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LIPOPOLYSACCHARIDE ACTIVATES THE SEA URCHIN IMMUNE SYSTEM

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☐**Abstract**—Profilin is a small, actin-binding protein that functions at the intersection of signal transduction and cytoskeletal modifications. Increases in the number of profilin messages per cell correlate with sea urchin coelomocyte activation in response to injury. Here we show that coelomocytes respond to immune challenge from lipopolysaccharide with significant elevations in profilin transcripts per coelomocyte.

☐**Keywords**—Sea urchin; *Strongylocentrotus purpuratus*; Coelomocyte activation; Profilin; LPS.

Nomenclature

LPS   lipopolysaccharide
cf   coelomic fluid

Introduction

Sea urchin coelomocytes are very sensitive to physiological perturbations of the internal milieu. They respond to such perturbation by activation processes that entail rapid alterations in motility, increases in phagocytosis or encapsula-

tion, and secretion or degranulation of toxic factors (1–4). We found earlier that coelomocyte activation in response to minor injury results in enhanced transcription of the profilin gene (5). Profilin is an important mediator of cytoskeletal modification triggered by signal transduction events [for recent review, see (6)]. Because of the competition between profilin and thymosinβ_4 for G-actin, slight variations in the profilin concentration greatly alter its effectiveness to catalyze the ADP–ATP exchange on G-actin. This nucleotide exchange promotes filament polymerization and modifies the cytoskeleton. Slight increases in the cytoplasmic profilin concentration occur as a result of signal transduction in the inositol triphosphate second messenger system where profilin interacts with phosphoinositol-(4,5)-bisphosphate and phospholipase Cγ1 (7). In this study, we have used profilin mRNA levels to indicate coelomocyte response to the bacterial product lipopolysaccharide (LPS). Previous studies showed that sea urchins efficiently clear bacteria from the coelomic cavity (8–10), and thus bacterial challenge would be predicted to be a strong stimulator of profilin gene expression. We show here that LPS causes significant cell activation, as measured by elevated levels of profilin message, and is a potent activator of the echinoderm immune system.

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Materials and Methods

Animals

Purple sea urchins, *Strongylocentrotus purpuratus*, were collected and housed as described (5,11,12). Cages for individual sea urchins were constructed from two small plastic baskets which were hinged and fastened together at the open ends with plastic cable ties (dimensions of two baskets together = $15 \times 10 \times 12$ cm). Injection protocols were as previously described (5), i.e. injections were given on days 1, 2, 4, and 5 using 25 gauge needles. On day 6, about 24 h after the last injection, all the coelomocytes from each animal were collected by removing "Aristotle's Lantern", or the mouth structure, and pouring the coelomic fluid into 10 mL of ice-cold calcium- and magnesium-free sea water (13) containing 30 mM ethylenediamine tetraacetic acid (EDTA) (5).

Initially, doses of the injected stimulators were determined by estimating the volume of coelomic fluid based on the formula of Yui and Bayne (9):

$$\text{weight of animal (g)} \times 0.35 - 4.2 = \text{mL coelomic fluid (cf)}.$$

However, this equation gave an overestimate of the true coelomic volume by an average of 11.0 ± 7.4 mL ($n = 18$). By measuring the volume of the coelomic fluid from the weighed animals, we found that a more reliable formula for *S. purpuratus* was:

$$\text{weight of animal (g)} \times 0.22 = \text{mL cf}.$$

The variability in the volume of the available coelomic cavity from one sea urchin to another was most likely due to the variation in gonad sizes. In order to calculate the actual dosages, coelomic fluid volumes were measured when the coelomocytes were collected after the final injection.

Bacteria

Vibrio parahemolyticus (ATCC #37969), originally isolated as a marine invertebrate pathogen (14), was reconstituted in 804 broth on a room temperature shaker overnight according to ATCC instructions. The culture was subsequently grown either in marine broth or on marine plates (Difco 2216) at room temperature, augmented with 5 g/L peptone and 3 g/L yeast extract (9). Bacteria were quantitated for injection by correlating spectrophotometric absorbance (OD_{600}) of the 16 h, 2216 broth culture with numbers of plated colonies from serial dilutions.

Other reagents

A stock solution (2 mg/mL) of LPS (from *Vibrio cholerae*; Sigma #L5262) was mixed in sterile artificial sea water (ASW) (13) for injections.

