

ECHINODERM IMMUNOLOGY: BACTERIAL CLEARANCE BY THE SEA URCHIN *STRONGYLOCENTROTUS PURPURATUS*

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ABSTRACT

Characteristics of bacterial clearance were investigated in the purple sea urchin, *Strongylocentrotus purpuratus* (Echinodermata: Echinoidea). Primary clearance kinetics were determined for three bacteria, a marine Gram negative motile rod, a marine Gram positive non-motile rod, and a Gram negative freshwater fish pathogen, *Aeromonas salmonicida*. Clearance kinetics differed for each of the three bacteria. Secondary clearance rates were not significantly different from primary clearance rates for any of the three bacteria, regardless of the time interval between inoculations (9–21 days), implying a probable absence of immunologic memory. During primary clearance, total coelomocyte counts declined 93% by 90 min post injection. All four coelomocyte types declined, however the relative proportions of each type changed during the six-hour sampling period. In cell-free coelomic fluid, viable counts of marine bacteria declined, with different kinetics for the two species. Viable counts in sea water controls did not change. Declines in viable counts may be due to bactericidal activity and/or agglutination, although bacterial agglutination was not observed.

INTRODUCTION

Despite recent advances (reviewed by Cooper, 1976; Manning and Turner, 1976; Marchalonis, 1977; Hildemann *et al.*, 1981), the phylogeny of immunity remains obscure. In particular, the mechanisms of invertebrate immunity are diverse, and many are poorly understood. The phylogenetic position of echinoderms makes them pivotal to the understanding of the phylogeny of immunity and the evolution of vertebrate immunity.

Allogeneic transplantation studies have shown that memory and specificity, two important characteristics of vertebrate immune responses, are possessed by echinoderms (Karp and Hildemann, 1976; Coffaro and Hinegardner, 1977; Coffaro, 1980), as well as by members of other invertebrate phyla (sponges, Hildemann *et al.*, 1979; cnidarians, Hildemann *et al.*, 1977; annelids, Cooper, 1970). Any role of memory and specificity in invertebrate internal defenses to potentially infectious agents has yet to be identified.

In this study, we sought to determine whether the echinoderm, *Strongylocentrotus purpuratus*, exhibits memory or altered reactivity on secondary contact with biologically relevant antigens, namely bacteria. Additionally, because the coelomic fluid of healthy echinoderms is generally aseptic (Bang and Lemma, 1962; Unkles, 1977; Kaneshiro and Karp, 1980), and few studies have been made on the characteristics and mechanisms of bacterial clearance in echinoderms (Johnson, 1969a, b; Johnson *et al.*, 1970; Johnson and Chapman, 1971; Wardlaw and Unkles, 1978),

Received 11 April 1983; accepted 25 July 1983.

we also studied some of the *in vivo* and *in vitro* primary interactions between echinoderm coelomocytes and cell-free coelomic fluid and bacteria.

MATERIALS AND METHODS

Collection and maintenance of animals

Strongylocentrotus purpuratus (80–150 g) were hand collected from intertidal surge channels at Yaquina Head, Newport, Oregon where the species is abundant. Care was taken to avoid damaging the urchins during collection and transportation to the 22,700 liter recirculating sea water system at Oregon State University in Corvallis, 70 km away. Water temperatures were 12–15°C, comparable to coastal temperatures. Urchins were fed macroalgae *ad libitum*.

Isolation and culture of bacteria

One Gram negative motile rod and one Gram positive non-motile rod were isolated from the coelomic fluid (CF) of moribund sea urchins. Selection was based on growth characteristics (rate of growth, colony color and morphology). All bacteria were grown in marine broth 2216E (Difco Laboratories), enriched with peptone (5 g l⁻¹) and yeast extract (3 g l⁻¹). Nutrient agar (1%) in marine broth was used for pour plate viable counts. For each experiment, bacteria were inoculated into fresh, enriched broth in triple baffle, side-arm flasks and grown at room temperature (20–23°C) on a rotary table (100 rpm). The bacteria were harvested during log phase, centrifuged for 10 min at 300 g, and resuspended in cold (10°C) sterile sea water to appropriate concentrations. Bacterial suspensions used for injections were serially diluted and plated to determine actual inoculation doses.

Because the urchins may previously have encountered the marine bacteria, a freshwater bacterium, *Aeromonas salmonicida*, was also used for some experiments. *A. salmonicida* is a salmonid fish pathogen, a Gram negative non-motile rod generally not present in the marine environment (although it has rarely been found in marine fish, Evelyn, 1971). These bacteria were grown in tryptic soy broth using methods already described.

