Invertebrate immune systems – not homogeneous, not simple, not well understood

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Summary

The approximate 30 extant invertebrate phyla have diversified along separate evolutionary trajectories for hundreds of millions of years. Although recent work understandably has emphasized the commonalities of innate defenses, there is also ample evidence, as from completed genome studies, to suggest that even members of the same invertebrate order have taken significantly different approaches to internal defense. These data suggest that novel immune capabilities will be found among the different phyla. Many invertebrates have intimate associations with symbionts that may play more of a role in internal defense than generally appreciated. Some invertebrates that are either long lived or have colonial body plans may diversify components of their defense systems via somatic mutation. Somatic diversification following pathogen exposure, as seen in plants, has been investigated little in invertebrates. Recent molecular studies of sponges, cnidarians, shrimp, mollusks, sea urchins, tunicates, and lancelets have found surprisingly diversified immune molecules, and a model is presented that supports the adaptive value of diversified non-self recognition molecules in invertebrates. Interactions between invertebrates and viruses also remain poorly understood. As we are in the midst of alarming losses of coral reefs, increased pathogen challenge to invertebrate aquaculture, and rampant invertebrate-transmitted parasites of humans and domestic animals, we need a better understanding of invertebrate immunology.

Vive la différence – invertebrates are a heterogeneous lot

One of the most remarkable developments in the recent history of immunology has been the discovery that the internal defense systems of invertebrates, vertebrates, and even plants share striking similarities. The similarities are most notably among signaling receptors of the Toll/interleukin-1 receptor family and in the signaling pathways involved in orchestrating an innate immune response. For very good reasons, this discovery has been big news: it has been important in revealing how a model invertebrate like *Drosophila melanogaster* defends itself from bacteria and fungi (1, 2); it is pointing the way to the discovery of a previously unknown and important innate defense system in mammals (3, 4), and it is revealing how

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innate immune responses regulate activation of adaptive immune responses (5). It is no wonder there has been an emphasis on stressing the similarities between vertebrate and invertebrate innate immune systems.

One goal of this article was to accentuate the fact that invertebrates are a heterogeneous lot and in aggregate remain relatively poorly understood from an immunological point of view. All of the 30 or so major lineages of animals that we formally recognize with a phylum name, with one exception, are exclusively invertebrate. The one exception, the Chordata, is also comprised in part of invertebrates. Many of these phyla were recognizably separate by the Cambrian explosion between 520 and 530 million years ago. The ages of the last common ancestors among major animal lineages, such as protostomes and deuterostomes and ecdysozoans and lophotrochozoans, are certainly considerably older, given that the origin of animals is 660 to 720 million years ago (6). Furthermore, invertebrates have proceeded along several disparate evolutionary trajectories: colonial or solitary; benthic or pelagic; short or long lived; and marine, freshwater, or terrestrial. Many invertebrates engage in elaborate and obligatory relationships with symbionts (7–9), and at times, it is hard to know where one partner stops and the other begins. *Buchnera*, obligate bacterial intracellular mutualists of aphids, are but one example. These bacteria exhibit a pattern of gene loss or reductive evolution that seems to complement similar processes occurring in its hosts (10).

Invertebrates encounter all the usual sorts of challenges to self-integrity. Their habitats are typically laden with infectious agents: viruses, bacteria, fungi, protists, and other animals. Many invertebrate groups have been in existence so long that they harbor their own specialized lineages of pathogens. For example, mollusks (especially gastropods) support the development of approximately 18,000 nominal species of digenetic trematodes that do great damage to these hosts (11). Invertebrates also suffer from neoplasia (12), and in addition, many substrate-inhabiting invertebrates have to compete for space with other members of their own species, a process that may be accompanied by aggressive encounters along lines of contact and by germ or somatic cell parasitism, a process whereby the genetic material of one organism invades and potentially takes over the body of another (13).

So, the term invertebrate embraces a spectacular diversity of body forms and lifestyles, reflective of long, independent evolutionary histories. Invertebrates continually confront an especially broad variety of challenges to self-integrity. It is important that we strive to understand how invertebrate body defenses operate. The hard coral cover of Caribbean reefs has been reduced by 80% in the past three decades (14), and compromised coral defense responses may well be part of the explanation. Invertebrate-transmitted diseases, such as malaria and schistosomiasis, remain common in the developing world (15, 16). Mass-reared invertebrate species, such as shrimps that are proving to be highly vulnerable to virus infection, provide an increasingly important source of human food (17). Although there are certainly important immunological commonalities among the diverse forms of invertebrates, we must retain open minds for differences and for the completely unforeseen. This point is underscored by recent genome studies that show that even relatively closely related invertebrates seem to have adopted quite different approaches to internal defense.

Comparisons among invertebrate defense systems from genome studies

With the completion of a genome sequence, we can begin to glimpse the realms of the possible with respect to an animal's defense capabilities. The sequence data alone leave much to be deciphered with respect to gene regulation and development, but still some striking points emerge, as shown by the following three brief comparisons.

The first example is of two ecdysozoans, the nematode *Caenorhabditis elegans* and the dipteran insect *D. melanogaster*. The study of invertebrate innate defense mechanisms has been greatly invigorated by use of the powerful *Drosophila* model, which yielded important insights, especially regarding the Toll-signaling pathway (18). The use of *C. elegans* to dissect immune pathways is in its infancy, but it will no doubt also prove to be a versatile model (19). Although several Toll pathway homologs are present in *C. elegans*, worms with mutations in several components of the pathway are not significantly affected relative to wildtype worms in response to pathogens (20). At least eight Toll receptors are present in *D. melanogaster*. Only one Toll-like receptor (TLR) (*tol-1*) gene, which has not been shown to have a direct role in anti-pathogen responses, has been found in *C. elegans* (21). In *C. elegans*, the p38 mitogen-activated protein kinase, programmed cell death and tissue growth factor-β-like pathways are involved in innate immunity (19). The worm also has a large number of C-type lectins that may play a role in pathogen recognition (22, 23), whereas the recognition molecules that have drawn most attention in *Drosophila* are members of a family of peptidoglycan-recognition proteins (24). Although both are ecdysozoans, it will hardly be surprising if the defense responses of these two model organisms continue to look so different. Their respective phyla have been separate for a long time. Also, a soil dwelling, bacteria-ingesting worm is bound to experience quite different immune challenges from a flying insect that thrives on yeast growing on fermenting fruit.

