PERSPECTIVE

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Invertebrate immunity and the limits of mechanistic immunology

Tom J Little, Dan Hultmark & Andrew F Read

Rapid progress is being made in elucidating the molecular mechanisms involved in invertebrate immunity. This search for molecules runs the risk of missing important phenomena. In vertebrates, acquired protection and pathogen-specific responses were demonstrated experimentally long before the mechanisms responsible were elucidated. Without analogous experiments, mechanism-driven work may not demonstrate the full richness of invertebrate immunity.

Many scientific endeavors assume that the world can be usefully interpreted with an approach that systematically demonstrates each building block that contributes to the whole. Thus, for example, knowledge of the 'suite' of cytokines influencing T cell production will ultimately help in fighting disease. Indeed, modern immunology is a paradigm of a reductionist approach, because it is largely if not wholly concerned with identifying the molecules, cells and functional cascades that respond to biological enemies as they invade hosts.

However, this fact about modern immunology belies its well known origins. The discipline of immunology grew out of the observation that people who had recovered from certain infectious diseases were then protected against later infection with those same diseases or closely related ones[1]. The defense against pathogens thus showed both memory and specificity. Vaccines also were discovered mainly through phenomenological studies; for example, Edward Jenner's observation that milkmaids do not get smallpox, or when Louis Pasteur accidentally vaccinated chickens against cholera and, fortunately for future generations, recognized the importance of his findings. The search for the mechanisms underlying these phenomena came much later. In many ways, this is how it needs to be; it is a natural progression of science. Until an interesting phenomenon is identified, for which mechanism does one probe?

We contend that using important phenomena as the starting point for the study of mechanism will lead to innovation. Genomics, postgenomics and microarrays are the buzzwords of our time, and the excitement surrounding these words is well earned. But however much reductionist approaches have taught us, they are also incomplete. For example, there is not yet a full understanding of what invertebrate immune responses can accomplish for the host. Moreover, trying to infer this from vertebrate mechanisms is often unhelpful. Whole-organism experimentation, like that which defined the field of vertebrate immunology, is long overdue and should be used to guide mechanistic approaches. Those few experiments that have been done suggest that invertebrate immunity can achieve much of what vertebrates can do.

Innate immune systems

A glance at a modern immunology textbook suggests that the field has only rarely applied itself to 99% of the immune systems on the planet. Not unexpectedly, the field of invertebrate immunology therefore lags far behind that of vertebrate immunology, although it does have a long history and is rapidly developing[1–4]. The invertebrate innate immune system comprises cellular responses (phagocytes and so on), a variety of inducible antibacterial peptides and a phenoloxidase cascade that produces melanin (used, for example, to encase parasitoids). Other important components of defense include nitric oxide synthase, clotting reactions and serine protease inhibitors.

Vertebrates have both an acquired response and an innate system of defense, and extensive homology between vertebrates and invertebrates[5] has been found only for the innate defense system. Those homologies have been justifiably greeted with considerable fanfare[6,7] because they bring greater relevance to the study of the invertebrate immune system and permit the study of innate immunity without the confounding forces of acquired immunity. However, extensive searches in invertebrate taxa for B cells, T cells and major histocompatibility complex molecules, the key ingredients of vertebrate acquired immunity, have not been successful. Does this mean that invertebrates lack immunological memory or specific immune responses? Here is an inevitable shortcoming of a reductionist approach: if invertebrates have systems analogous to specific or memory immunity but use completely different mechanisms, a search for homologous cells and proteins is bound to come up empty-handed.

And yet exciting studies of immune defense in invertebrates are now demonstrating phenomena that are functionally equivalent to that in vertebrates. As with the origins of vertebrate immunology, observations of whole-organism phenomena are providing a large part of the foundation. These studies are showing both immunity that is acquired and tremendous variation in the expression of disease that can be described as specificity.