Coelomic fluid volume estimates

Estimation of CF volumes was required for determining the quantity of bacteria to be injected for a specific concentration in the perivisceral coelom. The weight, test diameter, test height, and peristomium diameter were measured for 28 urchins. The CF was then drained through a cut in the peristomium and the volume measured. Each external parameter was regressed on the CF volume. Weight was found to be the best indicator of CF volume ($R^2 = 0.96$). The following equation defined the relationship and was used in all experiments:

$$\text{Coelomic fluid volume (ml)} = 0.35 \times \text{weight (g)} - 4.2.$$

Clearance experiments

Urchins were weighed, CF volumes estimated, and the dose of bacteria calculated for a given final concentration of bacteria in the CF. During experiments, urchins were kept in 20 liter plastic aquaria filled with aerated sea water at 10°C.

Before injections and sampling, the peristomium was washed several times with cold sterile sea water. Care was taken to avoid tearing tube feet during handling. The bacterial suspension (0.4–0.6 ml) was injected through the peristomial mem-

brane with a 26 gauge, 0.5-inch needle and 1 ml tuberculin syringe. Preliminary experiments showed that 40–90 min were required for even dispersal of bacteria. Because we wanted to sample within that time, half of the inoculum was injected, with the other half injected 180° from the initial injection site. Coelomic fluid samples (0.2–0.3 ml) were removed with a sterile 26 g, 0.5-inch needle and 1 ml syringe. Samples taken before the injection of bacteria were directly plated for sterility checks. Samples taken after injection of bacteria were serially diluted in sterile sea water and plated by pour plate methods for viable counts. Distinctive colony morphologies, colors, and growth rates were used to help ascertain that these bacteria were those previously injected.

Total and differential coelomocyte cell counts

At various times after urchins were injected with the marine Gram negative bacteria, or with an equal volume of sterile sea water, CF was removed with a 20 g, 1.5-inch needle into an equal volume of cold anticoagulant (30 mM EDTA in 0.3 M Hepes buffered sea water, after Bertheussen and Seljelid, 1978). Two differential counts were made and averaged using a Brightline hemacytometer. The four major coelomocyte types found in *Strongylocentrotus* spp. (Johnson, 1969a; Bertheussen and Seljelid, 1978), phagocytes (leukocytes), vibratile cells, and red and colorless spherule (morula) cells, were counted. Total counts were determined by adding the counts for the four cell types.

Humoral factors: In vitro effects of cell-free coelomic fluid on viable counts of bacteria

Coelomic fluid was removed with a 20 g, 1.5-inch needle or drained out through a cut in the peristomium into a sterile, cold beaker. The CF was immediately filtered by gentle passage through two Millipore prefilters then sterilized using a 0.22 μ m Millipore filter. Since clotting of CF (30 min, 10°C) before filtration does not seem to affect its bactericidal activity (Wardlaw and Unkles, 1978; Yui, 1982), we routinely filtered before clot formation.

Two ml of cell-free CF were placed in sterile glass vials. Sterile-filtered artificial sea water (Instant Ocean) or Hepes sea water medium (Bertheussen and Seljelid, 1978) was used as control fluid. Twenty μ l aliquots of the bacterial suspension were added to each vial, which was then held at 10°C. Samples (10 and 100 μ l) were removed and plated by pour plate methods for viable counts.

Humoral factors: Bacterial agglutinins

The CF (5–10 ml) was collected through a cut in the peristomium, filtered, added to the first well of a microtiter plate and serially diluted by one-half with 10°C artificial sea water (Instant Ocean). The last well was a sea water-only control. An equal volume of bacterial suspension in sea water was added to each well, then the plates were covered and placed on a rotary table (60 rpm) at 10°C. After 30 min to 20 h plates were inspected for agglutination.

RESULTS

Primary clearance

Gram negative bacteria. Six urchins injected with 3.3×10^7 marine Gram negative bacteria ml⁻¹ of CF rapidly reduced viable counts (v.c.) of bacteria in the CF

in the first hour post-injection (p.i.) by 95.7%, followed by a period of slower clearance (Fig. 1). Clearance continued after 6 h (Fig. 2), and bacteria were not detected 4–8 days p.i. To further characterize the initial 90 min of clearance, four or five samples were removed from each of nine urchins previously injected with 10^5 – 10^7 bacteria ml^{-1} of CF. Clearance was exponential. Lines were fitted to the log-transformed data using linear regression by calculating independent slopes for each urchin and obtaining a mean and standard error for those slopes. The slopes are equivalent to the “Phagocytic Index” or K value as defined by Biozzi *et al.* (1953) for particle clearance kinetics in mammals, and Renwrandt and Mohr (1978) for particle clearance in a land snail, *Helix*. For the first 90 min of clearance, a K value of $-0.0179 \pm 0.0016 \log_{10} \text{ bacteria min}^{-1}$ was obtained for the Gram negative ($R^2 = 0.963$). The slope was not strongly dose dependent (Yui, 1982).

