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REVIEW

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Immunogenetic aspects of the cellular immune response of *Drosophila* against parasitoids

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Abstract Host-parasite relationships represent integrating adaptations of considerable complexity involving the host's immune capacity to both recognize and destroy the parasite, and the latter's ability to successfully invade the host and to circumvent its immune response. Compatibility in *Drosophila*-parasitic wasp (parasitoid) associations has been shown to have a genetic basis, and to be both species and strain specific. Studies using resistant and susceptible strains of *Drosophila melanogaster* infected with virulent and avirulent strains of the wasp *Leptopilina boulardi* demonstrate that the success of the host cellular immune response depends on the genetic status of both host and parasitoid. Immunological, physiological, biochemical, and genetic data form the bases of a two-component model proposed here to account for the observed specificity and complexity of two coevolved adaptations, host nonself recognition and parasitoid virulence.

Keywords *Drosophila* · Cellular immunity · Parasitoid immune suppression

Introduction

With varying degrees of success, all organisms defend themselves against infection through genetically determined mechanisms that distinguish between indigenous (i.e., self) and foreign (i.e., nonself) materials. Two categories of immune responses are recognized, adaptive (or acquired) and innate (or natural). Adaptive immunity is characterized by specificity and memory. Specificity results from the clonal expansion of monospecific lymphocytes possessing antigen-specific cell surface receptors. Immune memory is evidenced when consecutive responses against a specific antigen are amplified and occur more rapidly than the initial response. The anticipatory nature of adaptive immunity necessitates a consummate repertoire of cell receptors in order to respond to all possible antigens. Innate immunity is neither anticipatory nor antigen specific. Innate immune effector mechanisms generate invariant responses against all types of infections, and are unaltered in their capacity to respond to subsequent challenges. The principal innate immune effector responses employed by vertebrates and invertebrates in their struggles against infectious agents include phagocytosis, the production of reactive intermediates of oxygen and nitrogen, the synthesis of antimicrobial peptides, encapsulation, and the formation of coagulation and complement-like cascades (Bulet et al. 1999; Nappi and Vass 2001). Comparative studies of innate immunity continue to document similarities in the effector mechanisms employed by vertebrates and invertebrates to eliminate pathogens and parasites, and implicate in the recognition processes regulation by similar molecular mechanisms (Hoffmann et al. 1999; Janeway 1989).

Vertebrate species typically exhibit both adaptive and innate immunity, whereas invertebrates have long been categorized as possessing only innate immunity. Despite numerous reports documenting their ability to selectively destroy a diversity of nonself components, and to effectively eliminate aberrant endogenous tissues (Bulet et al. 1999; Carton and Nappi 1997; Glinski and Jarosz 1997; Hoffmann 1995), invertebrates are said to be incapable of self-nonself discrimination because they lack vertebrate-like histoincompatibility responses (Klein 1999). While there may be

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justification for crediting organisms with a self-nonself discriminating immune system because they reject allografts, there is no acceptable rationale for categorizing organisms as incapable of self-nonself discrimination merely because they do not exhibit vertebrate-like histoincompatibility responses. Aside from the fact that histoincompatibility responses have been documented in invertebrates (George and Karp 1986), these responses alone are not valid immunological criteria. Clearly, immune effector responses, whether adaptive or innate, evolved as strategies to protect organisms against natural infections, not to combat surgically transplanted tissues or organs. The innate immune system is an evolutionary ancient system of host responses that employs pattern recognition receptors to differentiate between self and nonself (Medzhitov and Janeway 2000). Presumably, pattern recognition receptors recognize conserved pathogen-associated molecular patterns that are shared by various microbial pathogens. Once activated, pattern recognition receptors in turn activate conserved signaling pathways that control the expression of immune response genes. The current thinking is that pattern recognition receptors evolved as innate immune mechanisms to recognize pathogen-associated molecular patterns (Medzhitov and Janeway 2000).