What is more surprising are the differences that have come to light between two members of the same insect order (Diptera), *D. melanogaster* and the mosquito *Anopheles gambiae* (25, 26). The two lineages they represent have been separate for about 250 million years, and the blood-feeding habits of the latter impose unique demands, including exposure to pathogens like malaria parasites and arboviruses imbibed with blood meals. Whereas the Toll signal transduction pathway was found to be conserved relatively, surprisingly large differences were noted between the two insects with respect to the types of putative recognition molecules they possess. In general, a deficit of 1 : 1 orthologs and an over-representation of gene expansions were noted, suggestive of quite different responses to the circumstances in which they live. For example, the *Anopheles* genome contains a large expansion of genes (about 58 genes) encoding proteins with homology to the C-terminal domain of fibrinogen relative to the *Drosophila* genome (about 13 genes) (26). Fibrinogen domains have been implicated in non-self recognition in other invertebrates and vertebrates (27–30), and fibrinogen may play a role in hematophagy in mosquitoes by controlling bacteria ingested with blood meals or in defense against *Plasmodium* parasites (31).

A final example from genome studies is provided by a comparison of immunity-related genes from the ascidian *Ciona intestinalis*, a non-vertebrate chordate, and other members of the Chordata, namely the vertebrates (32). None of the pivotal genes implicated in the

adaptive immune response of vertebrates, such as those encoding T-cell receptors, immuno-globulins, or major histocompatibility complex class I or II molecules, were found in *Ciona*, although divergent orthologs may still prove to be present. Also lacking in *Ciona* were immunoproteosome-specific genes, suggesting that this invertebrate lacks an antigen-presenting system for T cells. A variety of genes likely to be involved in innate immunity were present in *Ciona*, including complement-like, lectin, and *TLR* genes. Even among putative genes of innate immunity, novelty was found; protein domains expected from such genes were found in unique combinations in *Ciona* (32). Jiang and Doolittle (33) searched the *Ciona* genome for 26 proteins involved in clotting or fibrinolysis in vertebrates, and they found no genuine orthologs, although paralogs and constituent domains were found. Gene duplication and shuffling of key modular domains are hypothesized to have occurred, accounting for the appearance of vertebrate-type blood coagulation during the 50–100 million year interval that separates the origins of ascidians and vertebrates.

Such comparisons lead us to expect that immune systems will show strong lineage-specific evolution based on processes such as gene duplication and domain shuffling, and that the specific ecological circumstances encountered will have a relatively large effect on the defenses employed and will transcend phylogenetic relatedness to some extent in dictating immune characteristics. Even apparently conserved and homologous systems, such as the nuclear factor-κB-signaling pathway of the innate immune systems of insects and mammals, seem to have evolved to acquire immune function independently in the two groups (34), further indicating the diverse paths taken to achieve internal defense.

How do invertebrates survive without an adaptive immune system?

An oft-asked question is how invertebrates survive in a pathogen-laden world without an anticipatory, specific, and lymphocyte-based immune system capable of clonal expansion, memory, and fine-tuned responses. Some invertebrates, like bivalves or colonial cnidarians, routinely live for decades (35), filtering or trapping all sorts of microorganisms from the water in which they live, including potential pathogens. So why is it that fast-evolving pathogens do not produce variants that eventually overcome the relatively static defenses of such hosts?

Part of the answer surely lies in the fact that innate defense systems, including the use of RNA interference (RNAi), pattern-recognition receptors (PRRs), anti-microbial peptides (AMPs), phagocytic cells, production of toxic oxygen and nitrogen metabolites, and melanization pathways, are brutally effective. The more we learn about these systems, the more we appreciate their capabilities, including the ability to recognize and inactivate viruses (36). The vast majority of multicellular organisms seem to make do with these capabilities, and hence the argument goes that they must be effective. It is further argued that PRRs recognize antigenic features, such as chitin, peptidoglycan, or lipopolysaccharides (LPSs), which are fundamental to microbial existence and simply cannot be modified. Similarly, AMPs must effectively interfere with some vital function or deliver such a lethal hit that invertebrate pathogens simply cannot recover. However, these arguments seem overly simplistic, given the extreme inventiveness of viruses, bacteria, and fungi in acquiring new genetic information, in generating variant surface antigens and novel mechanisms of

pathogenicity, and in establishing persistent infections in hosts as diverse as plants and vertebrates (37–39). There is no reason to expect these capabilities would be suspended in invertebrate hosts. As just one example, the ehrlichia *Anaplasma marginale* establishes persistent infections in ticks just as it does in cattle. A surface antigen known to be varied in cattle is also varied in the ticks, suggesting selective pressures occurring in the environment of the tick (40).

Another part of the answer is that there is likely to be more to invertebrate defenses than readily meets the eye. The development of microarrays will enable investigators to detect more systematically additional components of the innate immune response of which we are presently ignorant. For example, over 230 genes were shown to be induced in adult *Drosophila* following exposure to microbial infection, many with unknown functions (41). Below, we explore three phenomena not generally considered that might contribute significantly to invertebrate defense: collaboration with symbionts, mosaicism, and production of immune molecules that show a surprising degree of diversity.