Because of individual differences, primary and secondary clearance rates were determined for the same individual urchins. Injection doses of 10^6 – 10^7 bacteria ml^{-1} of CF were selected for further experiments because, at those doses, bacteria were:

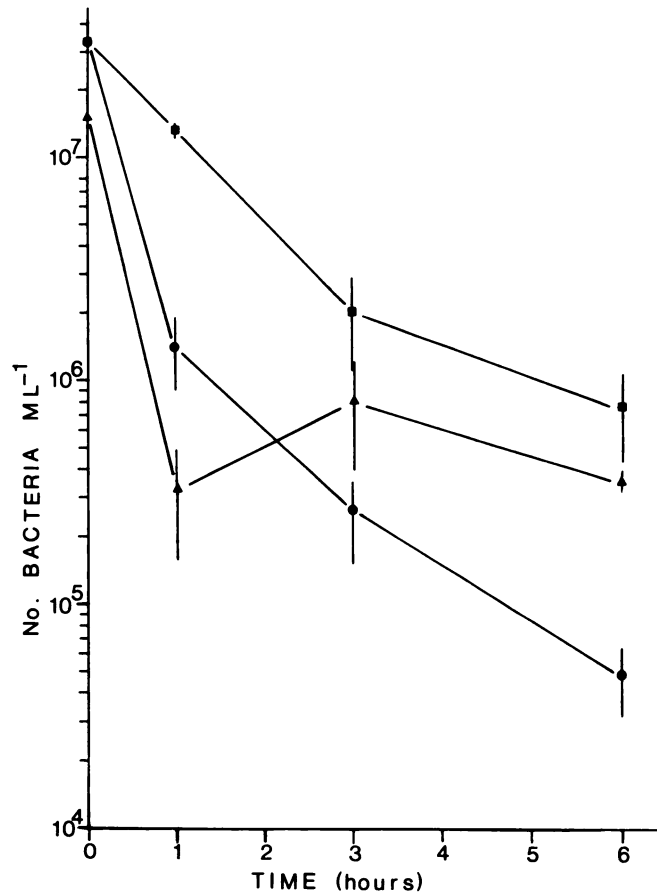


FIGURE 1. Primary clearance of the Gram negative and Gram positive marine bacteria, and *Aeromonas salmonicida*, sampled at 1, 3, and 6 hours post-injection (mean \pm standard error). ● Gram negative ($n = 6$), ▲ Gram positive ($n = 3$), ■ *Aeromonas salmonicida* ($n = 3$).

(a) cleared without mortality or obvious trauma, (b) detectable at high enough levels at 6 and 24 h p.i. that a decline upon secondary injection was still quantifiable even with small CF sample volumes, and (c) persistent in the CF for a period of 4–8 days. This persistence of viable bacteria in the CF was considered advantageous because sensitization and induction of memory may require a long period of exposure, as with graft rejection. Furthermore, the need for booster injections was precluded.

Gram positive bacteria. A rapid decline (97.7%) in v.c. also occurred within 1 h after injection of Gram positive bacteria (Fig. 1). However, unlike the results with the Gram negative bacteria, v.c. were slightly higher at 3 h with declines continuing to 6 and 24 h p.i. (Fig. 2). Bacteria were not detected 8–12 days p.i. The K value for the first 90 min was -0.0195 ± 0.0037 ($n = 3$), not significantly different from K for the Gram negative bacteria.

Aeromonas salmonicida. At 1 h p.i., v.c. dropped only 61.5%, a much lower initial rate of clearance than that of the marine bacteria (Fig. 1). Clearance continued at the same rate to 3 h and more slowly thereafter.

Primary versus secondary clearance

Because the first and second inoculation doses could not be made identical, the slopes of the lines were calculated from \log_{10} -transformed v.c. at time = 0 to 6 and

