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Invertebrate Immune Systems–Specific, Quasi-Specific, or Nonspecific?

Andrew F. Rowley¹ and Adam Powell

Until recently, it was widely accepted that invertebrates fail to show a high degree of specificity and memory in their immune strategies. Recent reports have challenged this view such that our understanding of the capabilities of the invertebrate immune systems needs to be reassessed. This account critically reviews the available evidence that suggests the existence of a high degree of memory and specificity in some invertebrates and seeks mechanistic explanations of such observations. It is postulated that elevated levels of phagocytosis may be a partial explanation for this phenomenon.

The immune defenses of invertebrates–a brief guide to the mechanisms

It must be remembered that because of the tremendous variety of body patterns, life histories, and ecological niches within the 1.3 million-plus species of living invertebrates, there is also a similar potential for diversity in their immune strategies. Hence, the immune strategy of a relatively long-lived aquatic crustacean such as the edible crab Cancer pagurus, which may survive for several years, may be very different from that in shorter-lived, terrestrial, social insects such as bees or wasps. Indeed, it could be argued that only long-lived animals would gain any evolutionary advantage from the development of an adaptive immune system capable of showing “memory.” The following section of this review concentrates on the arthropods (insects, crustaceans, and related forms), a highly successful group of protostome invertebrates of which a great deal is known of their immune systems and diseases. Wherever possible, two model animals are referenced: the fruit fly Drosophila melanogaster and the shrimp Penaeus/Litopenaeus spp.

Arthropods in general use a range of cellular and humoral defenses to protect themselves from disease agents that manage to gain access to their internal tissues by penetrating the exoskeleton/cuticle or alimentary canal. The cells principally involved are the circulating and sessile blood cells (correctly termed he-

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2 Abbreviations used in this paper: WSSV, white spot syndrome virus; AMP, antimicrobial peptide; Dscam, Down syndrome cell adhesion molecule; Imd, immune deficiency; PRP, pattern recognition protein.
cytes” this does not necessarily imply any evolutionary or functional relationship. Leukocytes, and although they share names such as “granulocytes,” these hemocytes are morphologically distinct from vertebrate leukocytes (2, 3), whereas shrimp produce penaeidins, crustins, and hecrins, Metchnikowins, defensins, attacins, cecropins, and drosocins. Examples of Drosophila AMPs include diptericins, drosomyelins, and drosocins, which are highly conserved. The importance of cellular (phagocytosis) vs humoral (AMP) defenses has been comprehensively reassessed in Drosophila (18). It was shown that double mutants of Drosophila larvae containing few or no circulating hemocytes but still with the ability to generate AMPs largely intact did not survive opportunistic bacterial or fungal infections (18), the implication being that greater emphasis needs to be placed on a molecular understanding of the cellular defenses such as phagocytosis and nodule formation.

The case for specific immunity in invertebrates

Even though the invertebrate immune system lacks lymphocytes and functional Ig’s, this should not rule out the potential for the existence of a unique form of an adaptive immune system that might have been discarded with the evolution of the first vertebrates. This section critically reviews the evidence for such specificity. Pioneers in evolutionary immunology such as Edwin Cooper and Bill Hildemann made use of the graft rejection models widely used by mammalian immunologists at the time to examine whether invertebrates also show a high degree of self-nonspecific recognition as seen in mammals and also whether second set grafts showed accelerated graft rejection (taken as a hallmark of memory). Cooper’s work (19) showed that earthworms could recognize and reject grafts from other earthworms and that they possessed the apparent ability to show faster rejection upon secondary exposure. To date, there is no tested mechanistic explanation for these important findings. A further interesting graft rejection model comes from colonial animals such as sponges and tunicates. In the case of the tunicate Botryllus schlosseri, the colonies are formed by a budding process to produce zooids that are genetically identical and share a common vascular system. When adjacent colonies of B. schlosseri grow close together, finger-like processes called ampullae from the zooids either fuse, leading to the exchange of blood cells, or are rejected postfusion, resulting in an inflammatory reaction and cell destruction. Our insight into this process has recently been strongly enhanced by the observations of Nyholm et al. (20), who identified the first invertebrate allorecognition receptor. Somatic diversification of this receptor can occur by alternative splicing, resulting in individual-specific forms within all tissues of the zooid. Interestingly, although potential homologues were found with other vertebrate immune system receptors, one interpretation of this work by Litman (21) highlighted that it may not be possible to explain the observation in this invertebrate in terms of what we know about allorecognition in mammals. Essentially, if invertebrates do show specificity and memory in their immune reactivity, it is probably a mistake to look for explanations centered on the mammalian immune system.