Anticipatory responses: forms of immunological priming

Pioneering work on earthworms, cockroaches and 'colonial animals' (which live in groups and are structurally joined; for example, corals) has

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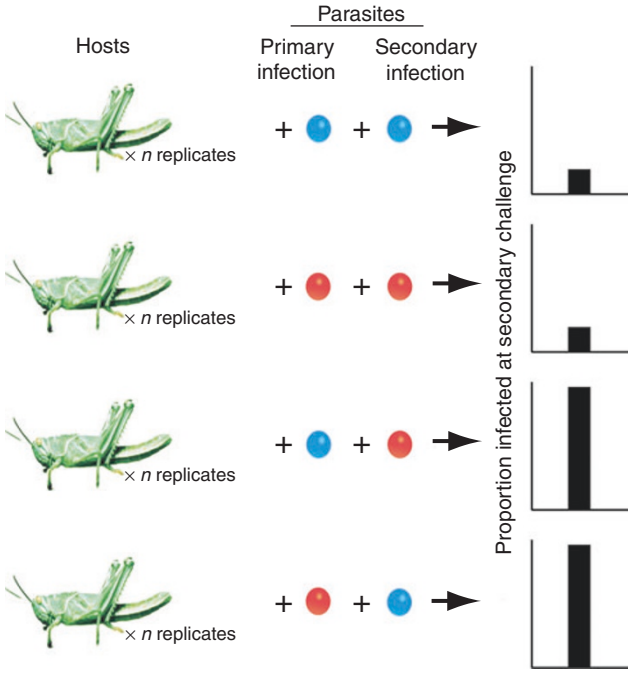

Figure 1 How to determine what an invertebrate immune system can do. One approach is to expose hosts to a parasite strain (Primary infection) and then administer a secondary challenge with either the same or a different strain (blue and red spheres indicate parasites of antigenically distinct strains). The final measurement is, for example, the proportion of hosts that become infected at the secondary challenge. Such studies are actually studies of variation and thus require replicates of each treatment to show that, on average, more infections result when primary and secondary challenges are heterologous.

shown that skin grafts from genetically distinct donors are more quickly rejected the second time the grafts are applied[8–11]. Thus, invertebrates have some kind of immunological memory. Many studies have now explicitly shown that primary exposure to pathogens may be prophylactic, providing hosts with protection during secondary encounters[12–17]. Typical experiments involve a primary challenge, a time delay, a secondary challenge and an assay (**Fig. 1** and **Table 1**). Critically, it seems that responses can be specific to particular pathogen genotypes[18–20]. For example, studies of a copepod have shown that the level of infection during secondary encounters with a parasitic worm is lower when primary and secondary encounters involve more closely related worms[18]. Similarly, 'vaccination' studies of a prawn have indicated different responses to two structural envelope proteins derived from white spot syndrome virus: primary exposure to protein VP28 provides protection against viral challenge, whereas primary exposure to VP19 does not[20]. In addition, both specific and general immunity can be passed from mother to offspring, endowing the offspring of pathogen-exposed parents with improved defense against infection[15,19]. For example, studies of maternal effects in the crustacean daphnia have shown that when mother and offspring are exposed to the same pathogen strain, offspring suffer less infection than when mother and offspring experience heterologous strains[19]. Given more relaxed ideas regarding what constitutes an immune response or considering relevant environmental variation that stimulates immune system upregulation, anticipatory responses in invertebrates seem to be widespread indeed[21–24]. The critical point, however, is that these whole-organism phenomenological studies let organisms show what invertebrate immune systems are capable of doing (**Fig. 1**).

Anticipatory responses in invertebrates are in the phenomenological sense analogous to acquired immunity in vertebrates[25]. There are of course key differences; unlike memory effects in vertebrates, these effects do not seem to be stronger during the second challenge and thus seem unlikely to be determined by the clonal expansion of memory cells. Indeed, invertebrate and vertebrate immunological priming are almost certainly under very different genetic and cellular control. The mechanistic basis of immunological priming in invertebrates is obscure, but it is important to bear in mind that the mechanistic field of immunology would not have predicted these priming effects after the unsuccessful search for major histocompatibility complex molecules or their precursors. Although the study of the phylogeny of vertebrate immune system molecules has greatly enhanced understanding of the evolution of immune systems, there is no logical reason to expect to be able to determine what invertebrate immunity can do, or how it does it, from what is known about the mechanistic basis of vertebrate immunity.

Anticipatory responses in arthropods have considerable importance. Aside from indicating unexpected complexity of the invertebrate immune system, the implication is that that host populations could become increasingly resistant as encounters with parasites become more frequent, and given genotype-specific interactions, become increasingly resistant to a specific parasite genotype as that genotype spreads through the population[19]. These are some of the same consequences brought about by vertebrate anticipatory responses and could alter the view of the way in which parasitic interactions influence evolutionary and epidemiological phenomena in invertebrates. These topics await further study.

