintaining the biological activity of the isolated molecules. Most of our knowledge on isolated hemolysins is based on results with Annelids and Echinoderms. Gel filtration performed on an AcA44 matrix separated the *E. foetida* coelomic fluid into 8 peaks, of which only peak number 4 showed hemolytic activity. SDS-PAGE of the concentrated fraction indicated that it consists of two different molecules with apparent molecular weight of 40,000 and 45,000 D[56]. By chromatofocusing, lytic activity can be shown at pH 6.3, 6.0,

and 5.95[57]. When tested individually, the coelomic fluid of *E. foetida* exhibits polymorphism of the lytic system. It appears to be made up to four lipoproteins or lipoprotein-bound substances, referred to as isoforms, characterized by their different isoelectric points, ranging from 5.9 to 6.3[55].

In *H. polii* a chromatographic method was first employed to isolate hemolysins. On a Bio gel A5m column, the coelomic fluid was resolved into three major components, the third of which showed lytic activity. Analyzed in SDS-PAGE under reducing conditions, the peak was found to include two polypeptide chains of about 90,000 and 68,000 D[9]. Satisfactory results in isolating hemolysins can be obtained when coelomocyte lysate is used instead of coelomic fluid. With an overlay technique two lytic bands which have different electrophoretic mobilities could be located by their biological activity in a polyacrylamide gel slab. The components were isolated and identified as hemolysin 1 (He1) and hemolysin 2 (He2). He1 is the calcium-dependent, heat-labile component, He2 the calcium-independent, heat-stable one. The two hemolysins are probably two isoforms sharing a serological identity, as demonstrated by immunodiffusion analyses, and the same molecular weight (80 KDa under non-reducing conditions; a doublet of 76 and 80 KDa under reducing conditions)[16].

Cells synthesizing hemolysins

The nature of the cells producing hemolysins has been well investigated in the annelid *E. foetida*. From the results of hemolytic plaque assays[29], indirect fluorescence, transmission electron microscopy using peroxidase labelling[67], and scanning electron microscopy[68], it was possible to state that both young chloragocytes and eleocytes synthesize and release hemolysins. In the mollusc *Mytilus edulis*, hemolytic molecules are released in a short-term hemocyte culture medium, which indicates that they are actively secreted by *Mytilus* blood cells[38]. Recently Canicatti et al.[18], using a sodium metrizoate discontinuous gradient, found that two hemolysin-producing cell populations can be separated from the total circulating coelomocytes of *H. polii*. Both consist of amebocytes. The amebocytes of population 1 are responsible for the production of the calcium-dependent and thermo-labile hemolysin, whereas those of population 2 produce the calcium-independent and temperature-stable hemolysin, corresponding, respectively, to the isolated He1 and He2.

Surface binding and mechanisms of action

In an attempt to identify the membrane components reacting with the hemolysins, inhibition experiments with saccharides were performed. As demonstrated by Roch et al.[56], the hemolytic system of *E. foetida andrei* is inhibited by various acetylated or methylated carbohydrates. Moreover, lipids also act as inhibitors of the hemolytic reaction[58]. Carbohydrates are also inhibitors of the sea urchin *Strongylocentrotus droebachiensis*. In this species amino sugars are potent inhibitors, whereas acetyl-substituted amino sugars are less efficient[6]. Sugars did not inhibit the *H. polii* hemolytic system. Lipids, on the other hand, displayed inhibitory activity. In fact, sphingomyelin, one of the principal membrane lipids, inhibited hemolysis, which suggests a specific involvement of this lipid in the lysis of the target cell[13,19]. Sphingomyelin is also an inhibitor of the *M. glacialis* hemolysin[18]. In other hemolytic systems, such as that of the sea urchin *Paracentrotus lividus*, neither sugars nor sphingomyelin inhibited the hemolytic activity of the coelomic fluid[12].