In the last few years, several groups of researchers have claimed to show the presence of some form of acquired (specific) immunity in invertebrates (Table I). Kurtz and Franz (22)
infected copepods (a crustacean) with two strains of its natural tapeworm parasite, Schistoscephalus solidus. Four days later they exposed these copepods to identical numbers of either the same or different strains of the parasite and subsequently on day 6 screened these to assess the reinfection rates. They found a significant reduction in the reinfection rate in those copepods previously exposed to the same strain of parasite. Their interpretation was that the immune system of the copepod was specifically “primed” by prior exposure to the parasite. Although this is an interesting observation, the short time scale of the experiment is of concern because the parasites remaining from the first exposure only 4 days later might have had some role in reinfection totally independent of the host defenses.

So-called “trans-generational immunological priming” has been reported in insects including mealworms (11) and bumblebees (10) and also in a crustacean, Daphnia (9). In the case of the study by Sadd et al. (10), Bombus terrestris queens were exposed to either the bacterium Arthrobacter globiformis or sterile saline. At a later time the progeny (offspring workers) from these queens were stimulated by LPS injection and 24 h later the antibacterial and phenoloxidase activities in the blood were measured. Although no significant differences were found in phenoloxidase levels, the antibacterial activity in the worker bees was significantly higher in those descendants from the queens that had been challenged with bacteria compared with those from the saline-challenged group. Unfortunately, the nature of the test agent (A. globiformis) used in the antibacterial assay was not revealed, so the possibility of the specificity of this reaction remains untested. Although similar results were also noted with mealworms, in that the antibacterial activity was significantly higher in the progeny from the adults previously exposed to LPS and the phenoloxidase levels were unaltered, the assay used to reach these conclusions was not strictly quantitative (11). More conclusive data were obtained by Little et al. (9) in their studies with the water flea Daphnia magna. Water fleas were artificially infected with either the pathogenic bacterium Pasteuria ramosa strain A or the P. ramosa strain G. The progeny of these two groups of animals were subsequently exposed to either strain A or G and their reproductive fecundity and survival postchallenge were determined. In both cases, exposure to homologous combinations (i.e., strain A followed by strain A or strain G followed by strain G) increased survival after the second challenge and increased reproductive fecundity. No mechanistic explanation of these observations was attempted.

More convincing evidence for a specific element in the immune response of any invertebrate comes from experiments with bumblebees (B. terrestris) where groups of these insects were initially exposed to the Gram-negative bacterium Pseudomonas fluorescens, two closely related Gram-positive bacteria, Paenibacillus alvei and Paenibacillus larvae, or saline (23). Either 8 or 22 days later the insects were given a secondary homologous or heterologous exposure, and their survival and ability to clear the three different species of bacteria from the blood was determined. This approach convincingly demonstrated that insects in the homologous re-exposure group (e.g., P. fluorescens injected at day 0 and either day 8 or 22) showed significantly higher survival rates than those given either saline or heterologous challenge. The authors found no evidence that this apparent specific protection involved AMPs; instead, they suggested (but did not test their hypothesis) that the homologue of Dscam formed by an alternatively spliced, hypervariable Ig domain-encoding gene recently elucidated in insects (12, 13) could be responsible for this specificity. Key humoral factors such as AMPs are not involved in this specific “immune priming,” the explanation of the specificity may be in the cellular reactivity of the hemocytes (e.g., phagocytosis or nodule formation) toward these bacteria. Finally, a recent report has shown that the immune system of Drosophila can be “primed” by exposure to a sublethal dose of Streptococcus pneumoniae that has some level of specificity and continued for “the life of the fly” (24). Although such specific protection could also be found for
other pathogens such as the entomopathogenic fungus *Beauveria bassiana*, rather surprisingly (and perhaps worryingly) the other bacteria tested yielded no enhancement in protection against later challenge (24).