The specificity gap

It is now apparent that interactions between invertebrate hosts and their pathogens may be extremely specific[18–20,26–29]. By this we mean that the severity of infection, or even if an infection occurs at all, is dependent on the genetic background of both host and parasite[30] (**Fig. 2**). These

Table 1 Whole-organism studies of invertebrates: anticipatory responses

Organism	Primary challenge	Time delay	Secondary challenge	Assay result
Copepod (*Macrocyclops albidus*)	Parasites from each of 24 'sibships'	4 d	Parasites from each of 24 'sibships'	Reduced infectivity when primary and secondary challenge are from homologous 'sibships'
Waterflea (*Daphnia magna*)	Parasite strain 1 or parasite strain 2	One generation	Parasite strain 1 or parasite strain 2	Reduced infectivity and virulence when primary and secondary challenge are homologous
Prawn (*Penaeus monodon*)	Protein VP28 or protein VP19	3–7 d	Exposure to virus	Reduced mortality after challenge with VP28 but no effect for VP19

These whole-organism studies of various invertebrates have shown phenomena analogous to anticipatory responses.

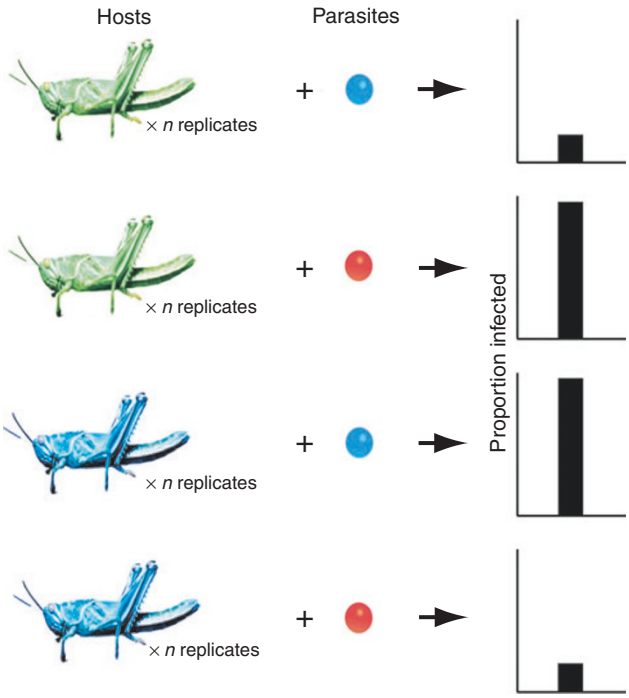

Figure 2 How to determine the level of genetic specificity in an invertebrate host-pathogen interaction. Experiments require two host genotypes (green and blue insects) and two parasite genotypes (blue and red spheres indicate parasites of antigenically distinct strains) at a minimum. Specificity is present where infection outcomes depends on the genotype of both the host and pathogen. As in **Figure 1**, such studies are actually studies of variation and thus require replication of each treatment to be informative. The phenomenon of specificity shown by such experiments needs no link to immunological memory.

observations come from studies of naturally occurring genetic variation and were made by evolutionary biologists and ecologists concerned with polymorphism. In studies of polymorphism, specific immunity is not equated with acquired immunity as it is in vertebrate immunology, because invertebrates can show specificity in the absence of 'memory' effects. Indeed, that memory and specificity are so bound in classic vertebrate immunology neatly illustrates how a mechanistic view comes to define a phenomenon. However, from a broader perspective, specificity need not be bound to acquired responses, or vice versa. This seems to differ from the view implying that ideas of specificity and anticipatory responses are not applicable to invertebrates if the molecular and cellular mechanisms do not show evolutionary homology with those of vertebrates[31].

