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HEMOLYSINS AND HEMAGGLUTININS IN THE COELOMIC FLUID OF A POLYCHAETE ANNELID, *GLYCERA DIBRANCHIATA*

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The specificity and anamnestic characteristics of immune responses of annelids, particularly cellular reactions to integrementary grafts between oligochaetes, have been the subject of considerable study and controversy (*e.g.*, Duprat, 1967; Cooper, 1969; Hostetter and Cooper, 1972, 1973; Parry, 1978; Dales, 1978a, b). There is little doubt that the amoebocytic coelomocytes of annelids can differentiate between material of self and nonself origin; these cells are active in phagocytosis, encapsulation, and wound healing (Dales, 1978c), and may actively follow chemotactic gradients toward bacteria and foreign tissues (Marks *et al.*, 1979). Considerably less is known of the humoral immune factors present in the coelomic fluid of annelids, especially polychaetes, and the extent of cooperation between humoral and cellular components of the immune response is uncertain. Oligochaete coelomic fluid contains natural hemolytic activity (Chateaureynaud-Duprat and Izoard, 1973; Roch, 1979) suggested to play a role in graft rejection (Chateaureynaud-Duprat and Izoard, 1977a), and hemagglutinins that also possibly serve as humoral recognition factors in various foreign-body responses (Cooper *et al.*, 1974). This paper presents a description of naturally occurring hemolysins and hemagglutinins in the coelomic fluid of *Glycera*, and results of attempts to induce these factors by several experimental protocols. It is probable that these factors participate in polychaete immune mechanisms.

In most annelids there are two separate fluid-filled compartments, the coelom and the vascular system. However, in *Glycera* and a few other polychaetes, a separate blood system has disappeared (Dales, 1970). The coelomic fluid contains several easily identified cell types: erythrocytes, amoebocytes, and occasionally gametes. The cells will settle out, or can be removed by centrifugation, to leave the pale straw-colored supernatant fluid used in these studies. There is no evidence of immunoglobulins in this fluid, or in the coelomic fluid or hemolymph of

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any invertebrate. However, other factors with immunological properties are present.

Materials and Methods

Animals and collection of coelomic fluid

Specimens of *Glycera dibranchiata* were purchased from the Maine Bait Company, Newcastle, Maine. They were maintained in recirculated-water aquaria containing Instant Ocean artificial sea water at 11°–13°C. The worms usually remained burrowed in the calcareous gravel substrate, emerging occasionally to swim about near the bottom.

Coelomic fluid was collected in a plastic petri plate as it flowed from a small incision penetrating the integrement near the anterior end of the worm. Each worm yielded 0.5–1.0 ml of coelomic fluid. Samples were not pooled, except where otherwise indicated. Clotting was minimal, although some clumping of the amoebocytes was observed. Attempts to withdraw coelomic fluid by hypodermic syringe usually caused considerable lysis of *Glycera* red blood cells; this did not occur in fluid obtained as outlined above. Cells were removed from the fluid phase by centrifugation at 525 × *g* for 4 min at 4°C.

Hemolysin assay

Test mammalian erythrocytes (1% in Hanks' balanced salts solution, 0.5 ml) were added to an equal volume of coelomic fluid, or coelomic fluid serially diluted with Hanks' balanced salts solution (BSS). This mixture was incubated 60 min at 21°C. Two milliliter Hanks' BSS were added and the tubes immediately centrifuged for 3 min at 750 × *g*. The optical density (O.D.) of the supernatant was determined at 541 nm. A coelomic-fluid color-control tube was prepared by following the above procedure, but substituting Hanks' BSS for the test erythrocyte suspension. All values were corrected by subtracting the O.D. of the coelomic fluid color control. Test erythrocytes, following the above procedure, were incubated in distilled water, and the resultant O.D., representing 100% lysis, was measured. Using this value, the corrected experimental O.D. was converted to percent lysis. This basic procedure was modified in several ways as described in the "Results" section. For example, the incubation time was varied to determine reaction kinetics, the coelomic fluid serially diluted to assay hemolysin titer, EDTA added to determine requirement for divalent cations, different types of test erythrocytes were used, or coelomic fluid was obtained from animals previously injected with erythrocytes.