Hemolytic molecules do not seem to act as enzymes[13,56]. Apparently hemolysis is mediated by the rapid binding of hemolytic molecules onto the target cells through sugars[56] or lipids[19]. As demonstrated by TEM observations[12], the cytolytic effect results in holes of different size (5 to 20 nm)[14]. Under improved experimental conditions, using coelomocyte lysate hemolysins, circular holes of 10 nm resembling vertebrate MAC could be observed on rabbit erythrocyte membranes lysed by the *H. polii* hemolytic system (Tschopp and Canicatti, unpublished). Recently, circular holes of about 10 nm could also be observed on negative-staining of rabbit erythrocyte membranes lysed by *E. foetida* coelomic fluid[58]. The analysis by 10% SDS-PAGE of the proteins associated with the membrane of rabbit erythrocytes, after lysis by ^{125}I *H. polii* coelomic fluid, revealed a pattern consisting of a high molecular weight band which did not penetrate into 10% gels, and a band with a mol.wt of about 27,000 D. A weak band with a mol.wt of 31,000 D was also present. Under reducing conditions the high mol.wt band disappeared and two strongly radioactive bands with molecular weights of about 80,500 and 64,000 D appeared instead. The 31,000 D band had a stronger intensity, whereas the 27,000 D one was lost, probably as a result of reduction[14]. Also in *E. foetida* a high molecular weight protein was detected by SDS-PAGE in sheep erythrocytes lysed by ^{125}I-labeled coelomic fluid. It was immunolabeled by anti-40 and 45 KDa hemolysin molecules[58] indicating that, as for *H. polii*[19], polymerization of the hemolytic molecules occurs on the target cell membrane during the hemolytic process.

Role

Hemolysins from the different invertebrate species so far examined are active against vertebrate erythrocytes. These cells represent a simple experimental surface model to test lytic principles, but do not solve the problem of the role of the lytic molecules present in invertebrates in vivo. The same lytic mechanisms are probably used against unwanted cells (i.e. self-transformed cells, protozoa, parasites and bacteria). Moreover, as in the lytic system of vertebrates[43], hemolysins could act as opsonic factors,

alone or through proteolytic fragments produced by their activation. A clear indication of an opsonic activity of the lytic proteins was demonstrated in the sea urchin *Strongilocentrotus droebachiensis*[6].

In *H. polii*, the engagement of hemolysins in the immune surveillance mechanisms was first indicated by injection experiments. When formalinized sheep erythrocytes were injected into the coelomic cavity, a decrease of the degree of hemolysis was registered in the injected samples compared to non-injected controls. This value increased steadily, reaching the control value on day 4[10]. The initial decrease is probably due to the involvement of hemolysins in the clearance of foreign materials. It was supposed[19] that these molecules – and not the hemagglutinin – could mediate, as opsonins, the formation of rosettes, which represents the initial step of the phagocytic phenomenon in the cellular response of *H. polii* to the injection of sheep erythrocytes[15].

Hemolysins and complement

A possible relation between the complement cascade and invertebrate hemolysins has been extensively debated. A complement-like activity was recognized in invertebrates on the basis of functions analogous to those of the complement counterpart[1, 26, 27, 36, 37, 49]. In the fall armyworm, a structural analogy was demonstrated between the major hemolymph protein and complement components[25]. In Echinoderms[6], the analogy to complement was deduced from the lytic action on rabbit erythrocytes that 'knowingly' activate the alternative pathway of the human complement system[50], and from the opsonic effect resulting from inhibition with various inhibitors of human complement.

For all these authors the presence of a complement cascade in invertebrates is conceivable. Some of them[7, 49] formulated the attractive idea that invertebrates can possess a C_3-like molecule, the key component in the vertebrate complement system. However, in *H. polii* the complement inhibitors do not seem to exert any inhibitory activity. Moreover, the nucleophils hydrazine and methylamine have no significant effect on the hemolytic activity of the coelomic fluid. Similar results were reported by Roch et al.[58] in the earthworm *E. foetida*. In mammals, these nucleophilous molecules covalently interact with the internal thioester bond of complement key-component C_3 leading to a loss of functional activity[33, 46]. In *H. polii* no covalent interaction was established between labeled methylamine and coelomic fluid proteins, as demonstrated by the experimental incorporation of [^{14}C] methylamine[13]. These results indicate that there is not a protein that, like C_3[60], possesses an intramolecular thioester bond responsible for the functional binding with the biological targets.

