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Natural selection on the *Drosophila* antimicrobial immune system

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Summary

The evolutionary dynamics of immune defenses have long attracted interest because of the special role the immune system plays in mediating the antagonistic interaction between hosts and pathogens. The antimicrobial immune system of the fruit fly *Drosophila melanogaster* is genetically well characterized and serves as a valuable model for studying insect and human innate immune defenses. I review here evolutionary and comparative genomic analyses of insect antimicrobial immune genes, with an emphasis on *Drosophila*. Core signal transduction pathways in the immune system are orthologously conserved across long evolutionary distances, but genes in these pathways evolve rapidly and adaptively at the amino acid sequence level. In contrast, families of genes encoding antimicrobial peptides are remarkably dynamic in genomic duplication and deletion, yet individual genes show little indication of adaptive sequence evolution. Pattern recognition receptors that trigger humoral immunity are evolutionarily rather static, but receptors required for phagocytosis show considerable genomic rearrangement and adaptive sequence divergence. The distinct evolutionary patterns exhibited by these various classes of immune system genes can be logically connected to the functions of the proteins they encode.

Introduction

Natural selection may act strongly on immune systems as hosts adapt to novel, diverse and co-evolving pathogens. Any effective host defense system must have the capacity to a) recognize potentially pathogenic infection, b) signal activation of the immune response, and c) kill the infectious agent. From a pathogen's perspective, surviving the immune defense is essential. This places an evolutionary premium on mechanisms to evade, resist or suppress host immunity. Every successful advance by the pathogen, however, creates selective pressure on the host to evolve reestablished immunity. The stage is thus set for coevolutionary interactions wherein hosts and pathogens reciprocally adapt to each other even though no major shifts in pathology are necessarily achieved [1,2]. It is well established that genes in the immune systems of vertebrates and *Drosophila* exhibit significantly elevated rates of amino acid evolution relative to non-immune genes [e.g., 3–6], indicative of molecular adaptation [7–9]. Not all immune response genes evolve equivalently, though, and we can draw inference regarding the nature of pathogen interaction with different components of the immune system through the distinct evolutionary histories and trajectories of various immune genes.

Insect antimicrobial immune responses consist primarily of defensive phagocytosis and extracellular circulation of potent antibiotic peptides. The fruit fly *Drosophila melanogaster* emerged as a valuable genetic model system for studying innate immunity in the mid-1990s

[well reviewed in 10]. The first evolutionary genetic analyses of *Drosophila* immunity genes quickly followed [11–13] and population genetic data on various components of the system have steadily accumulated since then. Complete genome sequencing of *D. melanogaster* and several other insects over the past few years, culminating last year with the landmark sequencing of 12 species of *Drosophila*, have now made comparative genomic analyses of insect immune systems possible [6,14–17]. In this review, I synthesize that literature to describe the molecular evolution of the *Drosophila* antimicrobial immune defense, relating gene function to evolutionary history and likely host-pathogen interactions.

Natural Selection on the Humoral Immune Response

The *D. melanogaster* humoral antimicrobial defense [10] is regulated by pattern recognition receptors (PRRs) such as peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs). These PRRs are exquisitely sensitive to ubiquitous and highly conserved microbial cell wall components such as bacterial peptidoglycans and fungal glucans. *Drosophila* humoral immune responses are initiated when activated PRRs trigger two primary signaling cascades, the Imd and Toll pathways, which ultimately drive the production of short, extracellularly secreted antimicrobial peptides (AMPs). Other PRRs play negative regulatory roles. Septic injury in *Drosophila* additionally activates wound healing through the JNK pathway, which bifurcates from the Imd pathway, and stress responses via the JAK/STAT signaling pathway. The *D. melanogaster* Toll pathway is also involved in embryonic development, hematopoiesis and possibly resistance to viruses and parasitoids. The genetic architecture of the *D. melanogaster* defense is stereotypical of insects, and homologous Imd, Toll, JAK/STAT and JNK signaling pathways are found even in vertebrates.