Hemagglutination assay

Hemagglutination assays were carried out in Cooke "U" well microtiter plates. The first well contained 100 μl coelomic fluid, 50 μl of which was then mixed with an equal volume of saline (0.9% NaCl) in the next well. In this way the coelomic fluid was serially diluted with a microdiluter through 10 wells. A control well contained saline only. Test erythrocytes (50 μl, 2% erythrocytes) were added to all wells and the microtiter plates covered and incubated for 60 min at 21°C. The hemagglutinin titers were read immediately after incubation.

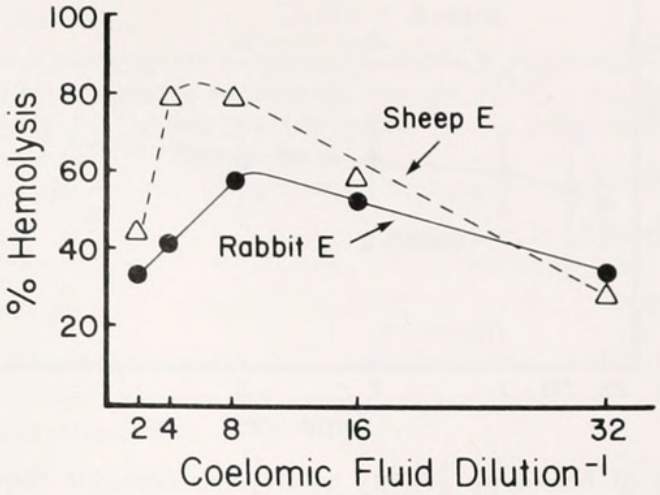

FIGURE 1. Effect of concentration on hemolytic activity of *Glycera* coelomic fluid. Erythrocytes (1%) were incubated with coelomic fluid for 60 min at 21°C. Each point is the mean of two separate determinations.

In certain cases, formalin-treated erythrocytes were used to measure hemagglutinin activity. These were prepared by exposure to 6% formaldehyde for 24–48 hr at 4°C. The cells were then washed five times and finally a 10% stock suspension in 0.15 M phosphate buffer (pH 7.0) was prepared. Aliquots of the stock solution were diluted to 2% for use in the hemagglutination assays.

Hemolysin and hemagglutinin induction

Hemolysin activity and hemagglutinin titer were determined in specimens of *Glycera* which had previously been injected with erythrocytes. The various injection schedules are described in the "Results" section. Each injection was intracoelomic and consisted of 0.1 ml of 10 or 50% erythrocyte suspension in sterile sea water. The injections had no effect on the behavior, morbidity, or mortality of the worms. At various intervals after injection, coelomic fluid was collected and hemolysin and hemagglutinin assays carried out as described above.

RESULTS

Hemolysis

Glycera. Coelomic fluid was shown to contain naturally-occurring hemolysin(s) against several mammalian erythrocytes (E). Maximal hemolysis was recorded at coelomic fluid dilutions of about 1/8 for rabbit E and 1/4–1/8 for sheep E. Sheep E were more susceptible than rabbit E to the lytic action of coelomic fluid at concentrations $> 1/16$ (Fig. 1). It was unusual to detect spectrophotometrically any E lysis at coelomic fluid dilutions greater than 1/128. The rate of sheep E hemolysis exceeded that of rabbit E (Fig. 2). The initial rate of reaction was rapid: In 1 hr about 90% of a standard suspension of sheep E was lysed by 1/8 coelomic fluid, while under identical conditions $\sim 60\%$ of a comparable suspension of rabbit E was lysed. Total hemolysis of the sheep E was reached by 2 hr; the percentage hemolysis of rabbit E increased very slowly to about 70% by 24 hr. Hemolysis assays were routinely carried out at room temperature (~ 21°C). Lowering the temperature of incubation to 4°C had no effect on hemolytic activity: When the study presented in Figure 2 was repeated at 4°C,