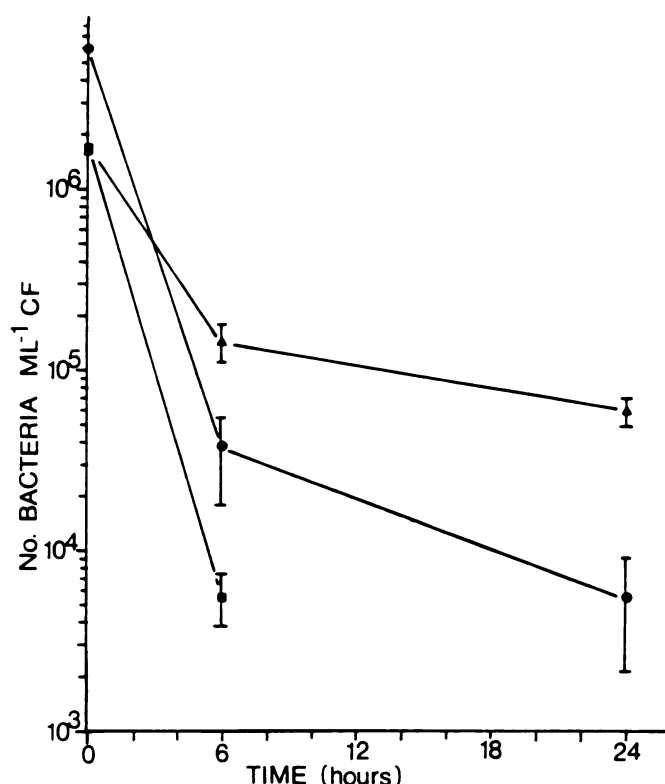


FIGURE 2. Primary clearance of the Gram negative and Gram positive marine bacteria, sampled at 6 and 24 hours post-injection, and *Aeromonas salmonicida*, sampled at 6 hours post-injection (mean \pm standard error). ● Gram negative ($n = 11$), ▲ Gram positive ($n = 12$), ■ *Aeromonas salmonicida* ($n = 6$).

24 h. The difference in slope between primary and secondary clearance for each urchin was then calculated and compared to zero (Table I).

Urchins injected with approximately 10^7 bacteria ml^{-1} of CF were challenged with a similar dose of the same bacteria 9, 14, and 21 days after the first injection of the two marine bacteria, and after 19 days with *Aeromonas salmonicida*. In no case was the mean difference in slope between primary and secondary clearance significantly different from zero. Even when data from the three injection times were pooled, the mean difference in slope was not significantly different from zero.

Total and differential counts of coelomocytes

The average number of coelomocytes ml^{-1} counted from uninjected urchins collected in August and September was $1.0 \pm 0.2 \times 10^7$ ($n = 6$). The majority of cells were phagocytes ($67.8 \pm 4.4\%$), followed by vibratiles ($16.8 \pm 3.8\%$), red spherules ($10.5 \pm 3.1\%$), and colorless spherules ($5.0 \pm 1.3\%$). These values are similar to those reported for *S. droebachiensis* (Bertheussen and Seljelid, 1978). In October and November, coelomocyte counts were lower, due predominantly to fewer phagocytes (Yui, 1982).

Sea water injected control urchins exhibited a sharp, brief decline in cell numbers during the first hour p.i., followed by rapid recovery to pre-injection values (Fig. 3). After injection of Gram negative bacteria, a sharp decline was seen during the first 1.5 h. Declines continued, more slowly, to about 5 h. The overall drop was from 8.8×10^6 to 6×10^5 coelomocytes ml^{-1} , a 93% decline.

After injection, all cell types declined (Fig. 3), with a change in the relative proportion of each cell type (Fig. 4). The percentages of phagocytes and red spherule

TABLE I

Mean difference in slope between clearance of primary (1°) and secondary (2°) injections of Gram negative and positive marine bacteria and *Aeromonas salmonicida**

1°-2° Interval (days)	Sample time (hours)	Mean difference in slope (1° minus 2°) $\bar{x} \pm \text{SD}$ (n)**		
		Gram negative	Gram positive	<i>Aeromonas salmonicida</i>
9	6	0.083 ± 0.106 (4)	-0.027 ± 0.108 (4)	—
	24	0.006 ± 0.018 (4)	0.011 ± 0.011 (4)	—
14	6	0.038 ± 0.190 (4)	0.027 ± 0.078 (4)	—
	24	-0.030 ± 0.051 (3)	-0.008 ± 0.016 (4)	—
21	6	0.032 ± 0.182 (4)	-0.063 ± 0.061 (4)	—
	24	0.013 ± 0.024 (4)	-0.020 ± 0.025 (4)	—
19	6	—	—	0.067 ± 0.090 (4)

* Approximately 1×10^7 bacteria ml^{-1} of coelomic fluid were injected for each clearance rate determination.

** A negative value indicates a more rapid 2° rate of clearance, a positive value, a less rapid 2° rate of clearance. None of these values were significantly different from zero using a Student's *t*-test.