**Mechanistic explanations for specific or quasi-specific immunity**

This section seeks to explore potential mechanisms that could account for the heightened and apparently specific protection observed in some of the recent studies already discussed. As previously described, cellular defense reactions are key players in protecting both insects and crustaceans from invading pathogens. Therefore, this is an appropriate starting point to look for mechanistic explanations of such changes. It is often forgotten that we have had evidence from studies performed over two decades ago (e.g., Ref. 25) for heightened phagocytic activity in the hemocytes of some invertebrates following previous exposure to foreign material. More recently, greater insight into such activities has been gained from elegant but simple approaches using a range of challenge regimes in the lobster *Homarus americanus* (26). What these authors found was that the injection of LPS into lobsters only acted as a nonspecific stimulator of phagocytic activity but that the challenge of these animals with whole, live pathogenic bacteria (*Aerococcus viridans* var. *homari*) induced marked increases in the in vitro phagocytic activity of lobster hemocytes, particularly against this challenge bacterium. Hence, there is evidence in the literature of enhanced phagocytic activity in “vaccinated” animals that shows some degree of specificity. Our knowledge of the recognition of microbial invaders by both insect and crustacean phagocytic hemocytes is surprisingly limited compared with that of the Imd (immune deficiency)/TLR pathways of AMP synthesis (see Refs. 2 and 3 for detailed reviews). What is clear is that the phagocytic hemocytes have both specific and nonspecific mechanisms of recognizing self from nonself (27–30). The nature of the pattern recognition proteins (PRPs) either in the plasma or directly associated with the phagocytic hemocytes that can specifically react with pathogen-associated molecular patterns (PAMPs) including peptidoglycan, LPS, dsRNA, and β-1,3-glucans is incompletely understood, although several PRPs have been identified in both insects and crustaceans (e.g., 29, 30), some of which involve plasma-derived lectins that bind to hemocytes via lectin receptors. Whether these PRPs hold a clue to the heightened phagocytic activity reported in “immunized” lobsters is uncertain, but a model that could explain this with some degree of specificity (as shown in the lobster studies) is illustrated in Fig. 2. The work of Watson et al. (12) on the heightened phagocytic activity in the hemocytes of some invertebrates following previous exposure to foreign material. 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(12) on the behavior of the Dscam homologue in *Drosophila* has profound bearing on this discussion in that some of the predicted 18,000 spliced forms of this molecule can bind bacteria (*Echerichia coli*), and the uptake of this bacterium by the phagocytic hemocytes is partially dependent on the presence of Dscam. An additional insight into the potential importance of Dscam variants in developing an explanation of how the innate system of invertebrates could show specificity emerges from work with the equivalent *Dscam* gene (*AgDscam*) in the mosquito *A. gambiae* (13). This gene is capable of producing in excess of 31,000 alternative splice forms to yield proteins with a variable range of binding capabilities to nonself material. The challenge of a mosquito-derived, hemocyte-like cell line with a range of different Gram-positive and Gram-negative bacteria, LPS, peptidoglycan, or two species of *Plasmodium (Plasmodium berghei* and *Plasmodium falciparum*) rapidly yielded different spliced forms of *AgDscam* such that their products were thought to have variable binding properties to these challenge agents. Both in vitro and in vivo challenge experimental approaches also revealed that exposure of mosquitoes to the two related parasites (*P. berghei* and *P. falciparum*) yielded different *AgDscam* variants, indicating a possible specificity in the manner in which the mosquito immune system could deal with these closely related parasites. When *AgDscam* gene silencing experiments were conducted, Dong et al. (13) found clear impairment of the immune defenses of mosquitoes such that they became susceptible to infections by opportunistic bacteria. Finally, these authors reported convincing evidence that the nature of the challenge pathogen was reflected in the resulting *AgDscam* splice form variants. These experiments with mosquitoes and fruit flies provide a plausible explanation of how bumblebees can show heightened specific responses following the second challenge with bacteria (23). The alternative splicing of *Dscam* produces a series of recognition elements (PRPs) in insects that could also yield sufficient specificity to explain the phagocytic stimulation seem in lobster hemocytes; however, whether all arthropods possess this gene remains to be elucidated. Further insight will be gained by a detailed examination of the nature of binding between Dscam variants and different closely related microorganisms or parasites.

