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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 re-assessed. 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 Journal of Immunology, 2007, 179: 7209–7214.

One of the key hallmarks of the mammalian immune system is the ability to generate clones of lymphocytes, each with its own unique ability to recognize and proliferate in the presence of a specific Ag. Hence, this immune system is said to have both memory (the ability to respond rapidly upon re-exposure to a particular Ag) and specificity. This feature of the immune system probably first evolved >500 million years ago with the evolution of the first jawed (gnathostome) vertebrates (1). Despite some initial indications >30 years ago that invertebrates also have a specific (adaptive) immune system based on the clonal expansion of activated lymphocytes, it has become the central dogma of evolutionary immunologists that invertebrates, in the absence of “true” lymphocytes and functional Ab, rely entirely on innate immunity as their primary mechanism of defense against parasites and pathogens. More recently, our knowledge of these innate defenses has flourished to the stage that we now have a detailed appreciation of both the cellular and humoral mechanisms in many invertebrates, but particularly in insects such as the dipteran fly Drosophila (2–4) and in crustaceans such as shrimp, Penaeus/Litopenaeus spp. (5). With the global aquaculture production of shrimp currently exceeding 2.4 million tons per annum (6) and an estimated loss of up to 25% of this production as a result of disease, there is a great need to understand the immune defenses of these commercially important animals. Reports on the potential development of vaccines to combat major viral pathogen of shrimp called white spot syndrome virus (WSSV)² (7, 8), together with studies that suggest the existence of specific or “primed” immunity in insects and crustaceans that in some instances can apparently be transferred from parent to offspring (9–11), have challenged this central dogma. These largely phenomenological studies offer little in terms of potential mechanisms to explain their novel observations. Most recently, however, detailed investigations into the action of a homologue of Down syndrome cell adhesion molecule (Dscam) in Drosophila (12) and the mosquito Anopheles gambiae (13) provide compelling explanations for how such specificity might be achieved at least in these invertebrates. This review aims to provide a critical overview of the findings to date and seeks potential mechanisms to explain these observations based on recent advances in our understanding of the mechanisms controlling the immune defenses of invertebrates.

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 protostomate 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-
cytes” this does not necessarily imply any evolutionary or functional relationship. Hemocytes perform a number of key actions, including the initiation of wound repair/blood coagulation to prevent pathogen ingress into the main body cavity termed the hemocoe. If this barrier is breached, the blood cells present in the hemocoe can phagocytose and digest small invaders such as protozoans, bacteria, fungi, and viruses and enmesh multicellular parasites in a thick wall of hemocytes, a process termed nodule formation or nodulation. Microorganisms are cleared from the hemocoel and become enmeshed in a central core of melanized hemocytes surrounded by a wall of flattened hemocytes, hence isolating such particles from the rest of the host. These cellular defenses are highly efficient, and early pioneering immunologists including Elie Metchnikoff and Serge Metchnikoff marveled at their potential to clear and kill extremely large numbers of pathogenic bacteria that would have proved fatal to more complex animals such as humans.

In terms of humoral mechanisms, the antimicrobial peptides (AMPs) are key effectors in the elimination and destruction of bacteria and fungi in invertebrates (Fig. 1). In insects such as Drosophila several distinct forms are synthesized by the fat body (the functional equivalent of the liver in insects), while in shrimp these AMPs are largely synthesized within the hemocytes (5). Examples of Drosophila AMPs include diptericins, drosomycins, Metchnikowsins, defensins, attacins, cecropins, and drosocins (2, 3), whereas shrimp produce penaeidins, crustins, and hemocyanin-derived peptides (5, 16). As well as AMPs, both insects and crustaceans use a variety of antimicrobial enzymes such as lysozyme. Lectins, either free in the blood (hemolymph) or associated with the hemocytes, may act as both recognizers and effectors of immunity. The prophenoloxidase cascade is thought to generate cytotoxic and opsonic factors together with melanin that is evident during the host response to foreign invaders (Fig. 1). In Drosophila at least, recent studies have shown that the activation of this system does not appear to have a major influence on the ability of these animals to survive microbial challenge (17), perhaps suggesting that this enzymatic cascade is of less overall importance than previously considered.

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 Igs, 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-nonself 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. If 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

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Table I. **Recent examples of reported acquired (specific) immunity in arthropoda**

<table>
<thead>
<tr>
<th>Event</th>
<th>Animals</th>
<th>Nature of Immunogen</th>
<th>Mechanistic Explanation</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Trans-generational enhanced immunity”</td>
<td>Insects including bumblebees, mealworms, and <em>Daphnia</em></td>
<td>Various</td>
<td>Enhanced antibacterial (humoral defenses) in progeny</td>
<td>Nature of antibacterial factors unknown; level of specificity in relation to challenge unknown</td>
<td>9–11</td>
</tr>
<tr>
<td>Apparent specificity in protective response against a natural parasite by pre-exposure</td>
<td>Copepods</td>
<td>Tapeworm larvae</td>
<td>Lectin binding</td>
<td>Short time scale between primary and secondary exposure may invalidate conclusions</td>
<td>22</td>
</tr>
<tr>
<td>“Vaccination” resulting in enhanced survival following challenge</td>
<td>Shrimp</td>
<td>Envelope proteins (VP19, VP28) of WSSV</td>
<td>None presented</td>
<td>Specificity of the reaction untested and overall duration limited to maximum tested of 25 days</td>
<td>7, 8</td>
</tr>
<tr>
<td>Specific “immune priming”</td>
<td>Bumblebees</td>
<td>Gram-positive and Gram-negative bacteria</td>
<td>Dscam homolog found on hemocytes</td>
<td>Shows specific and relatively “long-term” memory</td>
<td>23</td>
</tr>
<tr>
<td>Specific “immune priming”</td>
<td><em>Drosophila</em></td>
<td><em>S. pneumoniae</em> and <em>B. bassiana</em></td>
<td>Experiments imply the importance of phagocytic hemocytes and elements of the Toll pathway</td>
<td>Study unfortunately failed to assess the phagocytic activity of hemocytes following immune “priming”</td>
<td>24</td>
</tr>
</tbody>
</table>

*Note that most of these reports lack mechanistic explanations of their observations.*
other pathogens such as the entomopathogenic fungus *Beauveria bassiana*, rather surprisingly (and perhaps worriedly) 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. 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 beh...
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, Lemaître 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 non-specific 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 aquatic 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 reviewed 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 microbrial and macrobrial agents that are naturally found in the environment 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 parasites. If, as suggested by several studies, elevated phagocytosis may provide a mechanistic explanation for the specificity of immune 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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13. Dong, Y., H. E. Taylor, and G. Dimopoulos. 2006. AgDscam, a hypervariable immune 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.

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