Among invertebrates, the genetics and molecular biology of the antimicrobial response made by *Drosophila* have been extensively investigated and characterized, and interesting functional and evolutionary relationships have been proposed between these responses and the formation of certain acute-phase proteins in mammals (Hoffmann 1995; Hultmark 1993). Recently, specific antimicrobial responses have been demonstrated in *Drosophila* against various classes of microorganisms (Lemaitre et al. 1997). Parasites too large to be phagocytosed provoke an encapsulation response involving the adhesion of numerous hemocytes. The genetic basis for this cellular effector response, the mechanisms mediating the nonspecific recognition process, and the factors underlying target specificity have not been thoroughly investigated in insect host-parasitoid relationships. The associations of *Drosophila* species with the endoparasitic wasps *Leptopilina* and *Asobara* have been shown to be exceptionally good experimental models for addressing questions concerning self-nonself differentiation, and the genetic and physiological bases of host resistance and parasitoid virulence (Carton and Nappi 1997; Dupas and Carton 1999; Kraaijeveld and Godfray 1999).

In this paper, we analyze recent studies of the heritability of *Drosophila* host cellular immunity against endoparasitic wasps (parasitoids) that express varying degrees of virulence. In these host-parasitoid associations, compatibility is both species and strain specific (Dupas and Boscaro 1999; Poirie et al., 2000). Whether particular host genotypes are adapted to particular parasitoid genotypes, as in some plant-pathogen interactions (Briggs and Johal 1994), or whether resistance and virulence are simple one-dimensional traits, are important and still unresolved questions. Based on immunological, physiological, biochemical, and genetic data, a two-component model of host nonself recognition and parasitoid virulence is proposed to account for the observed specificity and complexity of the co-evolved adaptations (Fellowes and Godfray 2000; Kraaijeveld and Godfray 1999). Although there have been numerous studies of host-parasite interactions, very few investigations have focused on the immunogenetics of host resistance and parasite or pathogen virulence. Some of the unresolved questions concerning the co-evolution of these responses concern the extent to which opposing genotypes are co-adapted in various geographic areas, and whether host resistance and parasite virulence are simple one-dimensional traits (Henter 1995; Henter and Via 1995; Hufbauer and Via 1999). Current comparative studies of animal immunity addressing these issues are providing new insights into nonself recognition processes and immune cell signaling mechanisms.

Hemocyte-mediated immune response of *Drosophila*

In immune-competent (resistant) larvae of *Drosophila*, metazoan parasite eggs or larvae that invade the body cavity are rapidly sequestered and destroyed in capsules formed by numerous blood cells or hemocytes, which appear to be engaged in a type of communal phagocytosis (Vass and Nappi, 2000). The early stages of the encapsulation response are manifested in part by an increase in the number of circulating hemocytes, deposition of melanin on the surface of the parasite egg chorion, and a precocious morphological transformation of spherical plasmatocytes into large, flattened hemocytes termed lamellocytes (Fig. 1), both of which engage in capsule formation (Nappi and Streams 1969; Rizki and Rizki 1984; Russo et al., 2000; Walker 1959).

Normally, the transformation of plasmatocytes to lamellocytes occurs at the time of pupariation, but this response is precociously augmented in infected immune-reactive larvae, in larvae responding to implanted heterospecific tissues, and in mutants with abnormally developing endogenous tissues (Eslin and Prevost 1998; Nappi 1984; Nappi and Silvers 1984; Rizki and Rizki 1979, 1992). Since normally very few larval hemocytes circulate in the body cavity, the employment of large numbers of these cells in the encapsulation process necessitates their activation, mobilization, and acquisition of modified adhesive properties (Rizki et al. 1990; Russo et al., 2000). Presumably, the first hemocytes to make contact with the parasite recognize it as nonself and recruit, by as yet unidentified signaling molecules, numerous additional cells, which aggregate at the foreign surface. Activated hemocytes rapidly adhere to one another and to the