On the other hand, the finding that the purified *H. polii* hemolysin as well as the *E. foetida* one can produce a lytic effect on erythrocyte targets without the involvement of other proteins favored the idea that a system which does not need an activating cascade, like complement, exists in invertebrates. In vertebrates, molecules able to produce membrane damage on targets were isolated from natural killer cells and cytotoxic T-lymphocytes[39, 53]. They are the pore-forming protein of 75–76 KDa, perforin[52, 53] which, in the presence of Ca^{2+}, is able to form a tubular complex responsible for the transmembrane channel formation in target membranes[51]. The lesions produced, observed under the electron microscope, appear to be similar to the ones caused by complement[28].

Transmembrane channel formation is also produced by spontaneous or metal-dependent polymerization of C_9 (poly C_9)[61]. This component of the complement system appears to be closely related to perforin. In fact, antigenic cross-reactivity can be demonstrated between perforin and C_9[62, 73], indicating a probable common evolutionary origin. Considering all these aspects and the properties of the *H. polii* lytic system, Canicatti[19] hypothesized a functional analogy between the sea cucumber hemolysin, C_9 and perforin. Moreover, since in a phylogenetically unrelated species (i.e. earthworm) a similar analogy could be conceivable[58], the proposed evolutionary relationship between hemolysin and vertebrate pore-forming proteins could be extensible to other invertebrates.

Regulatory mechanisms

Whether lytic molecules occur naturally in or are released into body fluids of invertebrates, two spontaneous questions arise; what kind of mechanisms regulate their activation and, once activated, is there any protective control against lysis of self-cells? Unfortunately, in contrast to the complement system[43] and perforin[63, 64], for which several models of regulatory mechanisms have been proposed, nothing is known for the invertebrate lytic system. From the data accumulated on in vitro release of hemolytic molecules[16, 28, 72] it seems conceivable that, once released, hemolysins are able themselves to produce damage to target cell membranes and, at least for Ca^{++}-dependent hemolysis outside the cell, only calcium has a regulatory function. Therefore, if activating mechanisms exist, they are probably at cellular level and not in body fluids. As for many other cellular regulative functions[54], proteases could be implicated. Serine-esterase activity of the trypsin-like type has recently been shown in coelomocyte lysate and coelomic fluid of *H. polii*[21]. Trypsin-like as well as other serine esterase enzymes are present in lytic granules of cytotoxic cells[40, 41] where, as suggested by Goldfarb[31] and Masson and Tschopp[41] they could be implicated in the cytolytic activity.

Due to the lack of experimental evidence, it is more hazardous to propose a hypothesis on control of autolysis of the circulating hemolysins. In the case of perforin and complement, it has been suggested that self-killing may be inhibited by S-protein/vitronectin[64] or the lipid

portion of lipoproteins[63]. These proteins may represent an important mechanism of control of lytic molecules. In certain invertebrates[13,58] sphingomyelin inhibits hemolysis and, as reported in *H. polii*[13], the lack of this lipid fails to inhibit hemolysis. Moreover, the variable hemolytic potency of the sea cucumber hemolysin seems to reflect the variable content of sphingomyelin of the different vertebrate erythrocyte species (Canicatti, unpublished). It is therefore conceivable that the absence or masking of this lipid on the *H. polii* coelomocyte surface could contribute to the inhibition of self-killing.