Intuition might suggest that PRRs and AMPs should experience strong natural selective pressure because these proteins come into direct contact with invading microorganisms. Conversely, intracellular signaling genes might be expected to show little indication of adaptive evolution. In surprising defiance of this intuition, empirical data has consistently shown that signaling proteins evolve rapidly while PRRs and AMPs show little indication of adaptation at the amino acid sequence level. AMP and PRR genes show higher rates of genomic duplication and deletion than do signaling genes. These observations are illustrated in Figure 1 and discussed in detail below.

The genomic complement of antimicrobial peptides varies widely among insects, with many AMP gene families found in only a few closely related species. For example, *D. melanogaster* produces eight described classes of AMPs encoded by 28 distinct genes, but genes encoding only three of these peptide classes (defensins, cecropins and lysozymes) can be found in the genomes of bees, mosquitoes and beetles [14–17]. These other insects each produce their own unique AMPs. Genomic rearrangements of AMP gene families and acquisitions of novel AMPs are apparently frequent and rapid in insects, and such events occur even within the genus *Drosophila* [6,12,13,18–20] at a rate much higher than what is typical of *Drosophila* gene families [6].

Despite their rapid genomic turnover, extensive study has revealed no evidence of adaptive amino acid diversification in *Drosophila* AMPs [6,11,12,18,21,22], implying that minor adjustments in peptide function through altered amino acid sequence are not mediating host-pathogen coevolution. Most AMPs have simple and non-specific modes of antibiotic action, such as driving pathogen lysis through membrane disruption [23]. Even when microbes may be capable of evolving resistance to individual AMPs, the evolution of global resistance *in vivo* is probably limited by host employment of multiple AMPs with distinct activities. This strategy is analogous to the human attempts to forestall evolution of antibiotic resistance in clinical settings through simultaneous application of multiple antibiotics. The lack of adaptive

sequence evolution in *Drosophila* AMPs contrasts with the observation that AMP gene family radiation is frequently associated with amino acid diversification in vertebrates [24]. A mammalian defensin has recently been shown to have unexpected involvement in hair pigmentation [25], suggesting that mammalian AMPs may play roles outside immunity that place them under different selective pressure. Adaptive sequence radiation of a termite AMP has been observed, and the selection pressure was attributed to a major shift in host ecology [26].

PRRs are evolutionarily more stable across insects, with all of the insect genomes sequenced to date containing PGRP and GGBP multigene families. These show little indication of genomic rearrangement within the genus *Drosophila* [6] or between the mosquitoes *Aedes aegypti* and *Anopheles gambiae* [17]. PGRP and GGBP gene families have experienced considerable flux on the deeper lineages that separate flies, mosquitoes, bees and beetles, though, with numerous independent expansions and contractions in family size evident [6, 15–17]. Whether these genomic fluctuations mark adaptation to the distinct spectra of microbes faced by each of these insects remains to be determined.

PRRs could plausibly be expected to co-evolve with pathogens if pathogens escape host recognition through evolutionary modification of their cell walls, but neither population genetic nor comparative genomic analyses have revealed substantial evidence for adaptive evolution in *Drosophila* PGRP or GGBP amino acid sequences [4,6,27,28]. One exception is a two amino acid insertion in the recognition site of the PGRP-LCa splice variant of *Drosophila* species in the *melanogaster* subgroup. The insertion is predicted to alter the peptidoglycan binding properties of this PGRP-LCa isoform and appears to have been, along with several compensatory mutations, strongly favored by natural selection [6]. The observation that PRRs show little evidence of adaptive sequence evolution, however, seems to be generalizable across invertebrates including *Nasutitermes* termites [29], *Anopheles* mosquitoes [30], and cladoceran arthropods in the genus *Daphnia* [31]. Evasion of host recognition by massive cell wall modification is possible, as evidenced by the vertically transmitted endoparasitic bacterium *Spiroplasma poulsonii* [32], but the relative rarity of adaptive amino acid evolution in PRRs would seem to suggest that dispensing with or substantially modifying structural components of the cell wall may not be a viable evolutionary strategy for most insect microbial pathogens.