Transcript measurement

Procedures for coelomocyte collection, total RNA isolation, probe preparation, and probe excess transcript titration were as previously described (5,15). Briefly, total coelomocyte RNA was isolated from cells lysed in guanidinium thiocyanate followed by pelleting through cesium chloride. An antisense RNA probe was produced from the profilin cDNA and included the coding region and some of the 5' and 3' untranslated regions [see (5, Fig. 1)]. Excess probe was hybridized in solution to varying amounts of coelomocyte RNA, and the radioactive counts in the RNAse-resistant double-stranded hybrids were used to calculate the numbers of profilin transcripts per coelomocyte [for equations see (5,15)].

Results

Dose–Response Curves and Preliminary Studies

Bacteria. Yui and Bayne (9) showed that injections of 10^6–10^7 bacteria/mL cf could be cleared by *S. purpuratus* without mortality or obvious trauma. We introduced *V. parahemolyticus* at doses ranging from 60/mL to 10^6/mL to test coelomocyte activation in individual sea urchins. In general, this was much less than that used in the clearance studies of Yui and Bayne (9). Activation was assayed by titrating the profilin transcript content per coelomocyte in individual sea urchins, with the results shown in Figure 1. Animals receiving 350 or more bacteria/mL cf had elevated levels of transcripts per cell, although doses of up to 8×10^5 bacteria/mL cf did not result in increased numbers of profilin transcripts beyond the plateau of 50 or 60 mRNA molecules per cell. Doses of 60 bacteria/
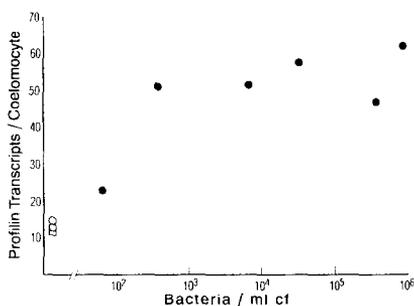

Figure 1. Dose–response curve: profilin transcripts per coelomocyte from sea urchins responding to a range of bacterial doses of *Vibrio parahemolyticus*. Sea urchins were injected with varying doses of bacteria on days 1, 2, 4, and 5, and the coelomocytes were collected on day 6 for RNA isolation and probe excess transcript titrations. Each point represents a single animal. Correlation coefficients calculated from the linear regressions from the transcript titrations ranged from 0.95097 to 0.99865. Animals receiving 360 or more bacterial/mL cf showed elevated numbers of profilin transcripts, indicating a cell activation in response to the bacterial challenge. Closed circles = bacterial injection; open square = ASW injection (injury control); open circles = normal controls.

mL cf did not elevate the profilin transcript level above the range seen in the controls reported here or previously (5). None of these sea urchins revealed any obvious pathogenic effects. They appeared healthy and continued to feed throughout the duration of the experiment. This was not surprising since there are between 1 and 5 million coelomocytes/mL cf, 65% of which are phagocytes (5), which means that one phagocyte in about 9×10^3 must phagocytose one bacterium per injection to clear this artificial infection. These results show that sea urchins respond to very minor bacterial challenges. In subsequent analyses we chose 3×10^4 bacteria/mL cf as our stimulatory dose.

In further experiments where sea urchins received doses of bacteria, elevations in profilin transcripts were seen ($x = 91.5 \pm 44.8$; $n = 9$); however, because of the large variability in the group receiving bacteria, statistically significant elevations of profilin transcripts could not be demonstrated when compared to the injury control group shown in Figure 2 ($x = 68.6 \pm 29.0$; $n = 17$; $p < 0.13$); yet it appeared that a few of the animals receiving bacteria were responding with greatly elevated levels of profilin message (for example, 120 and 138 messages per coelomocyte) similar to that seen previously in some experimentally manipulated animals [see (5, Fig. 8)]. This suggests that these preliminary experiments are qualitatively consistent with the data in Figure 1 and that *S. purpuratus* has the capacity to respond to live Gram-negative bacteria such as *Vibrio*, by activation of its coelomocytes.