1 Anderson, R. S., Day, N. K. B., and Good, R. A., Specific hemagglutinin and a modulator of complement in cockroach hemolymph. Inf. Immun. *5* (1972) 55–59.
2 Anderson, R. S., Hemolysin and hemagglutinins in the coelomic fluid of a polychaete annelid, *Glycera dibranchiata*. Biol. Bull. *159* (1980) 259–268.
3 Anderson, R. S., and Chain, B. M., Antibacterial activity in the coelomic fluid of a marine annelid, *Glycera dibranchiata*. J. Invertebr. Pathol. *40* (1982) 320–326.
4 Bernheimer, A. W., and Avigard, L. S., Properties of a toxin from the sea anemone *Stoichactis helianthus* including specific binding to sphingomyelin. Proc. natl Acad. Sci. USA *73* (1976) 467–471.
5 Bertheussen, K., The cytotoxic reaction in allogenic mixtures of Echinoid phagocytes. Exp. Cell. Res. *120* (1979) 373–381.
6 Bertheussen, K., Complement-like activity in sea urchin coelomic fluid. Devl comp. Immun. *7* (1983) 21–28.
7 Bertheussen, K., Complement and lysins in invertebrates. Devl comp. Immun. *3* (1984) 173–181.
8 Bretting, H., and Renwrantz, L., Untersuchungen von Invertebraten des Mittelmeeres auf ihren Gehalt an hämagglutinierenden Substanzen. Z. Immun. Forsch. *145* (1973) 242–249.
9 Canicatti, C., and Parrinello, N., Chromatographic separation of coelomic fluid from *Holothuria polii* (Echinodermata) and partial characterization of the fractions reacting with erythrocytes. Experientia *39* (1983) 764–766.
10 Canicatti, C., and Parrinello, N., Hemagglutinin and hemolysin levels in the coelomic fluid from *Holothuria polii* (Echinodermata) following sheep erythrocyte injection. Biol. Bull. *168* (1985) 175–182.
11 Canicatti, C., and Ciulla, D., Studies on *Holothuria polii* (Echinodermata) coelomocyte lysate. I. Hemolytic activity of coelomocyte hemolysins. Devl comp. Immun. *11* (1987) 705–712.
12 Canicatti, C., Evolution of the lytic system in Echinoderms. I. Naturally occurring hemolytic activity in *Paracentrotus lividus* (Echinoidea) coelomic fluid. Boll. Zool. *54* (1987) 325–329.
13 Canicatti, C., Parrinello, N., and Arizza, V., Inhibitory activity of sphingomyelin on hemolytic activity of coelomic fluid of *Holothuria polii* (Echinodermata). Devl comp. Immun. *11* (1987) 29–35.
14 Canicatti, C., Membrane damage by coelomic fluid from *Holothuria polii* (Echinodermata). Experientia *43* (1987) 611–614.
15 Canicatti, C., and D'Ancona, G., Cellular aspects of *Holothuria polii* immune response. J. Invertebr. Path. *53* (1988) 152–158.
16 Canicatti, C., and Ciulla, D., Studies on *Holothuria polii* (Echinodermata) coelomocyte lysate. II. Isolation of coelomocyte hemolysins. Devl comp. Immun. *12* (1988) 55–64.
17 Canicatti, C., and Grasso, M., Biodepressive effect of zinc on humoral effector of *Holothuria polii* immune response. Mar. Biol. *99* (1988) 393–396.
18 Canicatti, C., Ciulla, D., and Farina-Lipari, E., The hemolysin producer coelomocytes. Devl comp. Immun. *12* (1988) 729–736.
19 Canicatti, C., The lytic system of *Holothuria polii* (Echinodermata). A review. Boll. Zool. *55* (1988) 139–144.
20 Canicatti, C., Evolution of lytic system in Echinoderms. II. Naturally-occurring hemolytic activity in *Marthasterias glacialis* (Asteroidea). Comp. Biochem. Physiol. *93A* (1989) 587–591.
21 Canicatti, C., Protease activity in *Holothuria polii* coelomic fluid and coelomocyte lysate. Comp. Biochem. Physiol. (1989) in press.
22 Cenini, P., Comparative studies on haemagglutinins and haemolysins in an annelid and a primitive crustacean. Devl comp. Immun. *7* (1983) 637–640.
23 Cheng, T. C., and Rodrick, G. E., Identification and characterization of lysozymes from the hemolymph of the salt shelled clam *Mya arenaria*. Biol. Bull. *147* (1974) 311–320.
24 Cheng, T. C., Chorney, M. J., and Yoshino, T. P., Lysozyme-like activity in the hemolymph of *Biomphalasie glabrata* challenged with bacteria. J. Invertebr. Path. *29* (1977) 170–174.
25 Cruz, O. J. M., and Day, N. K., Structural and functional similarities between the major hemolymph protein of fall armyworm and cat C_4 binding protein of the complement system. Devl comp. Immun. *9* (1985) 541–550.