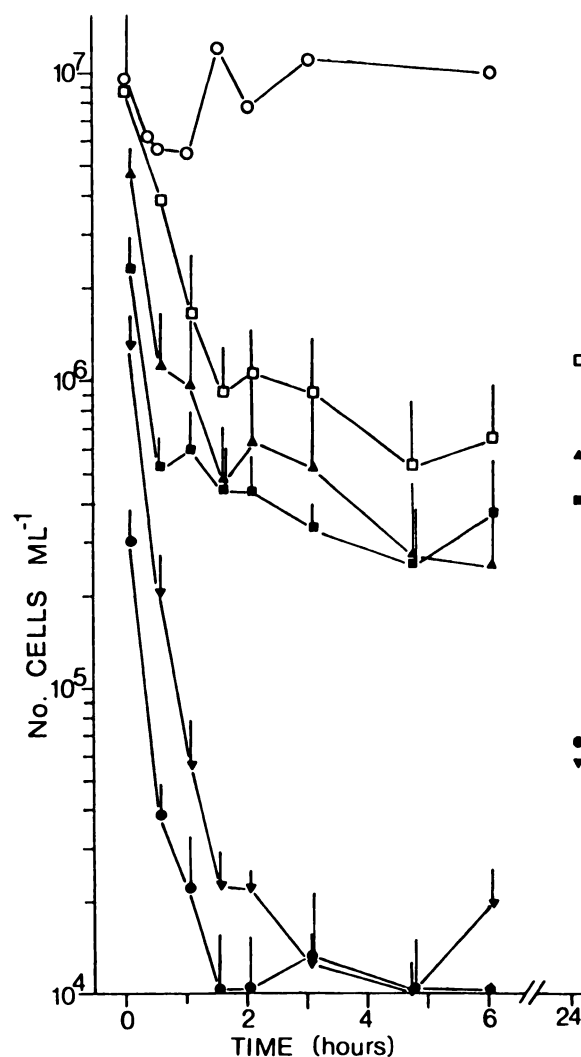


FIGURE 3. Total and differential coelomocyte counts (mean \pm standard error) after injection with 10^8 Gram negative bacteria ml^{-1} of coelomic fluid ($n = 3$). The 24-hour sample was from the only urchin sampled at that time. Also included are the total coelomocyte counts for the sea water-injected control ($n = 1$). Total coelomocyte counts: \circ sea water injected, \square bacteria injected; differential coelomocyte counts: \blacktriangle phagocytes, \blacksquare vibratiles, \blacktriangledown red spherules, \bullet colorless spherules.

cells declined while the percentage of vibratiles increased. The percentage of colorless spherule cells did not change appreciably, although the values were at very low levels throughout. In the one animal sampled at 24 h p.i., cell counts (Fig. 3) and relative proportions (Fig. 4) were approaching pre-injection values.

Humoral factors: In vitro effects of cell-free coelomic fluid on viable counts of bacteria

Gram negative bacteria. Coelomic fluids from six urchins, three injected 3 days earlier and three uninjected, were tested for their effects on viability of the marine

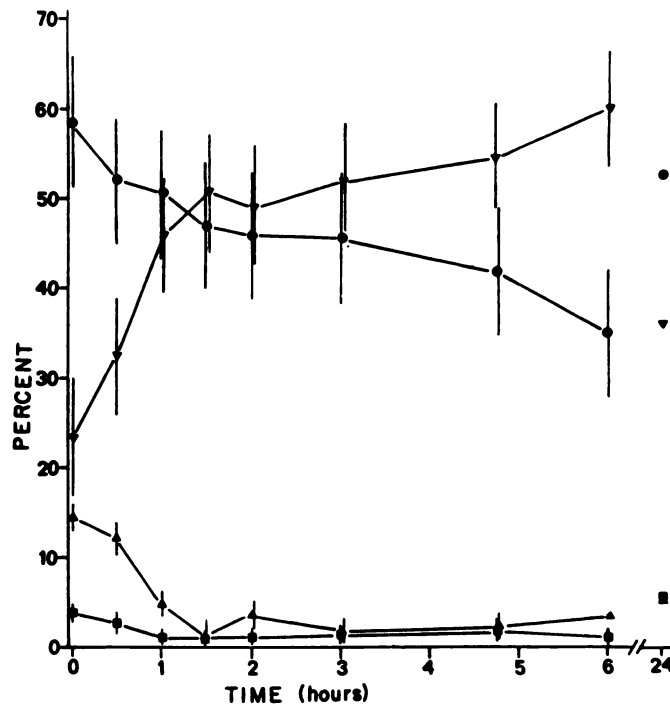


FIGURE 4. Relative proportions (mean % \pm standard error) of the four coelomocyte types after injection of 10^7 bacteria ml^{-1} of coelomic fluid ($n = 3$). The 24-hour sample was from only one of the three urchins. ● phagocytes, ▼ vibratiles, ▲ red spherules, ■ colorless spherules.

Gram negative bacteria. Sea water served as a control. No significant differences were noted between any of the three treatments at 15 and 45 min post-inoculation (one-sided Student's *t*-test) (Fig. 5). Although v.c. were lower in CF from preinjected than in uninjected urchins at 90 min or 5 h, the differences were not significant. V.c. were significantly lower in the CF from the six urchins than in sea water at 90 min ($P < 0.005$) and 5 h ($P < 0.01$).