If you can't beat 'em, join 'em – collaborating with symbionts in achieving defense

Multicellular animals evolved in a world in which bacteria and other microorganisms were already abundant and hence would have had pervasive interactions with them. Among the gamut of possible associations were some that were mutualistic, either for purposes of nutrition or defense from predators, competitors, or pathogens. Microbial symbionts are hypothesized to be the source of many of the interesting biologically active compounds isolated from marine invertebrates (42). One potential benefit to an animal of mutualistic associations with bacteria is that such bacteria could compete directly for space and resources and thus prevent colonization of the animal's body by potential pathogens. The adaptability of virulence mechanisms of bacterial pathogens could have been matched by equally adaptable defenses produced by mutualist prokaryotes. Thus, the rather static innate defenses of animals could have acquired a much more dynamic and responsive component. The presence of microflora on both external and internal epithelial surfaces is well known to prevent pathogen colonization (43). The surfaces of marine invertebrates are known to harbor species-specific microbial communities that are distinct from the surrounding environment (44). Another context in which mutualists are enlisted is in the deliberate coating of externally brooded eggs of the shrimp *Palaemon macrodactylus* with *Alteromonas* bacteria. The presence of the bacteria protects the eggs from overgrowth and destruction by *Lagenidium callinectes*, an oomycete crustacean pathogen (45). Experimental removal of bacterial symbionts is followed by rapid pathogen-mediated destruction of egg masses. Reconstitution of the symbiont on egg masses restored resistance to the oomycete. Several species of squid and cuttlefish have a special organ called the accessory nidamental gland associated with the female reproductive system. This gland houses symbiotic bacteria that colonize the layers of the cephalopod egg and protect them from overgrowth (46, 47).

These collaborations suggest that the animal partner can differentiate among different types of microbes and select or favor the mutualists to form a so-called exclusive contract (48),

recruit them to specific organs of residence, exclude other microbes from this site, and then prevent the favored symbiont from overrunning the body (9). Although it has nothing to do with defense from pathogens, one system that has been relatively well characterized is the association between *Vibrio fischeri* and the squid *Euprymna scolopes*. A virtual monoculture of the bioluminescent bacterium occupies the light-emitting organ of the squid, which is used to project false moonlight downward, thus preventing the formation of a shadow that could be detected by predators (48). Phagocytic defense cells of the squid police the lumen of the light organ and interact with, yet do not eliminate, *V. fischeri*. Extensive crosstalk between bacteria and tissue is evidenced by changes in the proteome of the squid that only occur in the presence of *V. fischeri* (49). The tissues that contain bacteria have high levels of a halide peroxidase, which probably functions not only in controlling *V. fischeri* but also in protecting squid from potential pathogens (50). Similar arrangements are likely to occur in the associations of solemyid clams and vestimentiferan worms with their chemoautotrophic bacterial symbionts (51, 52). The presence of specific, selective, and well-managed bacterial populations living within invertebrates suggests the presence of sophisticated recognition systems that go well beyond the capabilities of PRRs.

Giant bivalves of the genus *Tridacna* point out an opposite kind of effect with respect to internal defense that might result from an intimate association with mutualist symbionts. Tridacnids harbor photosynthetic dinoflagellate zooxanthellae living in a unique tubular system that arises from the clam's stomach and extends into the mantle (53). Compared to other bivalves, symbiont-containing tridacnids contain a distinctive type of hemocyte, the morula cell (54), that seems to engage with the symbionts in as yet uncharacterized interactions (55). Furthermore, hemocytes of *Tridacna crocea* have a diminished ability to produce superoxide anion, nitric oxide, and phenol-oxidase, relative to hemocytes of bivalves lacking zooxanthellae (56). This diminished cytotoxic capability may be critical to allowing the zooxanthellae to establish and persist in tridacnids, but it may make tridacnids vulnerable to parasites that exploit this mutualism. For example, *T. crocea* is known to be infected with an apicomplexan parasite that lives within the hemocytes (57). In the case of *Tridacna*, one wonders whether the symbiotic dinoflagellates, in addition to providing photo-synthetic products, might also contribute to defending their hosts from other pathogens. Similar considerations might apply to corals. Do corals that have undergone bleaching (lost their symbiotic zooxanthellae) become more vulnerable to pathogens because the protective contributions of their zooxanthellae have been lost?

Does mosaicism influence invertebrate susceptibility to pathogens?

Long-lived hosts, like bristlecone pine trees or some invertebrates, would seem to be at a profound disadvantage in defending themselves from fast-evolving pathogens. A factor that might improve the odds is the possibility that over time, these initially genetically homogeneous hosts become diversified due to the accumulation of spontaneous somatic mutations, which is called the genetic mosaicism hypothesis (58, 59). Thus, leaves on one branch of a plant might be very susceptible to a particular herbivore, but another branch is not, simply because the genes producing anti-herbivore chemicals have been mutated. The same considerations might apply to a colonial invertebrate with a modular body plan for which mosaic colonies are known to occur in nature (60). Clones produced by asexual

reproduction, because they have gone through many cycles of division and have had ample opportunities for spontaneous mutation, may not be as homogeneous as we once thought. Even in non-colonial animals, genes encoding soluble non-self recognition molecules might mutate differently in various parts of the body, and all the resultant gene products might contribute to the organism's aggregate systemic defense, if they were dispersed throughout the body via the circulatory system. The extent to which somatic mutations might actually influence the ability of animals to resist pathogens is poorly known. As noted by Gill *et al.* (59), quantitative or qualitative differences in defense may permit the long-lived host to minimize the impact of its destructive enemies. Although many animals follow the so-called Weismann doctrine (61) and have a strict separation of germinal and somatic lines such that mutations in the latter do not affect the former, members of at least 19 animal phyla do not (59). Included among animals for which mutations in somatic tissues could be passed on to progeny are cnidarians, platyhelminths, bryozoans, annelids, and entoprocts.

Although background mutation alone could by itself generate considerable somatic diversification in a long-lived animal (62), it is also possible that specialized mechanisms exist to increase the mutation rate in response to pathogens. Following exposure to the oomycete *Peronospora parasitica*, the vascular plant *Arabidopsis thaliana* responds by increasing the frequency of somatic recombination. This finding prompts the speculation that possible substrates for somatic recombination might be the large numbers of pathogen-resistance genes known to be present in plants (63). The result could be the generation of new pathogen specificities, some of which might be transmitted to progeny. Kovalchuk *et al.* (64) similarly noted a threefold increase in homologous recombination frequency in tobacco plants exposed to tobacco mosaic or oilseed rape mosaic virus. The results suggested the presence of a systemic recombination signal that is stimulated by virus infection and that can move through the plant, triggering potentially heritable genomic changes. The utility of such a response could be the generation of new specificities in pathogen-resistance genes.