The level of specificity noted in studies of genetic variation greatly differs from the level of specificity understood by mechanistic studies of innate immunity. The latter have suggested, for example, that drosophila can broadly distinguish between fungal and bacterial invaders or between Gram-negative and Gram-positive bacteria[32]. These are exciting discoveries by invertebrate immunologists, and it is encouraging to see that ideas of specificity and degeneracy in innate and acquired immunity are being broadened as mechanistic understanding of these processes increases[33]. Nevertheless, the discriminatory capacity of the innate system, as described by mechanistic studies, is limited and is of uncertain biological relevance. Indeed, it is possible that the antimicrobial response studied so far in drosophila is mainly a general defense against nonpathogenic saprophytes rather than a specific response to pathogens[34]. Certainly this level of specificity is coarse compared with, for example, the very different infection outcomes that occur when different genotypes of a single species of the crustacean daphnia are exposed to different strains of a single bacterial species isolated from within a single natural population[27]. The view from invertebrate immunology would not have predicted the finely tuned specificity seen in whole-organism phenomenological studies. We call this the specificity gap.

Genes of the immune system in the strict sense may not be the focus of specificity in invertebrates. Important genes might encode, for example, proteins on cells of host guts that parasites use to gain entry into hosts, although it is clear some cases of specificity result from induced responses[18–20]. As things now stand, involvement of the immune system in generating specificity in arthropods remains to be disproven, and it seems very premature to rigorously equate invertebrate immunity with a wholly general response system.

Notably, for plant pathogen systems there is no specificity gap, or at least there is a much smaller one. Phenotypic patterns of fine-scale specificity are established in plants, especially for the so-called 'gene-for-gene' system, which is a particular model of specificity for which the genetic control of infection outcomes was established through experimental exposure of plant strains to pathogen strains[35]. Moreover, the molecular underpinnings of this specificity are well understood[36–39]. One important reason why plant pathogen studies have obtained a stronger link between observations of specificity and its mechanisms may be that the rise of new pathogen variants is a grave concern for agriculture. Plant immunologists thus used the phenomenon of specificity (that is, new pathogen strains that had overcome previously resistant crop strains) as their starting point in the search for genes associated with a specific response. In other words, whole-organism studies showed what plant defense systems were capable of, and this seems to have been a fruitful starting point in the search for mechanism.

This is in contrast with the field of invertebrate immunology, which is dominated by the study of drosophila. Few are aware of new and spreading strains of fruit fly pathogens. The starting point of invertebrate immunology is typically a pin prick, the injection of a general 'immunoelicitor' or forced infection with a generalist or opportunistic microbe (such as a plant pathogen[40]). It is therefore not unexpected that the pathways uncovered in invertebrate immunology are of a broad spectrum. The exposure of anopheles mosquitoes to plasmodium[41,42] seems a more promising starting point for many issues, being based on a naturally coevolving system. In general, the study of organisms that at least cause pathogenesis, as was done in a study of drosophila mortality in response to a pathogen[43], seems to offer better opportunities for uncovering relevant immunological mechanisms or processes.

Conclusions and future directions

We still cannot dismiss the possibility that at least some invertebrates have an immune system that is functionally equivalent to the acquired response of vertebrates. The exciting finding of a new family of somatically rearranged receptors on lymphocyte-like cells in the sea lamprey[44] may explain observations of acquired immunity in agnathan vertebrates, which seem to lack recombining immunoglobulins and T cell receptors. Furthermore, a set of highly variable immunoglobulin 'superfamily' genes have been identified in a mollusc, and it was suggested that they may act as somatically diversified receptors of the immune defense[45]. Candidate innate immune system genes for specific immunity in invertebrates have been indicated by studies of molecular polymorphism[46–48], but these suggestions require verification.

The search for relevant molecules has barely begun, but mechanistic understanding of the invertebrate immune system is advancing rapidly[42,49,50]. This knowledge will only expand as an increasing number of invertebrate genomes are sequenced and the capacity to knock out genes swells. These approaches are unquestionably valuable, but phenomenological studies are demonstrating the true scale of invertebrate specificity and immunological memory, and this scale would not have been predicted from mechanistic studies alone. We would like to see the scrutiny of immunologists brought upon these phenomena. The specificity gap can be closed by searching for molecules while being guided by observations of polymorphism. Identifying the mechanistic basis of whole-organism observations of specificity, anticipatory responses and pathogenesis will greatly enhance understanding of disease in the tradition that gave birth to modern immunology. These arguments could apply to how science is approached generally.

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COMPETING INTERESTS STATEMENT

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