26 Day, N. K. B., Gewzy, H., Johannsen, R., Finstad, J., and Good, R. A., Complement and complement-like activity in lower vertebrates and invertebrates. J. exp. Med. *132* (1970) 941–951.
27 Day, N. K. B., Geiger, H., Finstad, J., and Good, R. A., A starfish hemolymph factor which activates vertebrate complement in the presence of Cobra venom factor. J. Immun. *109* (1972) 164–167.
28 Dourmashkin, R. R., Deteix, P., Simone, C. B., and Henkart, P. A., Electron microscopic demonstration of lesions in target cell membranes associated with antibody dependent cellular cytotoxicity. Clin. exp. Immun. *42* (1980) 554–563.
29 Du Pasquier, L., and Duprat, P., Aspects humoreaux et cellulaires d'une immunité naturelle non spécifique chez l'oligochète *Eisenia foetida* (Lumbricidae). C.r. Acad. Sci. Paris *266* (1968) 538–546.
30 Fusetani, N., Yasukawa, K., Matsunaga, W., and Hashimoto, K., Isolation and identification of three hemolysins from the hydroid *Solanderia secunda* (Inaba). Comp. Biochem. Physiol. *83B* (1986) 511–513.
31 Goldfarb, R. H., Role of proteases in NK activity, in: Mechanisms of Cytotoxicity by NK Cells, pp. 205–212. Ed. R. Haberman. Academic Press, London 1985.
32 Hültmark, O., Steiner, H., Rasmusson, T., and Boman, H. G., Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pulpae of *Hyalophora cecropia*. Eur. J. Biochem. *106* (1980) 7–16.
33 Janatova, J., Lorenz, P. W., Schechter, A. N., Prahl, J. W., and Tack, B. R., Third component of human complement. Appearance of a sulphydryl group following chemical or enzymatic inactivation. Biochemistry *19* (1980) 4471–4483.
34 Kamiya, H., Muramoto, K., and Goto, R., Cytotoxicity and hemolysis by an acqueous extract of a marine sponge, *Clathria* sp., Symp. Natural Toxins, China 1985.
35 Kauschke, E., and Mohring, W., Comparative analysis of hemolytic and hemagglutinating activities in the coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris* (Annelida, Lumbricidae). Devl comp. Immun. *11* (1987) 331–341.
36 Koch, C., and Nielsen, H. E., Activation of vertebrate complement by *Helix pomatia* haemolymph. Devl comp. Immun. *8* (1984) 15–22.
37 Laulan, A., Lestage, J., Chateaureynand-Duprat, E., and Fontaine, M., Mise en évidence de substances contenues dans le liquide coélomique de *Lumbricus terrestris*, possédant des fonctions communes avec celles de certains composants du complément humain. Annls Immun. *134* (1983) 223–241.
38 Leippe, M., and Renwrantz, J., Release of cytotoxic and agglutinating molecules by Mytilus hemocytes. Devl comp. Immun. *12* (1988) 297–308.
39 Masson, D., and Tschopp, J., Isolation of a lytic pore-forming protein (perforin) from cytolytic T-lymphocytes. J. biol. Chem. *260* (1985) 9069.
40 Masson, D., Nabholz, M., Estrade, C., and Tschopp, J., Granules of cytolytic T-lymphocytes contain two serine esterases. EMBO J. *5* (1986) 1595–1600.
41 Masson, D., and Tschopp, J., A family of serine esterases in lytic granules of cytolytic T-lymphocytes. Cell *49* (1987) 679–685.
42 Michaels, D. W., Membrane damage by a toxin from the sea anemone *Stoichactis helianthus*. I. Formation of transmembrane channels in lipid bilayers. Biochim. biophys. Acta *555* (1979) 67–78.
43 Müller-Eberhard, H. J., and Miescher, P. A., Complement. Springer Verlag, Berlin 1985.
44 Nigrelli, R. F., Stempien, M. F., Ruggieri, G. D., Liguori, U. R., and Cecil, J. T., Substances of potential biomedical importance from marine organisms. Fed Proc. *26* (1967) 1197–1205.
45 Norton, R. S., Bobek, G., Thomson, M., Moritz, R. L., and Simpson, R. S., Proteins with cardiac stimulatory and hemolytic activity from the sea anemone *Actinia tenebrosa*. Toxicon *7* (1989) 66–67.
46 Pangburn, M. K., and Müller-Eberhard, H. J., Relation of a putative thioester bound in C_3 to activation of the alternative pathway and the binding of C_{3b} to biological targets of complement. J. exp. Med. *152* (1980) 1102–1112.
47 Parrinello, N., Rindone, D., and Canicatti, C., Naturally occurring hemolysins in the coelomic fluid of *Holothuria polii* Delle Chiaje (Echinodermata). Devl comp. Immun. *3* (1979) 45–54.