It is also worth briefly evaluating whether the differential expression of AMPs following microbial challenge could lead to
quasi-specificity in immune reactivity upon the second encounter. There is some evidence from studies with *Drosophila* that the induction and expression of AMPs is related to the nature of the challenging infectious agent. Thus, Lemaitre et al. (31) reported that the challenge of fruit flies with a fungal agent resulted in the biosynthesis of anti-fungal AMPs, whereas an infection by a Gram-negative bacterium resulted in an increase in the levels of AMPs appropriate for the destruction of such bacteria. Unfortunately, this finding does not appear to be universal for other invertebrates and other pathogens. For instance, recent studies have either failed to observe an up-regulation of gene expression for AMPs following microbial challenge (32) or found that the nature of the challenge agent has no direct bearing on the resulting AMP profile (33).

The current model for the induction of AMP biosynthesis in *Drosophila* shows two distinct pathways, one using TLR(s) and a further one using Imd. Gram-positive bacteria and fungi principally stimulate the Toll pathway while Gram-negative bacteria mainly stimulate the Imd pathway (2, 3, 31). Despite the separate nature of these two pathways, some AMP genes such as those for defensin and Metchnikowin depend on both pathways. It seems unlikely that selective AMP gene up-regulation could provide a mechanistic model that could go anywhere toward explaining the specificity of protection claimed to be present in some insects. Therefore, selective induction of AMP biosynthesis on its own does not seem to be a promising avenue to explore in the search for a mechanistic explanation of acquired immunity in invertebrates. As in the mammalian immune system, there is evidence of interplay between cellular and humoral events in invertebrates. It has long been thought that there may be a link between phagocytic hemocytes and the fat body cells that are responsible for AMP biosynthesis. A recent report by Brennan et al. (34) identified a gene, *psidin*, that codes for a protein found in the lysosomes in the hemocytes of *Drosophila*. In *psidin* mutants the induction of defensin is severely hampered, suggesting the importance of hemocytes in controlling or stimulating AMP biosynthesis. One implication of this could be that hemocytes act in an analogous way to that of vertebrate APCs in that they either produce signals (cytokines?) that control the fat body or they digest complex Ags in lysosomes in such a way as to present components of these to the AMP-producing cells (35). If, as already discussed, the principal explanation for the observed “specific immunity” in invertebrates is an elevation of phagocytic activities of the hemocytes, a knock-on effect of this could involve the modulation of AMP biosynthesis.

**The future potential for vaccine development for invertebrates**

It may come as a surprise to immunologists who work with mammals that there is a need to develop vaccines to protect the health of invertebrates. Clearly there is no requirement to develop vaccines for the vast majority of invertebrates, particularly bearing in mind that some of these are pests to our agricultural crops or vectors of disease. Invertebrates of benefit to human-kind include honeybees that play a vital role in pollination and those animals subject to aquacultural development. In the case of shrimp aquaculture, which has been already highlighted in this review, during their larval development shrimp are highly susceptible to nonspecific vibrio infections while later on the adults are subject to serious acute viral diseases (36). To our knowledge, there is only one commercially available “vaccine” for invertebrates, namely AquaVac Vibromax, a multivalent vaccine from Schering-Plough Animal Health designed to give protection to shrimp larvae from a range of pathogenic vibrios. Although this vaccine appears to provide a demonstrable improvement in the survival and the “health status” of larvae, its mode of action is unknown, as is its specificity. Commercially available vaccines for protection of shrimp against WSSV are likely to appear in the very near future judging from recent encouraging reports of apparent enhanced survival of WSSV vaccine-treated shrimp (e.g., Ref. 8).