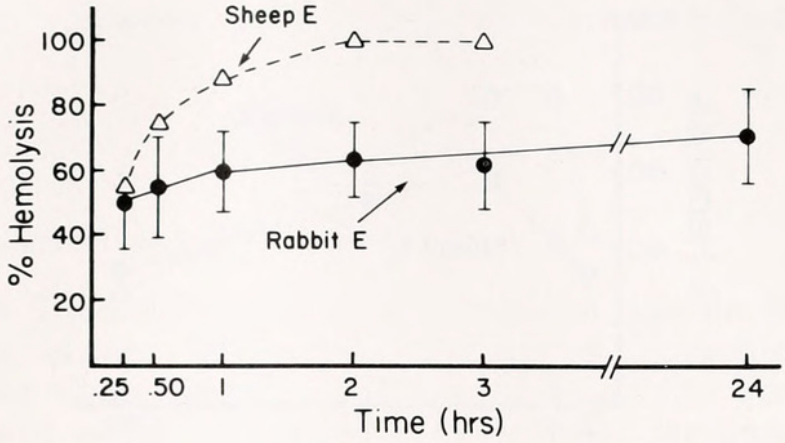

FIGURE 2. Kinetics of hemolytic activity of *Glycera* coelomic fluid. Erthyrocytes (1%) were incubated with 1/8 diluted coelomic fluid at 21°C. Means ± standard deviations (vertical lines) (N = 5) are given for rabbit erythrocytes; results of one representative experiment using sheep erythrocytes are included for comparative purposes.

none of the mean percent hemolysis values were statistically different from those recorded at 21°C. Attempts to preserve hemolytic activity at −80°C after quick freezing with ethanol and dry ice were unsuccessful; apparently biological activity was lost during freezing and thawing. The hemolysin was inactivated by heating to 56°C for 30 min. If the coelomic fluid was treated with > 1 mM EDTA (ethylenediamine tetraacetic acid, disodium salt) at neutral pH, its hemolytic activity was markedly inhibited (Fig. 3).

The hemolytic potential of coelomic fluid was considerably reduced by adsorption with erythrocytes (Table I). Adsorption with rabbit E reduced subsequent lysis of rabbit E or sheep E to a comparable extent. Sheep E were more efficient in adsorbing anti-rabbit and anti-sheep hemolysins than rabbit E. Hemolysis of sheep E was more inhibited by adsorption with autologous E than was hemolysis of rabbit E, following each of the first two adsorptions.

Hemolysin activity was determined daily for three days after a single injection of a 50% suspension of sheep or rabbit E (Table II). Injection with rabbit E produced no significant effect on anti-sheep hemolysin activity, but caused a reduction in the lysis of rabbit E, which lasted for the course of the experiment. Injection of sheep E caused a persistent reduction of both anti-sheep and anti-rabbit

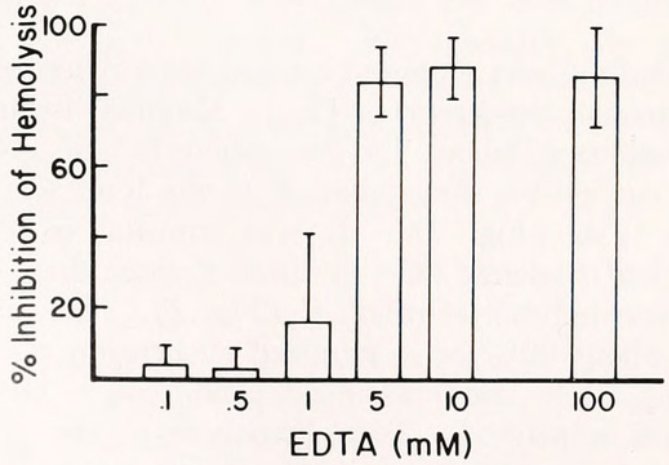

FIGURE 3. Inhibition of *Glycera* anti-rabbit-erythrocyte lysin by EDTA. Rabbit red blood cells (1%) were incubated with EDTA-treated coelomic fluid (1/8 dilution) for 60 min. Mean % inhibition ± standard deviation (vertical lines) (N = 5) are given for each EDTA concentration.