48 Parrinello, N., and Rindone, D., Studies on the natural hemolytic system of the annelid worm *Spirographis spallanzanii viviani* (Polychaeta). Devl comp. Immun. *5* (1981) 33–42.
49 Phipps, D., Menger, M., Chadwich, J. S., and Aston, W. P., A cobra venom factor (CVF)- induced C3 convertase activity in the hemolymph of *Gallesia mellonella*. Devl comp. Immun. *11* (1987) 37–47.
50 Platts-Mill, T. A., and Ishizaka, K., Activation of the alternative pathway of human complement by rabbit cells. J. Immun. *113* (1974) 348–358.
51 Podack, E. R., and Tschopp, J., Circular polymerization of the ninth component of complement. J. biol. Chem. *257* (1982) 15204–15212.
52 Podack, E. R., Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. Nature *302* (1983) 442–445.
53 Podack, E. R., Young, J. D. E., and Cohn, Z. A., Isolation and biochemical functional characterization of perforins from cytolytic T-cell granules. Proc. natl Acad. Sci. USA *82* (1985) 8629–8638.
54 Reich, E., Rifkin, D. B., and Shaw, E., Protease and biological control. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1975.
55 Roch, P., Protein analysis of earthworm coelomic fluid. I. Polymorphic system of the natural hemolysins of *Eisenia foetida Andrei*. Devl comp. Immun. *3* (1979) 588–608.
56 Roch, P., Valembois, P., Davant, N., and Lassegues, M., Protein analysis of earthworm coelomic fluid, II. Isolation and biochemical characterization of the *Eisenia foetida* Andrei factor (EFAF). Comp. Biochem. Physiol. *698* (1981) 829–836.
57 Roch, P., Davant, N., and Lassegues, M., Isolation of agglutinins and lysins form earthworm coelomic fluid by gel filtration followed by chromatofocusing. J. Chromat. *290* (1984) 231–235.
58 Roch, P., Canicatti, C., and Valembois, P., Interaction between the hemolytic system of the earthworm *Eisenia foetida Andrei* and the SRBC membrane. Biochim. biophys. Acta *983* (1989) 193–198.
59 Ryoyama, K., Studies on the biological properties of coelomic fluid of sea urchin. I. Naturally occurring hemolysin in sea urchin. Biochim. biophys. Acta *320* (1973) 157–165.
60 Tack, B. R., Harrison, R. A., Janatova, J., Thomas, M. L., and Prahl, J. W., Evidence for presence of an internal thioester bound in third component of human complement. Proc. natl Acad. Sci. *77* (1980) 5464–5472.
61 Tschopp, J., Circular polymerization of the membranolytic ninth component of complement. J. Biol. Chem. *259* (1984) 10569–10573.
62 Tschopp, J., Masson, D., and Stanley, K. K., Structural/function similarity between proteins involved in complement and cytotoxic T-lymphocyte-mediated cytolysis. Nature *322* (1986) 831–834.
63 Tschopp, J., Masson, D., and Schafer, S., Inhibition of the lytic activity of perforin by lipoproteins. J. Immun. *137* (1986) 1950–1953.
64 Tschopp, J., Masson, D., Schafer, S., Peitsch, M., and Preissner, K. T., The heparin binding domain of S/protein/Vitronectin binds to complement components C7, C8 and C9 and perforin from cytolytic T-cells and inhibits their lytic activities. Biochemistry *27* (1988) 4103–4109.