Gram positive bacteria. The change in v.c. of Gram positive bacteria in CF differed from that of the Gram negative (Fig. 6). V.c. had declined 2.5 orders of magnitude below those of the initial inoculum and the sea water control at 2 h post-inoculation. Test fluids from each of five urchins exhibited this large decline in v.c. at 2 h while none of the three controls exhibited a similar response. This result cannot reflect bactericidal activity since counts returned to values not significantly different from sea water controls by 5 h. At 20 h, v.c. in CF were lower than those in sea water ($P = 0.05$). Viable counts in CF were quite variable relative to sea water controls. This variability probably reflects differences in amount(s) of humoral factor(s) present in the CF rather than differences due to sampling methods.

Bacterial agglutinins

Cell-free CF samples from eight urchins were mixed with the Gram negative bacteria and CF samples from four urchins were tested with the Gram positive bacteria, in suspensions ranging from 5×10^4 to 5×10^8 bacteria ml^{-1} . Samples

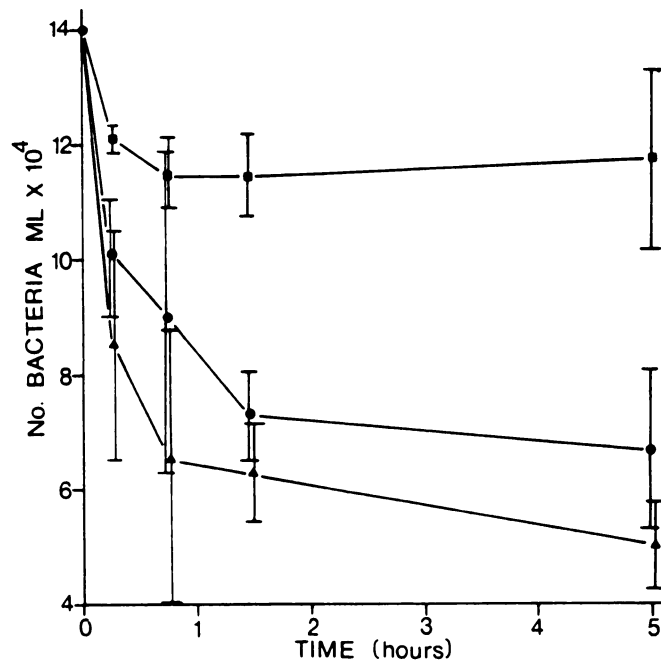


FIGURE 5. Viable counts (mean \pm standard error) for the Gram negative bacteria in sea water and cell-free coelomic fluid from injected and uninjected urchins. ■ sea water ($n = 3$), ● uninjected ($n = 3$), ▲ injected ($n = 3$).

were checked at various times from 30 min to 20 h. Neither of the two bacteria were agglutinated.

DISCUSSION

Primary clearance

Clearance from echinoderm coelomic fluid has been reported after the injection of bacteria (Wardlaw and Unkles, 1978; Kaneshiro and Karp, 1980; Bertheussen, 1981), bacteriophage T₄ (Coffaro, 1978), red blood cells, latex beads and yeast cells (Bertheussen, 1981), xenogeneic cells and carborundum (Reinisch and Bang, 1971), and bovine and human serum albumin (Hilgard and Phillips, 1968). However, the kinetics of particle clearance have not been followed, and the fates of these particles in echinoderms remain unknown.

Strongylocentrotus purpuratus efficiently cleared all three bacteria from its coelomic fluid. Viable counts were reduced 90–99% in 3–6 h after injection of doses of approximately 10^6 – 10^7 bacteria ml^{-1} of CF. Clearance occurred in at least two phases, with the rate of clearance of one of the bacteria different from that of the others during each phase. Clearance was approximately exponential for the first 1.5 h p.i. for the Gram negative bacteria. Both marine bacteria were cleared at similar rates over the first 1.5 h while the initial rate of clearance of *A. salmonicida* was slower than that of the marine bacteria.

The second phase of clearance was slower. However, by 6 h p.i., *A. salmonicida* had been cleared almost as well as the marine Gram negative bacteria despite the slower initial rate, suggesting a higher rate of clearance after 1 h compared to the