LPS. The major surface component of Gram negative bacteria is LPS which, in mammals, is known to be a potent immune system activator. In order to analyze the molecular response of sea urchin coelomocytes to challenge with LPS, this substance was injected into the
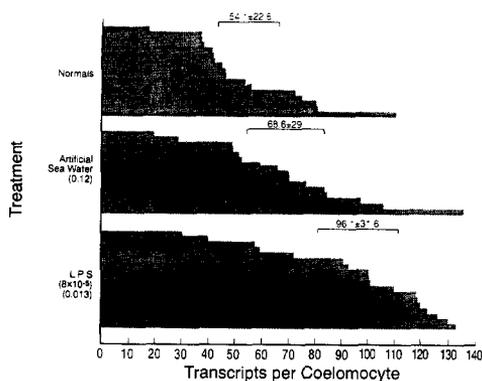
Figure 2. Profilin transcripts per coelomocyte in individual sea urchins. Each horizontal bar represents the probe excess transcript titration for an individual sea urchin. Means and standard deviations for each group are indicated in the figure. *p* values for each group compared to the normals are indicated under each treatment. The two *p* values indicated under the LPS group correspond to comparisons with the normals and the ASW controls, respectively. The single *p* value under the ASW group is the comparison with the normals. Injections and cell collections were performed as in the previous experiments and in (5). Correlation coefficients calculated from the transcript titrations ranged from 0.92138 to 0.9993. Doses of LPS = 1.21 ± 0.31 μg/mL cf; ASW control = 1.78 ± 0.31 μL/mL cf (approximately the same volume as the LPS injections).

coelomic cavities, in the same way as the live bacteria used in the previous experiments. We tried LPS concentrations ranging from 2 to 140 μg/mL cf. The results indicated that profilin transcripts were significantly elevated ($x = 41.95 \pm 4.09$; $n = 4$) compared to normal ($x = 17.25 \pm 0.07$; $n = 2$) and injury (13.8; $n = 1$) controls over the entire range of LPS doses tried. In the following experiments we used 2 μg LPS/mL cf.

Coelomocyte Responses to LPS

To refine better these preliminary analyses of sea urchin responses to LPS, experiments were performed using more animals. Results are shown in Figure 2. Pairwise statistical comparisons between groups were done using Student's two-

tailed *t*-test for equal variances. The group receiving LPS ($n = 18$) showed significantly elevated profilin transcripts per coelomocyte with respect to either control group, i.e. the uninjected normal animals ($p < 8.5 \times 10^{-5}$), or the ASW injected (minor injury) controls ($p < 0.013$). Of the 17 animals tested in this experiment, however, five did not appear to respond.

We found that the data obtained in this study were quite variable. Figure 2 shows that in both the uninjected control sample and the injury control (i.e. ASW injected) sample there is a heterogeneous pattern of profilin expression. That is, three of the 16 animals in the ASW sample, and at least one animal out of the 17 in the uninjected sample, displayed levels of profilin transcripts that we would associate with immune activation. These animals may well have been dealing with immune system challenges other than those we imposed experimentally. An examination of the normal group shown in Figure 2 reveals a general elevation in the profilin transcript levels per cell when this group is compared to the normals in Figure 1 and the normal groups published previously (5, Fig. 8). This suggests that these animals were responding to an unknown substance that was either in the sea water being pumped through the Caltech marine lab at Corona del Mar, CA, or originated from the subsection of the sea urchin housing system in which these particular animals were kept. There were no variations in the protocols between our previous studies (5) and this current one, both studies being conducted in the fall and early winter, and in both studies the animals had been housed for long periods of time before being used. We assume that all groups shown in Figure 2 were equally affected. The responses to the injected LPS and the unknown stimulus would then be additive and may function through the same coelomocyte activation system. Clearly, our ability to detect incipient, recently defended or ongoing pathological episodes

in the sea urchins we used for these studies is very limited. The variation in our control groups required that fairly large numbers of animals be tested in order for statistically significant results to be obtained. However, the experiments of Figure 2 clearly demonstrate that *S. purpuratus* coelomocytes respond to LPS.

Discussion

We show here that the sea urchin immune system is remarkably sensitive, responding to as few as 360 bacteria/mL cf (Fig. 1) with elevations in profilin transcript levels beyond those previously associated with simple injury (5). Furthermore, maximal coelomocyte activation occurs in response to LPS at concentrations as low as 2 µg/mL cf. For comparison, in mammalian cell culture, typical LPS concentrations used for cell activation or proliferation are 2 to 25 µg/mL of media (16). Measurement of the profilin message is a very sensitive molecular indicator for activation because profilin functions as a regulator of actin filament polymerization, and the mechanisms that coelomocytes employ to clear microbes from the coelom require shape changes in the cytoskeleton (17). These include such activities as motility, phagocytosis, secretion, degranulation, and clot formation [for review, see (18)]. It is clear from the data presented here that the sea urchin immune system is highly reactive to bacterial challenge and may be "pre-armed" to respond quickly to incipient microbial infection.