Production of diverse immune molecules by invertebrates – some theoretical and experimental views

It is not known whether invertebrates have mechanisms of enhanced somatic recombination upon pathogen exposure similar to those coming to light in plants. If such a mechanism exists to generate pathogen-recognition molecules, there is an obvious advantage with respect to increasing the likelihood of binding a rare pathogen epitope or of matching a pathogen that is undergoing antigenic variation. There is also the obvious disadvantage that a recognition molecule that recognizes self could be generated. One possible response would be to eliminate cells producing self-reactive recognition molecules, but perhaps a more realistic scenario would simply be to use a self-recognition system to override any signal provided by a mutated recognition molecule that happened to bind to a self cell. Another reason to wonder whether the ability to produce somatically diversified recognition molecules would be advantageous is simply whether or not a sufficient quantity of the molecules could be produced to be of any value. Without the ability to produce expanded clones of cells that generate unique and relevant recognition capabilities, would somatic diversification be worth the effort?

Under appropriate circumstances, which we explore below with the aid of a mathematical model, the answer is affirmative: diversification, even in the absence of clonal selection, can be advantageous. First, however, we shall re-examine the potential role of diversification in innate immunity with respect to its much more familiar role in adaptive immunity.

In adaptive immunity, diversification is extremely thorough, generating receptors that are essentially random, covering the whole space of potential epitopes. This production is consistent with their role as recognition molecules, which must identify non-self and signal its presence to the rest of the immune system. Holes in the repertoire, under these circumstances, could represent fatal vulnerabilities. Given this state of affairs, the number of cells bearing receptors appropriate for any given epitope will be very small, and amplification by clonal selection will be necessary to accomplish this task. Finally, such expansion could be devastating if directed against host cells. Tolerance to self, another task of selection, becomes another indispensable corollary of diversification.

There is another role of immunoglobulins that may be more relevant in the context of invertebrate immunity. They are effector-enhancement molecules that link effectors of innate immunity, such as phagocytes and complement components, to microbes. In this context, they are not recognition molecules. Indeed, the effectors must already be present and activated. Serum immunoglobulin by itself is not particularly dangerous and is cleared effectively in a short time, so that self-reactivity, if it should arise as a side effect of infection, will not persist.

Suppose there is such a bridge, or effector-enhancement molecule, in an invertebrate. When, if ever, would diversification prove advantageous? Random changes will likely lead to enhancement of effectiveness in a minority of molecules and no change or diminution in the rest. When would this particular tradeoff prove worthwhile? Let us formalize this question and state it mathematically. For the case of a receptor, the on- and off-rates for binding to its ligand are key determinants of its effectiveness. We shall focus attention on the off-rate or its inverse, the mean lifetime of the receptor–ligand complex, τ, but similar arguments can be developed for multiple traits as well. The concentration density of molecules with inverse off-rate τ is designated by $c(\tau)$, and the effectiveness of molecules with inverse off-rate τ is designated by $f(\tau)$. Effectiveness is intended to be a very general term standing in for any number of possible quantitative measures of immunological competence. The mathematical definition of the effectiveness function will depend on the detailed mechanism of the process in which the molecules participate. We provide one such possible definition below. For the sake of simplicity in the argument, if we assume that the molecules act independently of each other, the total effectiveness is given by the following integral expression:

$$f_{\mathrm{T}} = \int c(\tau) f(\tau) d\tau$$

This formula can be regarded as a weighted average effectiveness, with weights being the relative concentrations. Random diversification corresponds to increasing the spread of the distribution $c(\tau)$, without changing its location. The variance of τ increases, but the mean remains unchanged. A population with no diversity has variance zero. The question we ask is: when does increasing the variance from zero increase the total effectiveness? That is,

when is the derivative of f_T with respect to the variance of τ positive? Under very mild assumptions, the answer is that f_T increases with the variance, when the second derivative of f with respect to τ (evaluated at the mean value τ_0) is positive (Fig. 1).

In many common situations, this second derivative is negative, due to the prevalence of saturating functions in biology and the fact that these functions must have negative second derivatives (at least somewhere and maybe everywhere). There is an important class of processes where the second derivative is generically positive. These are compound processes in which the dissociation of the molecule in question can occur at several places in the chain of reactions. Suppose, for example, that a process involves the following sequence of reactions: (i) the host defense molecule (L) binds/unbinds receptor (R) on the microbial surface; (ii) the host effector (E) binds to the host defense molecule on the surface; and (iii) the host effector neutralizes the microbe. The chemical reaction scheme below illustrates this process:

$$E + L + R \xrightarrow{\alpha} E + LR \xrightarrow{\beta} ELR$$
$$\downarrow \tau^{-1} \qquad\qquad \downarrow \tau^{-1}$$
$$E + L + R \qquad EL + R$$
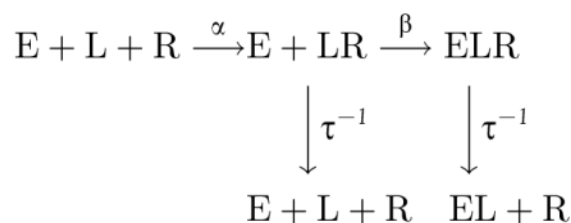
A natural measure of effectiveness for this process is the equilibrium number of complete ELR complexes. A straightforward computation yields the following:

$$[\mathrm{ELR}] = \frac{\alpha\beta[\mathrm{E}][\mathrm{L_0}][\mathrm{R}]\tau}{(1+\tau\beta[\mathrm{R}])(1+\tau\alpha[\mathrm{E}])}$$

The second derivative is positive for as long as τ is less than some threshold value. That is, if the molecule already binds very well, then diversification alone will not increase the effectiveness. If it binds only moderately well or poorly, random diversification could, even without subsequent selection, improve its performance.