As well as these “vaccines,” several types of potential immunostimulants have been investigated in a variety of crustaceans of importance to the growing aquaculture industry. These include bacterial products (e.g., LPS and peptidoglycans), animal-, plant-, alga-, and yeast-derived complex carbohydrates (various glucans, Ergosan, and chitin), and “probiotic” bacteria (e.g., *Lactobacillus plantarum*) (e.g., Refs. 37–39). By definition, immunostimulants act to nonspecifically stimulate immune potential, for instance by enhancing the total number or killing potential of hemocytes and/or stimulating the expression of AMPs (Fig. 1). Although some recent reports provide good evidence of such events in crustaceans following the dietary application of bacterial peptidoglycan as an immunostimulant (40), a recent key review of the immunostimulants used in crustacean aquaculture has questioned the evidence of clear health benefits from their delivery and has suggested that some factors could even over-stimulate the immune system to the detriment of the host (37).

Overall, consistent evidence that putative vaccines give enhanced and specific protection to invertebrates is currently lacking.

**Closing remarks**

There is mounting evidence that at least some invertebrates show a high level of specificity in their immune response to different pathogens such that subsequent re-exposure results in enhanced protection. Whether these observations prove the existence of an analogous adaptive immune system with levels of specificity and memory with equivalent status to that in jawed vertebrates is still very much unanswered. Also, there is a large gap between the phenomenological observations made in some animals such as honeybees and *Daphnia* and the rapid advances in our understanding of potential molecular mechanisms exemplified by the important observations in *Drosophila* and *Anopheles* (12, 13). What is surely needed is the ability to unequivocally prove the existence of immune mechanisms in selected invertebrates that both yield a memory component and have specificity in their mode of action. Furthermore, a drive to reconcile phenomena with the mechanism in one or two model species is wanting. Perhaps the first stage in a determined quest to prove the existence of some form of acquired immunity in invertebrates is to find appropriate model animals. Within the protostome invertebrates, either shrimp of *Drosophila* would appear to be good candidates for such approaches as they both have well-defined immune systems. Also, because there are two main evolutionary lineages within the animal kingdom, namely the deuterostomes and the protostomes, it would also be appropriate to examine such events in a deuterostome model organism. The recent genome analyses of two deuterostome invertebrates, the sea squirt *Ciona intestinalis* and the purple sea urchin *Strongylocentrotus purpuratus*, and the initial interpretations of these studies regarding immune genes (41–43) would make
them ideal for such goals. Importantly, both of these animals are relatively abundant in the aquatic environment, have large numbers of blood cells, and are fairly easily maintained under aquarium conditions, hence permitting long-term primary and secondary challenge experiments. Once suitable model species have been identified, greater emphasis on experimental design is needed. For instance the time scale and the putative specificity of the response need to be carefully examined. Some studies re-viewed have used very short periods between primary and secondary challenge such that a simple elevation in hemocyte numbers, as occurs following wounding, could explain their findings. The nature of the immunogen used also requires careful selection where it is important to choose appropriate micro-bial and macrobial agents that are naturally found in the envi-ronment with the particular animal under study. Finally, care is needed to ensure that the specificity of the putative changes in immune reactivity is fully addressed by secondary challenge with a wide range of related and unrelated pathogens or para-sites. If, as suggested by several studies, elevated phagocytosis may provide a mechanistic explanation for the specificity of im-mune reactivity (13, 25, 26), it would be very easy to assess this in a systematic manner in an appropriate animal model. To date this is still lacking.

Disclosures
The authors have no financial conflict of interest.

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