TABLE I

Effect of adsorption with erythrocytes on Glycera *hemolysin. Each adsorption consisted of 15 min incubation at 1°C of 1 vol freshly packed erythrocytes resuspended in 2 vol coelomic fluid. Means and standard deviations are given for the first two adsorptions (N = 3, for each value); three adsorptions were carried out on only one pool of coelomic fluid.*

Coelomic fluid adsorbed with:	Hemolysis (% of unadsorbed coelomic fluid)	
	vs. rabbit erythrocytes	*vs.* sheep erythrocytes
Rabbit erythrocytes		
1x	59.5 ± 20.6	58.7 ± 0.6
2x	40.4 ± 13.6	30.3 ± 9.9
3x	19.7	17.1
Sheep erythrocytes		
1x	44.2 ± 17.3	29.1 ± 6.5
2x	13.4 ± 10.8	5.1 ± 1.6
3x	3.9	3.0

hemolysin. In all cases following a single injection of E, there was a gradual tendency for the decreased levels of hemolysins to return to control levels with time. The hemolysin values from uninjected animals were valid controls for this study because the mean hemolytic activity in uninjected animals was not significantly different from that of *Glycera* injected with sterile sea water.

In three studies comparable to that reported in Table II, hemolytic activity was followed after a single 0.1 ml injection of a 10% suspension of sheep E or

TABLE II

Hemolysin activity (% hemolysis) in Glycera *coelomic fluid after injection of mammalian erythrocytes (E), 0.1 ml, 50% E in sterile sea saline. Mean ± SD (N). Probability* (P) *values from two-tailed* t *test comparing % hemolysis mediated by coelomic fluid of injected animals to controls.*

Indicator E	Percent hemolysis by coelomic fluid			
	Uninjected	Time after intracoelomic injection of rabbit E		
		24 hr	48 hr	72 hr
Sheep E	73.9 ± 16.7 (25)	60.2 ± 28.3 (6) N.S.	64.7 ± 22.4 (6) N.S.	71.0 ± 27.2 (6) N.S.
Rabbit E	58.5 ± 8.7 (25)	32.3 ± 19.3 (6) $P < 0.01$	30.3 ± 18.9 (6) $P < 0.01$	36.0 ± 15.0 (6) $P < 0.01$
	Uninjected	Time after intracoelomic injection of sheep E		
		24 hr	48 hr	72 hr
Sheep E	73.9 ± 16.7 (25)	37.8 ± 22.7 (6) $P < 0.01$	41.4 ± 11.8 (6) $P < 0.01$	55.4 ± 15.4 (6) $P < 0.05$
Rabbit E	58.5 ± 8.7 (25)	21.0 ± 20.5 (6) $P < 0.01$	25.6 ± 12.0 (6) $P < 0.01$	26.9 ± 10.4 (6) $P < 0.01$

TABLE III

Effect of multiple intracoelomic injections of mammalian erythrocytes (0.1 ml, 50% E in sterile sea saline) on hemolysin activity in Glycera *coelomic fluid. Mean ± SD (N). Two-tailed* t *test comparing % hemolysis mediated by coelomic fluid of injected animals to controls; for all comparisons the value of* t *is at the 0.01 level of significance. Regimen I:* Glycera *injected at 0 hr, 24 hr, and coelomic fluid sampled at 48 hr. Regimen II:* Glycera *injected at 0 hr, 24 hr, 72 hr, and coelomic fluid sampled at 96 hr.*