65 Tuan, T. L., and Yoshino, T. P., Natural hemolysin in the hemolymph of freshwater bivalve *Corbicula fluminea*. Devl comp. Immun. suppl 3 (1984) 193.
66 Tuckova, L., Rejnek, J., Sima, P., and Ondiejova, R., Lytic activities in coelomic fluid of *Eisenia foetida* and *Lumbricus terrestris*. Devl comp. Immun. *10* (1986) 181–189.
67 Valembois, P., Roch, P., Lassegues, M., and Cassand, P., Antibacterial activity of the hemolytic system from the earthworm *Eisenia foetida Andrei*. J. Invertebr. Path. *40* (1982) 21–27.
68 Valembois, P., Lassegues, M., Roch, P., and Vaillier, J., Scanning electron microscopic study of the involvement of coelomic cells in earthworm antibacterial defence. Cell Tissue Res. *240* (1985) 479–484.
69 Valembois, P., Roch, P., and Lassegues, M., Antibacterial molecules in Annelids, in: Immunity in Invertebrates, pp. 14–93. Ed. M. Brehelin. Springer Verlag, Berlin (1986).
70 Weinheimer, P. F., Evans, E. E., Stround, R. M., Acton, R. T., and Painter, B., Comparative immunology: Natural hemolytic system of the spiny lobster, *Panulirus arguus*. Proc. Soc. exp. Biol. Med. *130* (1969) 322–326.
71 Weinheimer, P. F., Acton, R. T., Cushing, J. E., and Evans, E. E., Reactions of sipunculid fluid with erythrocytes. Life Sci. *9* (1970) 145–151.
72 Yoshino, T. P., and Tuan, T. L., Soluble mediators of cytolytic activity in hemocytes of the asian clam *Corbicula fluminea*. Devl comp. Immun. *9* (1985) 515–522.
73 Young, J. D. E., Cohn, Z. A., and Podack, E. R., The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T-cells: Structural, immunological and functional similarities. Science *233* (1986) 184–190.
74 Ziccardi, R. J., The three components of human complement (C1): Activation and control, in: Complement, pp. 167–184. Eds H. J. Müller-Eberhard and P. A. Miescher. Springer Verlag, Berlin 1985.

0014-4754/90/030239-06$1.50 + 0.20/0

Research Articles

The morphology of the lateral line system in 3 species of Pacific cottoid fishes occupying disparate habitats

H. A. Vischer

Neurobiology Unit, A-002, Scripps Institution of Oceanography, University of California at San Diego, La Jolla (California 92093, USA)
Received 30 June 1989; accepted 24 August 1989

Summary. The superficial neuromasts in *Leptocottus armatus* (Pacific staghorn sculpin) and *Oligocottus maculosus* (tidepool sculpin) are depressed below the rim of an epidermal collar, whereas the superficial neuromasts in *Psychrolutes paradoxus* (tadpole sculpin) are surrounded by a pair of hillocks. The lateral line canal system appears identical across these species, whereas the system of superficial neuromasts shows some intraspecific variation. The sand-living *Leptocottus* has a more highly developed system of superficial neuromasts than *Oligocottus*, which lives in a rocky habitat. The present results support the correlation between lateral line system morphology and habitat features previously postulated by Dijkgraaf[1].
Key words. Cottoid fishes; mechanoreceptive lateral line system; neuromasts; scanning electron microscopy.