There is striking genomic conservation across insects in core immune signaling pathways. Nearly every gene in the Toll, Imd, JAK/STAT and JNK signaling cascades is found in perfect orthology between species of *Drosophila* [6], mosquitoes [14,17], the honey bee [15] and the red flour beetle [16]. In spite of this remarkable maintenance of orthology, signaling proteins in these pathways are rapidly and adaptively diverging at the amino acid sequence level [4,6, 17,33]. The Relish cleavage complex of the Imd signaling pathway provides a particularly striking example. Relish, an NF- κ B family transcription factor, is cytoplasmically bound by an autoinhibitory domain in the absence of infection. Upon immune stimulation, a caspase-mediated cleavage complex degrades the inhibitory domain and the activated transcription factor is translocated to the nucleus. Several proteins in the cleavage complex (Dredd, dFADD, IKK β , and Relish itself) show strong evidence adaptive evolution [3,6,33,34]. The putatively adaptive amino acid substitutions are systematically overrepresented in the *Relish* autoinhibitory domain and cleaved linker, the *Dredd* caspase domain, the dFADD death domain, and the IKK β kinase domain [6,33,34]. There thus is compelling evidence for adaptive evolution in the Relish cleavage complex as a whole, with adaptive substitutions found in protein domains functionally important for releasing the active Relish transcription factor. This adaptive evolution appears to be restricted to taxa in the *melanogaster* species group and is not characteristic of all species in the genus *Drosophila* [6,35], but in an interesting convergence, *Relish* also shows credible evidence of adaptive evolution in *Nasutitermes* termites, again with putatively adaptive substitutions localized in and around the caspase cleavage site and the linker

[29]. The direct agent of selection on the Relish cleavage complex has not been determined in either *Drosophila* or *Nasutitermes*, but one hypothesis is that the complex is engaged in a coevolutionary arms race with pathogens capable of interfering with host immune signaling [34], such as bacteria that inject immunomodulatory molecules into host cells via Type III Secretion Systems [36–37], immunosuppressive fungi [38], and parasitoid mutualistic polydnaviruses [39]. The requisite physical interactions among proteins in a signaling cascade may mean that host mutations to escape pathogen interference are fixed in conjunction with compensatory mutations in the same or other proteins, accelerating amino acid divergence and enhancing the signature of selection in the entire pathway [17,40].

The Cellular Immune Response

The *Drosophila* cellular immune response to bacterial infection consists primarily of defensive phagocytosis by circulating and stationary hemocytes [10]. Bacteria to be phagocytosed are captured by membrane surface receptors such as those in the Eater/Nimrod and scavenger receptor families, then internalized in a membrane enveloped phagosome where they are killed. Phagocytosis may be facilitated by extracellularly secreted opsonins, such as insect thioester containing proteins (TEPs), which are hypothesized to bind to both microbial and eukaryotic pathogens and enhance phagocytosis.

Phagocytosis receptors show considerable genomic diversification across the genus *Drosophila*. For instance, *eater* and *nimC1* have each independently expanded in multiple *Drosophila* species lineages and the *nimrod*-related gene *hemese* is unique to the *melanogaster* species group [6]. Likewise, class C scavenger receptors have expanded from one gene in basal *Drosophila* species to four in the *melanogaster* group [6,41], although class B scavenger receptors have retained orthology between *Drosophila* and mosquitoes [6,17]. Class C scavenger receptors also show exceptionally high rates of amino acid divergence within the *melanogaster* group [41], and several *nimrod*-related genes and scavenger receptors have been shown to evolve under recent adaptive evolution at the amino acid level [6,33,41]. The *TEP* family of opsonin genes has also undergone frequent genomic turnover in copy number between *Drosophila* and mosquitoes, honey bees, and *Tribolium* [14–17]. Like phagocytic receptors, *TEP* genes frequently show evidence of adaptive sequence evolution in *Drosophila* [6,28], *Anopheles* [30], and *Daphnia* [31], with selected sites predominantly found in and around a domain that is proteolytically cleaved to activate the TEP protein. It is unknown whether the proteases that perform TEP cleavage are produced by the host or by microbes, so it is difficult to infer whether *TEP* adaptation is a response to diversity in pathogen proteases or to pathogen interference with *TEP* function. Overall, however, the receptors and opsonins mediating phagocytosis are evolutionarily remarkably dynamic with considerable evidence of ongoing positive selection. Phagocytic receptors may bind to diverse or evolutionarily labile pathogen molecules, in contrast to evolutionarily static PRRs which recognize highly conserved microbial compounds, and may be subject to interference by pathogen proteins.