Indicator E	Percent hemolysis by coelomic fluid		
	Uninjected	Intracoelomic injection of rabbit E	
		Regimen I	Regimen II
Sheep E	73.9 ± 16.7 (25)	52.9 ± 12.9 (6)	49.1 ± 19.3 (6)
Rabbit E	58.5 ± 8.7 (25)	18.7 ± 14.3 (6)	21.5 ± 15.6 (6)
	Uninjected	Intracoelomic injection of sheep E	
		Regimen I	Regimen II
Sheep E	73.9 ± 16.7 (25)	33.1 ± 20.1 (6)	28.4 ± 25.9 (6)
Rabbit E	58.5 ± 8.7 (25)	12.5 ± 10.0 (6)	18.5 ± 17.2 (6)

rabbit E. The previously observed decreases in hemolytic activities after injection of 0.1 ml 50% E were not seen. Hemolysin activity in 10%-E-injected animals was not significantly different from controls.

Table III shows the effect of multiple intracoelomic injections of E on *Glycera* hemolysin. In one set of experiments, E was injected at 0 and 24 hr, and coelomic fluid sampled at 48 hr. In another set, E was injected at 9, 24, and 72 hr, and coelomic fluid was sampled at 96 hr. Repeated E injections, regardless of which regimen was used, reduced the hemolysin activity more than a single E injection. Injecting rabbit E according to these schedules significantly inhibited anti-sheep hemolysin, an effect not produced by a single injection.

Hemagglutinin

Glycera coelomic fluid contained hemagglutinating factor(s) active against both rabbit and sheep erythrocytes (Table IV). Rabbit E were agglutinated at significantly ($P < .005$) lower concentration of coelomic fluid than were sheep E.

TABLE IV

Hemagglutinin activity (titer) in Glycera *coelomic fluid against formaldehyde-treated erythrocytes (f E) and untreated erythrocytes (E). Titer = Log_2^{-1} $\bar{x}$ lowest coelomic fluid concentration giving visible hemagglutination ± SD (N).*

Indicator E	Hemagglutinin titer
Sheep E	2.9 ± 0.3 (10)
f Sheep E	6.9 ± 1.2 (20)
Rabbit E	4.6 ± 0.7 (10)
f Rabbit E	4.4 ± 0.6 (20)

Erythrocytes pretreated with formaldehyde were more strongly agglutinated at any given dilution of coelomic fluid than untreated E. Furthermore, anti-formalin-treated sheep-E hemagglutinin titers were significantly ($P < 0.005$) higher than those of untreated sheep E. Formalin treatment of rabbit E did not increase hemagglutinin titers.

The hemagglutinin did not appear to require divalent cations for activity; EDTA at 50 mM or less had negligible effect on its activity. Freezing and thawing had no effect on the hemagglutinating activity of coelomic fluid. However, samples heated to 56°C for 30 min had only 5–10% of the hemagglutinating capacity of untreated coelomic fluid.

Coelomic fluid was adsorbed three times with test Es, as described in Table I. Adsorption with either sheep E or rabbit E caused a reduction of both anti-sheep-E and anti-rabbit-E hemagglutinin; neither E type was more effective than the other as a hemagglutinin-adsorbing agent.

Attempts to alter hemagglutinin activity by prior intracoelomic injection of mammalian erythrocytes were ineffective. *Glycera* were injected with either sheep E or rabbit E; hemagglutinin titers were determined according to the schedules described previously for the hemolysin assays. Animals were injected with 0.1 ml suspension of either 10% or 50% E; neither anti-sheep-E nor anti-rabbit-E hemagglutinin titers showed significant change over a 72-hr period. Typical data comparing control values to 24-hr post-E injection titers are given in Table V. Multiple injections of 0.1 ml of 50% E suspension, administered according to the protocols given in the hemolysin section, also produced no increase in hemagglutinin titer.