Clearance studies have shown that echinoderms efficiently remove a variety of foreign particles from the coelomic cavity (19–21). Bacterial doses ranging from 1×10^6 to 3.5×10^7 marine gram negative bacteria/mL cf are quickly eliminated from the coelomic cavity [90% to 99% in 2–3 h; 100% in 1–8 days; (8–10)]. Within minutes of a bacterial injection, the phagocytes change their morphology and begin phagocytosis and small clot formation to remove and encapsulate the bacteria (22). In vitro, phagocytes and red spherulous cells have been observed to be chemotactically attracted towards bacteria with subsequent wall or barrier formation by the phagocytes, and degranulation of echinochrome A by the red spherulous cells (1). Echinochrome A has been shown to be an effective bactericidal compound (3,23). Similar coelomocyte reactions have been noted around microbial infections of the epidermis. Thus phagocytes and red spherulous cells accumulate around skin and spine infections, form walls and degranulate echinochrome A (2,24). These coelomocyte responses combine to restrict the spread of the microbial infection, and eventually to reverse it.

A major surface component of gram negative bacteria is LPS. Although in higher vertebrates LPS is a potent immune activator, reactions to LPS have been demonstrated in only a few invertebrate deuterostomes. Injection of LPS induces hemocyte secretion in the tunicate, *Halocynthia roretzi* (25). Both *H. roretzi* and the sea urchin, *Paracentrotus lividus*, have a hemagglutinin in their coelomic fluid that binds LPS (26,27). When LPS is added to cultures of coelomic fluid including the coelomocytes from the sea cucumber, *Parastichopus californica*, the LPS is neutralized in 4 h (27), an activity similar to that identified for an LPS binding protein in *Limulus* (29). These reports suggest that invertebrate deuterostomes have humoral proteins and perhaps cell surface receptor(s) that bind bacteria and LPS. These functions might be of central importance for activating the defense systems of these animals against infection.

Mammals mount multiple immediate cellular responses to bacterial infection, which are collectively termed inflammation or the acute phase response [for review, see (30)]. The macrophage is the primary responding cell in mammals and

Table 1. Functions of Cells Mediating Non-adaptive Immunity.

	Mammals	Echinoderms
Primary cell responders	Macrophages, platelets	Phagocytes, red spherulous cells
Respond to	Infection, injury	Infection, injury
Response type	Immediate, non-specific, without memory	Immediate, non-specific, without memory
Secretion of cytokines	IL-1, IL-6, TNF-α, others	Diffusible molecules with cytokine-like activity
Cell response to cytokines	Activation: chemotaxis, phagocytosis, encapsulation, secretion, degranulation, proliferation, differentiation	Activation: chemotaxis, phagocytosis, encapsulation, secretion, degranulation, proliferation, differentiation(?)

Compiled from the citations found in the text.

initiates the cascade of events. A variety of LPS receptors has been identified on macrophages (31,32). Activation of these receptors signals the secretion of cytokines, including interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF-α), and others. These diffusible molecules initiate and mediate the acute phase response. The invertebrate deuterostome immune response may also use cytokine-like molecules for intercellular communication and general amplification of the immune response [for review, see (33)]. Diffusible factors have been isolated from both echinoderms and tunicates that are produced by coelomocytes or hemocytes, and that cause cell proliferation and migration, increase phagocytosis, and induce encapsulation (34–40). Such factors would appear to play a central role in up-regulating the immune response in invertebrate deuterostomes. Unfortunately, the responsible molecules have never been characterized at the sequence level.

Many aspects of the non-specific immune response of echinoderms appear similar to the primary and initiating responses of higher vertebrates (Table 1). Both are non-specific responses to infection and injury that are immediate (i.e. no clonal amplification of the responding cells); there is no augmentation upon secondary challenge (i.e. no immune memory); and both are mediated by phagocytic cells. Higher vertebrates utilize cytokines to amplify the response by activating cells, attracting distant cells to the site, and initiating proliferation and differentiation of additional cells. Echinoderms (and ascidians) may also have diffusible ligands that function as cytokines to amplify their immune responses (37–39). Although very little is yet understood about the mechanisms that regulate the invertebrate deuterostome immune system, these features may be shared mechanistic characters that descend from the primordial common ancestors of echinoderms and chordates.

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