The evolutionary properties of the *Drosophila* cellular response have not been studied beyond pathogen recognition. It is well known that many bacteria are capable of manipulating proteins involved in cytoskeletal rearrangement either to inhibit phagocytosis [e.g., 42,43] or to promote bacterial invasion of host cells [e.g., 44]. To my knowledge, however, no studies have examined the evolutionary dynamics of insect genes involved in intracellular aspects of phagocytosis and bacterial killing. Neither have any studies addressed natural selection on *DSCAM*, a hypervariable phagocytic receptor gene potentially capable of generating through alternative splicing tens of thousands of isoforms that may be either secreted or membrane bound. Exposure of insect cells to bacteria specifically enhances the production of some isoforms, and silencing of *DSCAM* by RNAi reduces the efficiency of phagocytosis in *Drosophila* and

mosquitoes [45,46]. Like the vertebrate *major histocompatibility complex (MHC)* [7], *DSCAM* may prove to be both evolutionarily and somatically diverse.

Conclusions

The recent wealth of population genetic and comparative genomic data allows us to draw some comprehensive conclusions regarding the evolution of the antimicrobial immune response in *Drosophila* and other insects. In the humoral antimicrobial defense, PRRs and AMPs show little evidence of adaptive amino acid diversification, although the independent AMP gene family radiations in several insect lineages may reflect adaptation to distinct pathogen suites. In contrast, however, adaptive sequence evolution is pervasive in orthologously conserved intracellular signaling molecules, suggesting that pathogen interference with host immune induction may drive co-evolution between insects and pathogenic microbes. This model seems plausible if it is evolutionarily difficult for pathogens to evade detection by PRRs or to resist killing by AMPs. Disruption of signaling obviates the need for evasion or resistance, and the orthologous maintenance of core signaling pathways across distantly related insects may make these pathways attractive targets for pathogen interference. Pathogen receptors utilized in phagocytosis are much more diverse and adaptively evolving, suggesting that the recognition properties of these proteins may be quite distinct from those of PRRs. The evolutionary properties of intracellular proteins required for phagocytosis and phagosomal killing remain to be explored.

Critically, most studies to date have focused on long term evolutionary properties of immune system genes. There has been far too little emphasis on short term evolutionary dynamics, including recent adaptation to local pathogens or environmental conditions [but see 47]. Even when data are suggestive of adaptive evolution, we are critically impaired in our ability to infer the proximal agent of selection because we know so little about the epidemiology of infection in natural *Drosophila* populations. Not only do we have little information about the diversity of microbes that infect *Drosophila* in the field, we know precious little about virulence mechanisms that natural pathogens employ, or how natural pathogens interact with the host immune system. Biologists interested in the evolution of insect immune system genes have a strong mandate to identify and characterize ecologically relevant pathogens. At the same time, there is pressing need to study the molecular evolution of immune systems in insects whose ecologies are better characterized than and are distinct from that of *D. melanogaster*.

Despite its shortcomings as an ecological system, the *Drosophila* model has proven fantastic for establishing the basic rules governing the functional and evolutionary genetics of insect immune systems. A series of careful and comprehensive population genetic studies combined with thoughtful whole genome comparisons both within the genus *Drosophila* and between *Drosophila* and other insects have given insight into the evolutionary dynamics of innate immunity that is unparalleled in other physiological systems or organisms.

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Figure 1. A schematic illustration of an idealized *D. melanogaster* immune responsive cell illustrating prominent proteins required for the activation of a humoral immune response and receptors involved in defensive phagocytosis. Proteins whose gene families have experienced considerable genomic turnover among *Drosophila*, *Anopheles*, *Aedes*, *Apis* and *Tribolium* are outlined in heavy blue [6,14–17]. Proteins whose gene families have experienced considerable genomic turnover within the genus *Drosophila* as well as between *Drosophila* and the other insects are outlined in heavy green [6]. Red shaded proteins have been implicated as evolving adaptively at the amino acid sequence level in *D. melanogaster* and/or *D. simulans* [4,6,33, 41].