Discussion

The coelomic fluid of *Glycera* contained naturally occurring lytic and agglutinating factors active against both sheep and rabbit erythrocytes. Based on their activity in the presence of EDTA, *Glycera* hemolysin required Ca^{2+} or Mg^{2+} for activity, whereas the hemagglutinin did not. Similar findings had been reported for the hemolysin and hemagglutinin of *Lumbricus terrestris* (Cooper *et al.*, 1974) and *Amphitrite ornata* (Garte and Russell, 1976), although Roch (1979) reported no EDTA inhibition of *Eisenia fetida* hemolysin. Many invertebrate hemagglutinins require Ca^{2+} for biological activity; for example, those from *Limulus* (Marchalonis and Edelman, 1968), lobsters (Hall and Rowlands, 1974), oysters (Acton *et al.*, 1969), and tunicates (Anderson and Good, 1975). However, other invertebrate lectins do not show a dependency on divalent cations; oyster hemagglutinin is active against sheep E and rabbit E in the absence of exogenous Ca^{2+} (McDade and Tripp, 1967), and insectan hemagglutinin is active in the presence

Table V

Hemagglutinin titer in Glycera *coelomic fluid 24 hr after an injection of erythrocytes (0.1 ml, 10% E suspension). Titer* = Log_2^{-1} $\bar{x}$ *lowest coelomic fluid concentration giving visible agglutination of formaldehyde-treated erythrocytes (f E)* ± *SD (N).*

Indicator E	Uninjected	Rabbit E injected	Sheep E injected
f Sheep E	7.2 ± 0.9 (10)	7.5 ± 1.1 (10)	7.3 ± 0.9 (10)
f Rabbit E	4.7 ± 0.8 (10)	4.7 ± 2.1 (10)	4.4 ± 0.8 (10)

of EDTA (Anderson *et al.*, 1972). In lower vertebrates including sharks, rays, and paddlefish, and in higher vertebrates such as guinea pigs and man, agglutinins are not inhibited by EDTA; however, hemolysins are EDTA-sensitive (Gewurz *et al.*, 1966).

Both *Glycera* hemolysin and hemagglutinin were heat-inactivated by holding at 56°C for 30–60 min, as was the case for these humoral factors in oligochaetes (Cooper *et al.*, 1974; Roch, 1979). Garte and Russell (1976) report that a polychaete hemagglutinin, amphitritin, is active at temperatures below 85°C. Heat stability above ~ 70°C is uncommon for invertebrate lectins or hemolysins. Incubation of E with *Glycera* hemolysin at temperatures of 4°–30° had little effect on the rate or extent of lysis. Sipunculid worm coelomic fluid also contains a hemolysin that has unaltered activity when incubated with E over a 0°–25° temperature range (Weinheimer *et al.*, 1970). In this regard, it is interesting that the activity of hemolysins of poikilothermic vertebrates is increased at lower temperatures (Cushing, 1945; Gewurz *et al.*, 1966). Considerable activity of *Glycera* hemolysin is lost after freezing and thawing, a reaction not typical of hemolysins from other invertebrates such as *Mercenaria mercenaria* (Anderson, unpublished observation), the spiny lobster (Weinheimer *et al.*, 1969), and the earthworm (Roch, 1979). The hemagglutinin of *Glycera* does not lose activity after storage at − 80°C; this is typical of most invertebrate lectins (Pauley, 1974).

Since both hemolysins and agglutinins are postulated to play a role in the immune reactions of invertebrates, their target cell specificity should be considered. Based on our observations, both factors could react with E from several mammalian species; however, the intensity of the reaction against a particular type of indicator cell was different from that against another. Also, the hemolysin adsorption studies showed that, although there was considerable cross-reactivity, there was some evidence of specificity. While adsorption with either sheep E or rabbit E reduced hemolysin activity against both kinds of E, adsorption with sheep E reduced anti-sheep E lysin more than anti-rabbit E lysin. A similar directed effect on *Glycera* lysins was not seen after adsorption with rabbit E. Adsorption of coelomic fluid with either E type reduced agglutination of both equally.