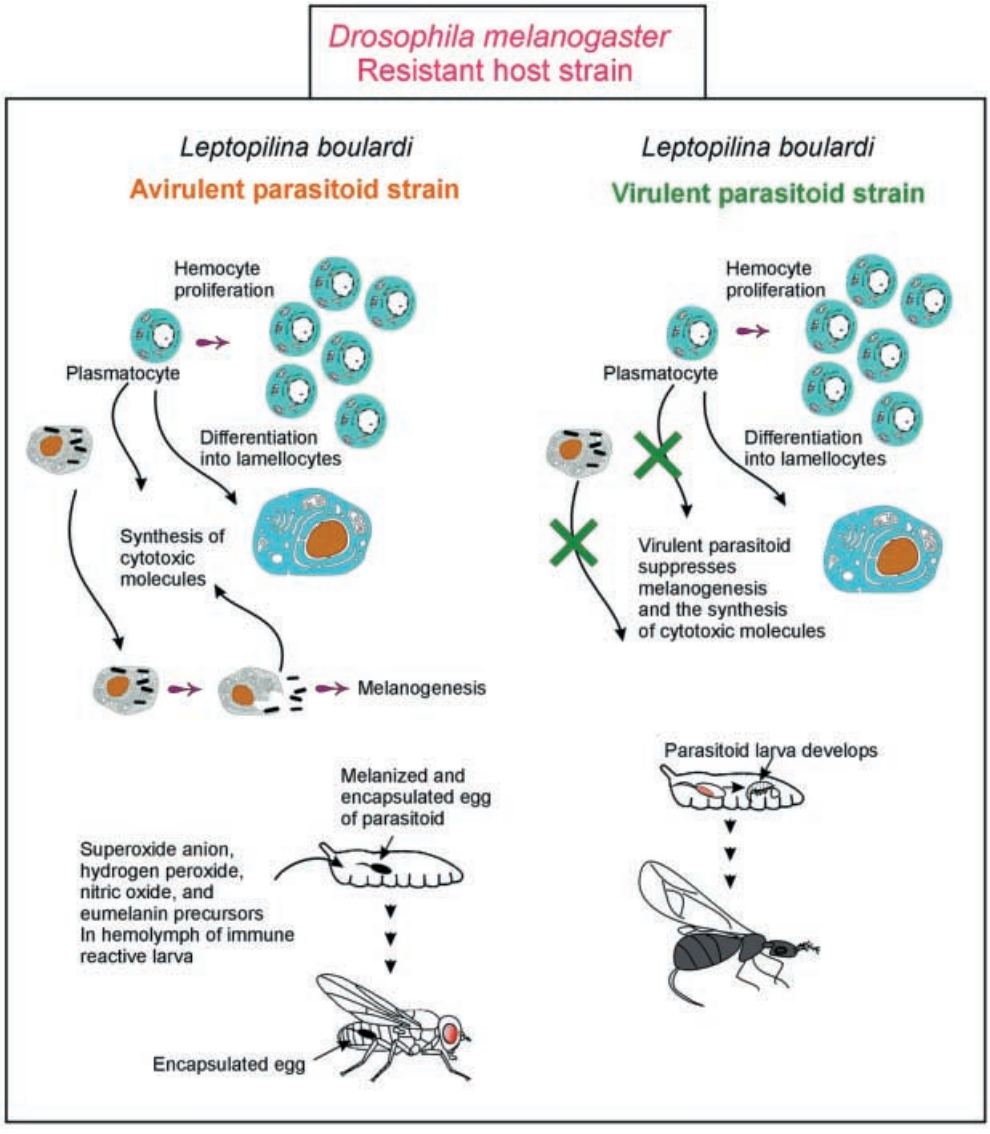

Fig. 1 Diagram illustrating the contrasting cellular and biochemical responses manifested in resistant (Rlb^+/Rlb^+) *Drosophila melanogaster* host larvae when infected by the avirulent (Ism^-/Ism^-) and virulent strain (Ism^+/Ism^+) of the parasitoid *Leptopilina boulardi*. Hemocyte proliferation and differentiation occur immediately after infection, and are manifested against eggs of both strains of the parasitoid. However, subsequent host responses, which include melanogenesis and the production of cytotoxic molecules, are abrogated totally by virulent parasitoids