One can further elucidate this observation by taking it to its natural limit. Suppose a process absolutely requires an inverse rate constant above some threshold value for it to have any effectiveness at all and that τ_0 is below that rate. Then in the non-diversified state, the process is completely ineffective. All diversification has to do is produce a few molecules with $\tau > \tau_0$ to improve this situation.

This result has salutary implications for the evolutionary origins of diversification mechanisms in immunity. Such mechanisms can arise even in the absence of (and therefore prior to) sophisticated mechanisms for clonal expansion and tolerance induction, which may then have arisen later and co-evolved alongside a progressively more extensive and tightly regulated diversity-generating mechanism.

The existence of active diversification in invertebrates could help resolve an additional puzzle in the evolution of immunity. It seems as if all mechanisms of somatic diversification arose at the same point, some time between the divergence of the vertebrates and of the cartilaginous fishes, as all jawed vertebrates possess V(D)J recombination, TdT, and somatic hypermutation or gene conversion, or both (65). This sudden burst of genetic innovation has been dubbed the big bang of immunology (66, 67). However, if somatic mutation arose prior to and independently of the acquisition of the recombination activation genes (*RAG-1* and *RAG-2*) (68), then the apparent coincidence loses some of its mystery. Perhaps, a pre-existing host-defense mutation mechanism was simply co-opted and enhanced in the novel context of obligatory combinatorial diversification.

Several recent experimental findings suggest that the above may be more than just a theoretical exercise. Now that molecular methods for cloning and sequencing have become more widely available, increasingly detailed analyses have disclosed a diversity of factors involved in the innate immunity of a variety of organisms. Counter to expectations based on the model of pattern recognition in which a limited set of PRRs sufficiently recognizes and responds to groups of pathogens (69), factors that function in the context of innate immunity have been shown to display sequence diversity in vertebrates and even more so in invertebrates. The innate immune system of rainbow trout employs three variants of complement factor C3 (70). Carp and other teleost species also have isoforms of C3 and additional complement factors. These additional iso-forms are hypothesized to increase the range of epitopes that can be recognized (71, 72). Diversity has also been documented from invertebrate deuterostomes. Five different families of diverse V-region-containing chitin-binding proteins that may serve in non-self recognition were described from the proto-chordate *Amphioxus* (73). Among echinoderms, the genome of the sea urchin *Strongylocentrotus purpuratus* was predicted to harbor as many as 1200 scavenger receptor cysteine-rich genes, for which expression patterns differ between individuals (74, 75). Additionally, extensive diversity was evident in a group of related sequences that made up 94% of clones recovered from a cDNA library derived from LPS-stimulated coelomocytes of *S. purpuratus* (76). At the other end of the spectrum of animal phylogeny, extensive diversity of receptors for non-self recognition governing fusion/rejection interactions and internal defense occurs in sponges (*Geodia cydonium*) (77, 78) and colonial cnidarians (79). As expected, similar diversity occurs also in the other major lineage of animals, the protostomes. The crustacean *Penaeus monodon* (Ecdysozoa) expresses variant sequences of AMPs such as peneaidins and crustins (80–83). A representative of the Lophotrochozoa, the gastropod *Biomphalaria glabrata,* expresses diverse parasite-reactive hemolymph lectins in response to infection. This response includes fibrinogen-related proteins (FREPs) that are comprised of N-terminal immunoglobulin superfamily (IgSF) domains and fibrinogen-related sequences at the C-terminus (27). FREP sequences display diversity due to point mutations and alternative splicing. Thirteen different *FREP* gene subfamilies have been recognized, based on the criterion that members of a subfamily share 86% or higher sequence identity of the N-terminal IgSF sequence (29, 76, 84, 85).

We are at an interesting juncture with respect to interpretation of the diversity revealed by such studies. Genome sequences are not yet available for most of the organisms used, and many of these observations have been based on medium-to-large-scale application of

polymerase chain reaction (PCR)-based amplification and sequencing methods. Nucleotide misincorporations occur even when DNA polymerases of the highest fidelity are used (86). Furthermore, amplification of a target sequence that is a member of a family of related genes can potentially generate sequences comprised of portions of the different family members, a PCR artifact known as template switching (87). The observed sequence diversity of PCR amplicons can be falsely increased. These nagging sources of artifact pose a distinct challenge to investigators seeking to document diversity of immune molecules in animals that may otherwise be poorly known. Nonetheless, several laboratories using a variety of methods with quite different experimental subjects are converging on the discovery of surprisingly diverse immune molecules, strongly suggesting that at least some part of the diversity is real. These observations need not be considered inimical to the concept of recognition of pathogen-associated molecular patterns mediated through a set of PRRs of limited diversity. Rather, variable recognition or effector molecules could play an important complementary role in innate defense.

Invertebrates and viruses – a surprising gap in our knowledge

Like all organisms, invertebrates are plagued by viruses that not only cause mortality but also inflict subtle fitness penalties (88). Some of these viruses are of great concern, because they directly threaten the continued vitality of enterprises such as shrimp farming (17, 89) or commercial harvesting of edible bivalves (90). Others, such as the baculoviruses, have been used in the biocontrol of lepidopteran pests (91) or have been developed as important expression vectors of eukaryotic proteins. Over 500 known varieties of arboviruses are exceptionally versatile in that they can infect both cells of their invertebrate vector host and of a vertebrate host (92). One such virus, West Nile virus (93), is currently epidemic in the United States. Although many invertebrate viruses have made themselves conspicuous in one context or another, the overall extent of our knowledge of the diversity and biology of invertebrate viruses is rather scant. We are also surprisingly ignorant about how invertebrates control or eliminate viruses. Vertebrates deal with viruses in multiple ways. Viral infection results in the induction of interferons, which in turn upregulate the transcription of many genes that protect cells from damage and death. Infection may also be countered by the activation of potentially lytic natural killer (NK) cells, production of antibodies to block viral receptors and to promote phagocytosis by opsonization, and use of cytotoxic T cells to kill virally infected cells.