It is not known if *Glycera* hemagglutinins and hemolysins are single entities with rather broad specificities or if they exist in multiple forms with more limited specificities. Current data suggest that both of these factors in other annelids may exist in multiple forms. Garte and Russell (1976) isolated and purified a hemagglutinin from the polychaete *Amphitrite ornata*. Sephadex G-100 fractionation yielded three active fractions with molecular weight of 30,000, 54,000, and 100,000. These fractions showed parallel specificity toward certain E, but different patterns of agglutination for other E types. The 30,000 dalton glycoprotein fraction (Amphitritin) probably was a subunit of the higher molecular weight agglutinin. Earthworm hemolysin was shown by the use of isoelectric focusing to be composed of four lipoprotein isoforms (Roch, 1979). Each isoform had natural lytic activity against sheep erythrocytes. One isoform was found in all *Eisenia fetida* studied. The other isoforms were variably present. It was suggested that the natural hemolysin of *Eisenia* was induced and played a role during second set graft rejection (Chateaureynaud-Duprat and Izoard, 1977a). However, Roch (1979) found no qualitative difference in the pattern of hemolytic proteins (isoforms) in grafted animals; the patterns remained stable regardless of grafting, wounding, starvation, or age of the worms.

It has been reported that hemolysins and hemagglutinins may be induced in terrestrial annelids by grafting or by the injection of erythrocytes (Cooper *et al.*, 1974; Chateaureynaud-Duprat and Izoard, 1977b). We were unable to induce in *Glycera* either hemolysins or hemagglutinins by either single or multiple intracoelomic injections of erythrocytes. Cooper *et al.* (1974) injected *Lumbricus* with 0.1 ml of a 10% E suspension; 24 hr later, lysis and agglutination of both sheep and rabbit E were enhanced. Chateaureynaud-Duprat and Izoard (1977b) reported that *Lumbricus* coelomic fluid had no agglutinating or lysing activity until 3–4 days after the injection of sheep E. None of our procedures involving single or multiple injections of 0.1 ml of 10 or 50% mammalian E caused any significant change in *Glycera* hemagglutinin titers over a 72–96 hr period of observation. No induction of hemolytic activity was achieved by our protocols; in most cases, hemolysin activity was significantly decreased following E injection. This reduction was more pronounced in the coelomic fluid of those worms that received multiple E injections, suggesting that the hemolysin was simply removed from circulation by adsorption to the membranes of the injected erythrocytes.

Studies of graft rejection and other manifestations of immunological memory among polychaetes have not been undertaken. However, our studies suggest that the naturally occurring hemolysins and hemagglutinins of *Glycera* are not inducible. Therefore, one would not predict that these humoral factors would play a significant role in anamnestic immune reactions. This is not to exclude the possibility that the factors may have an important function in a more primitive immunological recognition system.

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Summary

1. Naturally occurring hemolysins and hemagglutinins active against erythrocytes from two mammalian species were found in the coelomic fluid of the polychaete *Glycera dibranchiata*.

2. The hemolysin required divalent cations and was inactivated by freezing and thawing; the hemagglutinin retained activity after freezing and was active in the presence of EDTA. Both factors were thermolabile above 56°C.

3. The rate of reaction and degree of intensity of lysis or agglutination was different for each type of test erythrocyte. These differences were found in all individuals studied, and were considered to be characteristic of the species.

4. Both hemolysin and hemagglutinin were readily adsorbed from coelomic fluid by the addition of erythrocytes. Adsorption with a particular type of erythrocyte resulted not only in reduced lytic and agglutinating activity against that cell type, but also in reduced activity against red blood cells from other species.

5. The hemolysins and hemagglutinins of *Glycera* were not induced by single or multiple intracoelomic injections of erythrocytes. These experimental treatments had no significant effect on hemagglutinin titers, and usually caused a marked reduction in hemolysin activity. The consequence of the apparent lack of inducible humoral factors on possible anamnestic immune mechanisms, such as graft recognition and destruction, has yet to be evaluated.

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