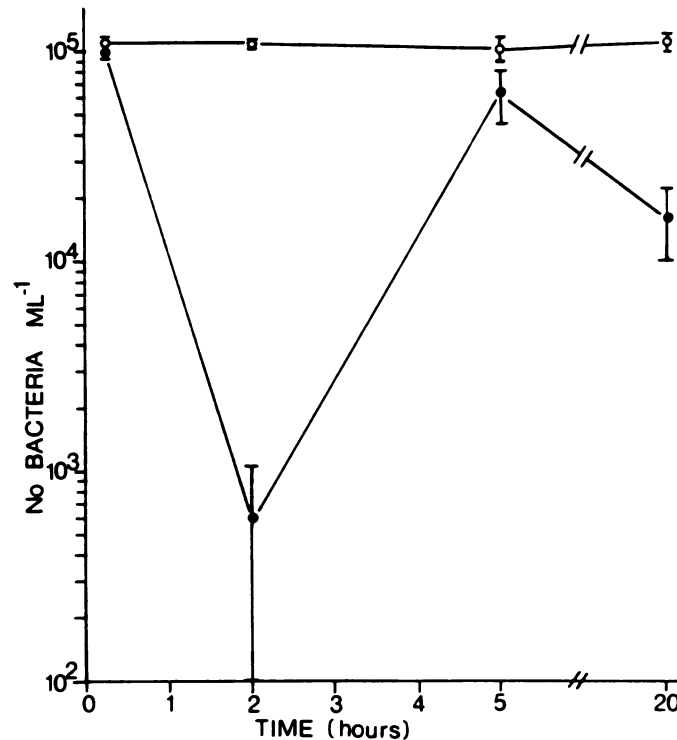


FIGURE 6. Viable counts for the Gram positive bacteria in sea water and cell-free coelomic fluid (mean \pm standard error). \circ sea water ($n = 3$), \bullet cell-free coelomic fluid ($n = 5$).

other bacteria. The marine Gram positive, on the other hand, had a significantly lower reduction in v.c. at 6 h p.i. compared with the marine Gram negative bacteria and *A. salmonicida*. At 24 h the overall percent reduction of Gram positive bacteria was still lower than that of the Gram negative.

The clearance we observed resembles the equivalent process in other invertebrates and in mammals. Generally, the first phase of clearance in mammals is rapid and exponential, with 90–99.9% reduction in circulating bacteria (Rogers, 1960). This phase is relatively independent of the nature of the microbe, the animal under study, and the subsequent outcome of injection. During the second phase the microbe either persists at lower concentrations or is slowly removed over several hours or days. This phase differs considerably with different bacteria.

Similarly, four mollusc species (Bayne and Kime, 1970; Pauley *et al.*, 1971; Bayne, 1973; van der Knaap *et al.*, 1981) and a crustacean (Smith and Ratcliffe, 1980) cleared 90–99% of injected bacteria (10^6 – 10^9 bacteria per animal) in the first 2–3 h p.i., with slower subsequent declines after 3 h. In some species Gram positive and Gram negative bacteria may be cleared equally well (Smith and Ratcliffe, 1980), while in others, Gram positives may be cleared more rapidly than Gram negatives (van der Knaap *et al.*, 1981). Gram negative and Gram positive bacteria may be recognized by different coelomocyte subpopulations in the marine annelid, *Arenicola marina* (Fitzgerald and Ratcliffe, 1982). In *Strongylocentrotus* spp., Johnson (1969b) noted more active phagocytosis of Gram positive than Gram negative bacteria, in

contrast with this study. This difference may be due to the different bacteria used or because phagocytosis alone does not determine the overall rate of bacterial clearance.

Total and differential cell counts indicated a 93% reduction in numbers of all four coelomocyte types accompanying primary clearance of Gram negative bacteria. Similar declines in circulating hemocyte numbers have also been observed in crustaceans (Cornick and Stewart, 1968; Smith and Ratcliffe, 1980), insects (Wittig, 1965; Gagen and Ratcliffe, 1976), molluscs (Bayne and Kime, 1970; Pauley *et al.*, 1971; van der Knapp *et al.*, 1981; Renwrautz *et al.*, 1981), and in leukocyte numbers in mammals (Rogers, 1960).

Clotting, due to aggregation of phagocytes (Johnson, 1969a), no doubt contributes to some of the observed decline in numbers of cells and bacteria. Cellular clots were often observed in coelomic fluid samples which were taken after injection of bacteria. Bertheussen (1981) noted clot formation in *S. droebachiensis* only after injection of bacteria and not after injection of other particles (red blood cells, yeast, latex). Cellular aggregation and/or attachment to epithelia may enhance phagocytosis by providing a substrate for trapping bacteria. Vertebrate leukocytes trap and phagocytose encapsulated bacteria more easily when attached to substrates (Wood, 1960).

Cellular clotting may also explain the observation that red spherule cells declined proportionately with phagocytes while vibratile cells increased proportionately. Red spherules are relatively non-motile and they may therefore be passively caught with aggregating phagocytes as observed in hanging drops (Johnson, 1969a). Although the percentage of non-motile colorless spherules did not appear to change, actual cell counts were very low so changes were difficult to detect. Vibratiles, being highly motile, may extricate themselves from the clots or may be recruited more rapidly from elsewhere, causing the observed increase in relative abundance.