NK cells, antibodies, and cytotoxic T cells are all lacking in invertebrates. No invertebrate has been shown to have obvious homologs of interferon (65, 94), although an important component of a pathway activated by interferon in mammals, the enzyme $2′,5′$-oligo A synthetase, has been reported in sponges (95, 96). In mammals, this enzyme activates an RNA-digesting enzyme that can destroy viral RNA and also reduce viral and host cell mRNA levels. The extent to which it functions in protection from viruses in sponges is not known. So how do invertebrates respond to viruses?

Work with model invertebrates and their anti-viral responses has thus far not proceeded from the perspective of basic immunology. These investigations are undertaken mostly from the perspective of trying to understand the basic raison d'être for the phenomenon of RNAi and

its role in gene silencing (97). It is not the purpose of this article to review RNAi but rather to try to place it in the general context of invertebrate immunology. RNAi is a sequence-specific response initially triggered by double-stranded RNA (dsRNA) that subsequently targets any cytoplasmic RNA species sharing homology with the triggering sequence. It is conserved across plants, some fungi, and animals, and it is increasingly viewed as a basic trans-eukaryotic genome defense against molecular parasites like viruses and transposons (37, 98, 99). Work with both *C. elegans* and *Drosophila* has contributed significantly to unraveling the general properties of RNAi, and an anti-viral context has been shown specifically for *Drosophila* (100). These authors showed that flock house virus, an RNA virus that can infect both vertebrate and invertebrate hosts, is both an initiator and target of RNAi in *Drosophila* host cells. It encodes the protein B2 that also operates as an RNA-silencing suppressor, suggesting that RNAi is a potent host defense mechanism that must be circumvented by the virus (100). In general, the role of RNAi as a viral defense mechanism is better understood in plants than animals. Several plant viruses are also known to have elaborated a variety of RNAi counter measures (37).

Many viruses of invertebrates have either single-stranded RNA or dsRNA genomes, and as noted by Ahlquist (101), RNA viruses replicate their genomes through complementary strands, resulting in dsRNA replication intermediates that make them potentially vulnerable to RNAi. For instance, positive sense single-strand RNA viruses from plants have shown to be vulnerable to RNAi (102). Thus, one would expect RNAi to be an important defense mechanism of invertebrates against potentially all RNA viruses. But what about DNA viruses? Some of the important DNA viruses of invertebrates are iridoviruses, baculoviruses, entomopox viruses, and densoviruses. In general, our knowledge of the relationships between RNAi and DNA viruses is still rudimentary. Some DNA viruses of plants have been shown to be silenced by RNAi, possibly because dsRNA was fortuitously produced during their transcription or amplification (37). Other intracellular anti-viral mechanisms, such as the production of RNA-editing enzymes that mutate viral genomes (103), may also be operative in invertebrates.

Given that several arboviruses, like yellow fever, dengue, and West Nile viruses, are important human pathogens, it would be especially valuable to know more about their biology within the cells of their mosquito hosts. Adelman *et al.* (104) pre-exposed *Aedes aegypti* to Sindbis virus engineered to contain dengue sequences, and *Ae. aegypti* were subsequently shown to be highly resistant to challenge with dengue isolates containing homologous sequences, an effect attributed to RNAi. Similar results were obtained by Caplen *et al.* (105) using Semliki Forest virus and dengue in mosquito cell lines. These studies suggest novel ways to prevent replication of arboviruses in arthropod vectors, such as development of transformed mosquito lines that express viral RNAs that would stimulate RNAi and effectively prevent natural infection with viruses containing homologous sequences (106).

Although it seems very likely that RNAi is an important defense against viruses, do invertebrates have mechanisms other than intracellular ones to protect themselves from viral attack? The state of our very limited knowledge is perhaps best exemplified with respect to anti-viral immunity in marine crustaceans. There are about 20 different viruses that

collectively comprise the greatest threat to the world's shrimp industry (17, 107), yet we know very little about anti-viral mechanisms in shrimps. White spot syndrome virus (WSSV), which has had the greatest impact on shrimp culture, causes a sharp reduction in hemocyte counts of *P. monodon*, but the underlying reasons for this reaction are unclear (108). Pan *et al*. (109) found that the tissue extracts obtained from blue crabs (*Callinectes sapidus*), shrimp (*Penaeus setiferus*), and crayfish (*Procambarus clarkii*) possess anti-viral activities that inhibit Sindbis, vaccinia, vesicular stomatitis, mengo, banzi, and poliomyelitis viruses. The blue crab inhibitor prevents viruses from attaching to cells and has a lipid component, but beyond that it remains uncharacterized. Although we are ignorant of the mechanisms, antiviral defenses are clearly present in shrimp and can be enhanced by selection, as shown by the presence of a shrimp strain specifically resistant to taura syndrome virus (110). Also, it has been shown that shrimp can be protected from virulent WSSV by pre-exposure to a generally avirulent infectious hypodermal and hematopoietic necrosis virus, but the underlying mechanism for the interference is not known (111).

Studies of TLRs in mammals have shown that TLR3 recognizes dsRNA and thus could have an anti-viral role (112). TLR9 might also be involved in anti-viral defense (113). There are no data to suggest that invertebrates have comparable receptors capable of recognizing viruses. Also lacking is evidence that defined lectins with anti-viral activity exist in invertebrates. An important mechanism for control of viral infections in invertebrates could be induction of apoptosis before the virus has a chance to complete its development (114), but if so, the molecules involved in detecting viral infection are not known.

Some specific questions

Fig. 2 provides a more extravagant model than normally envisioned for the operation of invertebrate immune systems. This schema is presented more for the purpose of highlighting some of the specific questions that await resolution rather than to promulgate it in preference to simpler and perhaps more fundamentally correct models. As we emphasized above, it is not realistic to expect that all invertebrate immune systems function in the same way, and hence some of the questions we ask below may apply to some groups more than others.

Do invertebrates rely on identification of self as a way to prevent self-reactivity and to help delineate non-self?

NK cells of vertebrates have receptors that transfer inhibitory signals to the cell when a self-signal is received. The model in Fig. 2 illustrates self signals that interact with self receptors to inhibit hemocyte autoreactivity. Although it is clear that invertebrates can clear non-self particles from their blood and react to grafts or to conspecifics competing for space, the extent to which such reactions are dependent on the absence of an inhibitory self signal, as opposed to the recognition of non-self, is not clear. Quesenberry *et al*. (115) have noted that tunicates express lectins able to recognize self and are therefore potentially able to recognize missing self or induced or altered self. Work with *C. elegans* or *Drosophila* has yet to suggest the presence of a self-recognition system. The nature of the molecules that indicate self or of the receptor that interacts with the self signal, if they exist, is unknown for any invertebrate. Progress in identifying such molecules would represent a significant advance in

our understanding of invertebrate defenses. They might, for example, hold the key for understanding the basis of host specificity shown by many invertebrate pathogens.

Can invertebrates diversify their non-self recognition molecules?

With respect to recognition of non-self, the predominant paradigm is for the presence of both circulating and cell membrane-associated pattern-recognition molecules capable of recognizing determinants characteristic of broad groups of pathogens. Abundant evidence for these molecules now exists, especially in *Drosophila*, and they could prove to be sufficient to account for all non-self recognition. There have been no reports of the existence of unusual mechanisms to diversify known invertebrate pattern-recognition molecules, but there is a growing body of literature to suggest that invertebrates can produce diversified immune molecules. The extent to which diversified non-self recognition molecules occur in invertebrates, which might be generated by unusual processes such as gene conversion or somatic mutation, is a high priority for study. While being mindful of the possibility of PCR-related artifacts, some of the challenges awaiting such studies are the following: to reveal the mechanisms for generating diversity, to learn whether pathogen exposure provokes diversification, and to determine whether this diversity is relevant in defense. The presence of a self-recognition system would prevent the problem of autoreactivity, which could potentially result from production of diversified receptors.

Fig. 2 suggests one possible way that diversified antigen receptors might work, borrowing from a model proposed by Richards and Renwrantz (116) based on their study of phagocytosis of opsonized particles by hemocytes of the snail *Helix pomatia*. A hemocyte-associated receptor may be capable of binding to several different humoral recognition factors, but only after the latter have bound non-self structures and have been modified. The modification might be an enzymatic cleavage or conformational change that exposes a ligand site that can be bound by the hemocyte-associated receptor, resulting in phagocytosis of the opsonized structure. The appeal of this model is that a multipurpose membrane-associated receptor could interact with a variety of different humoral recognition molecules.

Do invertebrates have specialized mechanisms to identify and destroy viruses or virally infected cells?

Another purpose for the model provided was to highlight our ignorance about how invertebrates cope with viral infections. We are now rapidly learning about RNAi, and perhaps this and other intracellular mechanisms are all that invertebrates have available for combating viruses. However, it seems premature to conclude that effective means do not exist for blocking viral entry into cells, for promoting their aggregation and phagocytosis, and for identifying and destroying virus-infected cells. Are the mechanisms involved part of the standard invertebrate package of pattern-recognition and phagocytosis, or might more specialized systems for eliminating viruses exist? We have much to learn in this area.

Do invertebrates with abundant symbiont populations use them in internal defense?

For the many invertebrate species that harbor dense symbiont populations, do the symbionts play a role other than in nutrition? Whether symbionts might aid hosts by co-evolving with

pathogens and matching virulence factors with anti-virulence factors remains an intriguing subject for future study.

Conclusions

Invertebrates have become popular subjects in the context of ecological immunology, because of the simple mechanisms underpinning their innate immune systems (117). The use of invertebrates as models in all kinds of studies is certainly to be encouraged, but at the same time, it is critically important not to underestimate, over-simplify, or take for granted their immune capabilities. It is not at all clear that studies attempting to measure immune competence by some unidimensional parameter, such as melanin content or hemocyte counts, will conclude anything meaningful. When claims about the immune capabilities of invertebrates are made, such as in the recent study providing evidence of immunological memory in copepod crustaceans (118), they need to be backed up by explicit information about the antigenic nature of the parasites employed. The host responses inferred to be present need be identified and their efficacy ascertained.

The term invertebrate is dangerously broad, so we should expect that the solutions for internal defense in one group would be different from the solutions obtained in other groups. Although invertebrates lack many of the well-known complicated features of vertebrate immunity, they may well employ alternative means to generate diverse and complex responses. In fact, there is much about their immunobiology that we do not fundamentally grasp, and this lack makes the future study of invertebrate immunobiology compelling. As noted above, the impetus for learning more is strongly driven by the urgent need to understand several applied problems that relate to invertebrate defenses.

Acknowledgments

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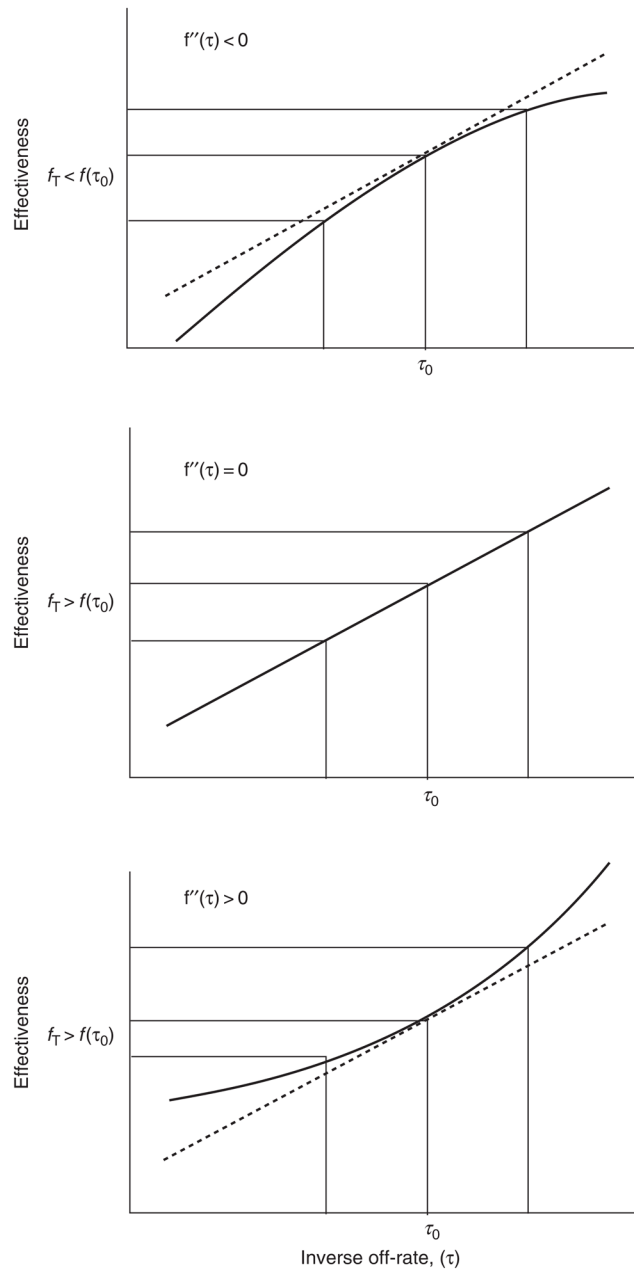
Fig. 1. An illustration of the conditions under which diversification without subsequent selection is likely to have a positive effect