Cell-free coelomic fluid contains one or more humoral factor(s) active against both Gram negative and Gram positive bacteria. To the contrary, Wardlaw and Unkles (1978) found that coelomocytes were required for bactericidal activity. Because the quantity of humoral factor(s) would be limited and not renewable in a cell-free *in vitro* system, the number of bacteria per given volume of coelomic fluid would be critical in the detection of activity. In addition, observable activity may depend upon the species of bacteria used and the species of echinoderm from which test fluids are obtained.

Declines of 6 to 9×10^4 v.c. ml^{-1} of CF from 6 urchins were observed *in vitro* by 2 h after inoculation with Gram negative bacteria, enough to account for at least some of the decline observed *in vivo*. It is possible that the decrease in v.c. was due to agglutination rather than bacterial killing, although agglutination was not detected in this study. Prior injection with bacteria did not accelerate the decline.

Results obtained with Gram positive bacteria in cell-free CF are difficult to explain since agglutinins were not found. Large declines in v.c. were noted in the 2 h samples from the CF from all 5 urchins tested. These declines could not be explained by bacterial killing because v.c. returned to control levels by 5 h p.i. and these bacteria do not grow that quickly at 10°C. These results, however, do correspond with the lower v.c. noted at 1 h than at 3 h in *in vivo* clearance of the Gram positive. Although v.c. in cell-free CF *in vitro* were not significantly lower than in sea water at 5 h, v.c. at 20 h were considerably lower than the sea water controls and the initial inoculation dose.

Although bacterial agglutination was not observed, these experiments do not rule out the possibility that agglutinins function in clearance of bacteria. In fact, the

results of the bactericidal test using the Gram positive suggest that agglutination may occur temporarily at 2 h p.i. Relative proportions of bacteria to concentration of active molecules in the CF may have been inappropriate for the observation of agglutination.

Differences in the rates of clearance of the three bacteria may depend on (1) differences in specific or non-specific cellular recognition and response, (2) the quantity, rate of release and effectiveness of humoral bactericidal substances, agglutinins, and/or opsonins, and (3) the rate of bacterial reproduction. Based on our studies of *in vivo* and *in vitro* primary clearance, it appears that both cellular and humoral factors are involved in bacterial clearance. Clotting and the overall decline of coelomocytes paralleled bacterial clearance. Bacteria were observed in the cellular clots, and within phagocytes after injection (Yui, 1982). Different quantities of CF factor(s) or numbers of responding cells may cause the large individual variation observed between urchins.

The two stages of bacterial clearance in *S. purpuratus* are probably the result of a decline in active cells and/or molecules in the first few hours with further clearance being dependent on the level of cell recruitment or activation and/or release of active molecules.

Primary versus secondary clearance

Our failure to find accelerated secondary clearance of bacteria is consistent with the few other attempts to induce elevated responses in echinoderms to foreign materials, other than grafts. Coffaro (1978) was unable to elicit accelerated clearance of the bacteriophage T₄ in *Lytechinus pictus*. No increase in the rate of foreign protein uptake was detected in *S. purpuratus* after injection of four doses of protein at 18 h, 3 days, 7 days, and 2 months after primary injection (Hilgard and Phillips, 1968). *Asterias vulgaris* was inoculated with 5×10^5 *Arbacia* cells six times, with no change in the rate of cell disappearance (Reinisch and Bang, 1971).

Secondary responses to bacteria have been elevated in other phyla, including both deuterostomes (e.g., vertebrates) and protostomes, in which molluscs (see Bayne *et al.*, 1980 for review; Bayne, 1980; van der Knapp, 1980), sipunculids (Evans *et al.*, 1969), crustaceans (Evans *et al.*, 1968; Acton *et al.*, 1969; McKay and Jenkin, 1969; Stewart and Zwicker, 1974) and insects (Boman *et al.*, 1972; Boman *et al.*, 1974) have yielded positive results. However, in the invertebrate examples, either enhancement has been non-specific or the extent of specificity has not been thoroughly tested, and mechanisms of enhancement are seldom known.

The lack of accelerated secondary clearance in this study can be explained in the following ways:

- (1) There is no memory component to bacterial clearance, although memory does appear to exist in responses to grafts and may exist in response to other infectious organisms.
- (2) Memory does exist but was undetected due to:
 - (a) previous exposure to the same or similar bacteria sharing certain surface characteristics or molecules critical for recognition and response, or
 - (b) lack of sensitivity in detection of memory or sub-optimal enhancement of secondary responses.

Despite such considerations, there is a need to conduct experiments to identify the conditions under which echinoderms will respond optimally to immunologic

challenge. Without such studies, the mechanisms of echinoderm internal defenses and the evolutionary history of immune responsiveness will remain elusive.

ACKNOWLEDGMENTS

We thank E. S. Loker and C. A. Boswell for suggestions on the manuscript, and J. A. Longmate for assistance collecting urchins. The research was supported by grants from the Oregon State University Zoology Department Research Fund.

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