The abscissa is the inverse off-rate and the ordinate is the effectiveness of the response. Suppose that τ_0 is the current value of the inverse off-rate. The three panels show the different behaviors that can occur in the neighborhood of τ_0. The top panel shows the case of a negative second derivative. Note that this condition diminishes the advantage of increasing τ and exacerbates the disadvantage of decreasing τ. Thus, the expected value in the presence of diversification, f_T, is less than the current value of the effectiveness $f(\tau_0)$. The middle panel shows the case where the second derivative is zero, where potential advantage is exactly balanced by potential disadvantage, so that $f_T = f(\tau_0)$. Finally, the bottom panel

shows the case of particular interest: positive second derivative, which enhances the advantage of increasing τ and moderates the disadvantage of decreasing τ. Under these circumstances, the expected value under random diversification is greater than the present value, so that diversification becomes a good bet, even without selection.
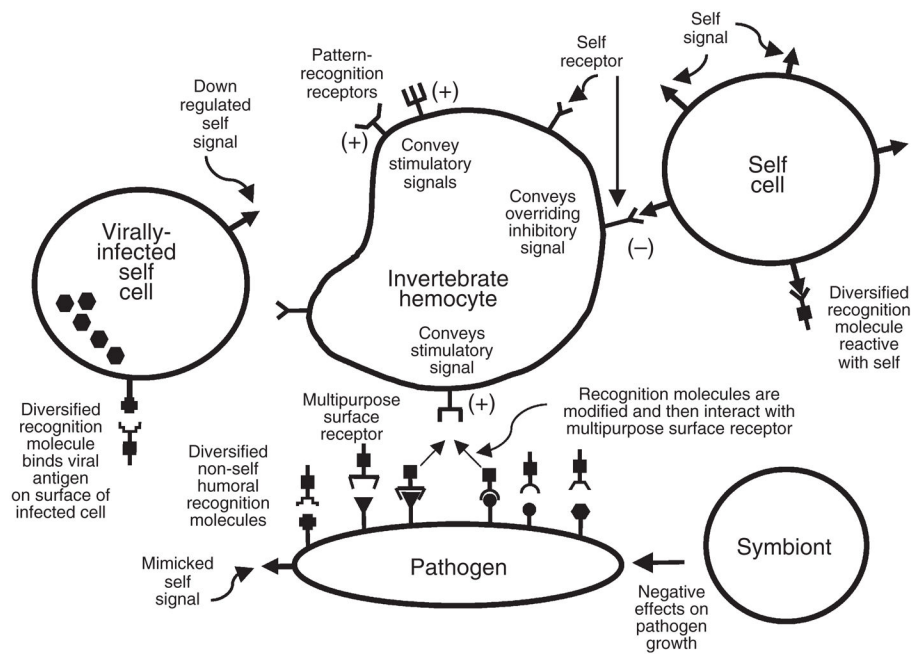
Fig. 2. A general model indicating hypothetical interactions between the major component of the invertebrate defense system, a multipurpose hemocyte, and self, non-self (pathogen), and virally infected self cells

This model emphasizes the recognition phase of the interaction and does not consider the signaling pathways or effector mechanisms employed by the hemocyte in actually killing a pathogen. Note that the version presented involves a system of dual recognition, featuring both detection of a specific self signal, which is found on self cells, and non-self signals, which are on pathogens or virally infected self cells. Pathogens are assumed to be capable of generating diversified antigens. Receipt of a self signal conveys an inhibitory signal to the hemocyte and downregulates a response, analogous to natural killer (NK) cells, although no strict homology with NK self receptors is implied. In addition to a self receptor, two different kinds of non-self receptors are illustrated, both of which convey stimulatory signals to hemocytes. The response of the hemocyte might depend on the integration of both inhibitory and stimulatory signals. Non-self receptors of the first category are the conventional pattern-recognition receptors such as the surface-associated peptidoglycan-recognition protein receptors of *Drosophila* (24). These would interact with a repetitive pattern on a bacterium, fungus, or possibly a virus. Also shown is a hemocyte-associated multipurpose receptor that interacts with a set of diversified humoral non-self recognition molecules. These humoral factors might be diversified by processes such as somatic mutation and gene conversion. Once these recognition molecules have bound a pathogen-associated antigen, they are either modified or undergo a conformational change that allows them to interact with the multipurpose hemocyte receptor. Note that some of these diversified recognition molecules might react with components on self cells, creating the possibility of autoreactivity. This tendency could be overridden by simultaneous receipt of the self signal by the self receptor. Note also that the pathogen may bear a mimicked self signal that enables it to escape detection. Another pathogen strategy not shown here is the production of factors that directly harm or interfere with defense cells or molecules. Self

cells infected with a virus might either undergo downregulation of self signals or upregulation of membrane-associated viral antigens, tipping the balance in favor of destruction of the infected cell. Depending on the invertebrate involved, symbionts may also play a role in producing anti-microbial peptides or other biologically active compounds that inhibit pathogen growth.