parasite, over the surface of which the cells flatten and spread. The rapid increase in the number of circulating hemocytes in immune-reactive larvae may result from their proliferation within, and their release from hemopoietic organs (lymph glands). Eventually, the attachment of hemocytes diminishes with the formation of a compact capsule comprised of several overlapping layers of cells. The virtual disappearance of crystal cells (i.e., oenocytoids) during the initial stages of encapsulation (Fig. 1) suggests that these hemocytes, which presumably contain pigment precursors, lyse on contact with the parasite surface and contribute to the formation of melanotic capsules (Rizki and Rizki 1984). Although melanization is a characteristic feature of insect encapsulation responses, the origin and functional significance of pigment in the cellular immune response remains to be established (Nappi and Vass 1993). Melanization was recently demonstrated to occur in larvae of the domino mutant of *Drosophila* that lacks blood cells (Braun et al. 1998), suggesting that other cells may be involved in pigment biosynthesis, as well as alternate melanogenic pathways involving enzymes other than tyrosinase (e.g., peroxidase) and substrates other than phenylalanine-derived tyrosine (e.g., tryptophan). The metabolism of dopamine has been implicated as a contributing factor in the cellular immune response of *Drosophila* against *Leptopilina boulardi*. Using as hosts larvae of a dopa decarboxylase temperature-sensitive mutant, immune capacity was shown to be dependent on gene expression of this enzyme, which converts L-dopa to dopamine (Nappi et al. 1992a). These data indicated that successful cellular immune responses in *Drosophila* involve an alternate pathway for the synthesis of eumelanin involving the decarboxylation of dopa, and the subsequent oxidation of dopamine-derived indole quinones. It is of interest to note that the activation of hemocytes and their recruitment and localization at foreign surfaces are reactions also manifested in cuticular wound healing,

in the sequestration of abnormally developing endogenous tissues as observed in certain melanotic tumor mutants of *Drosophila* (Nappi 1984; Rizki and Rizki 1979), and in response to transplanted allogenetic tissues (Rizki and Rizki 1980).

Little is known of the cytotoxic substances generated during melanotic encapsulation reactions in insects. Implicated as killing molecules are quinones, semiquinones, quinone methides, and reactive intermediates of oxygen and nitrogen (Nappi and Vass 2001). Recent studies have shown that during melanotic encapsulation, *Drosophila* larvae produce superoxide anion (Nappi et al. 1995), hydrogen peroxide (Nappi and Vass 1998), and nitric oxide (Nappi et al. 2000). The role proposed for these reactive molecules is one of potentiating the formation of the hydroxyl radical, the most cytotoxic reactive oxygen species. However, these reactive intermediates of oxygen and nitrogen may also serve in immune cell signaling. The indole and trihydroxyphenyl precursors of eumelanin are extremely reactive molecules that can cross-link proteins and function in killing the parasitoid. The pigment comprising hemocytic capsules may also serve as a radical trap, localizing cytotoxic reactions at foreign surfaces, thereby preventing the distribution of these potentially damaging substances throughout the insect's open circulatory system.

Parasitoid strategies that circumvent the *Drosophila* immune response

Any discussion of host compatibility must take into consideration the fact that parasitoids also manifest considerable genetic diversity, with virulent forms killing high percentages of their hosts, and avirulent individuals experiencing a high percentage of mortality due to cellular encapsulation (Dupas et al. 1996). Successful parasitoids circumvent host immunity by passive or active mechanisms. Passive protection occurs if the parasitoid develops in locations inaccessible to host hemocytes, or if its surface displays a type of molecular mimicry that prevents hemocytes from recognizing it as nonself (Asgari et al. 1998; Kinuthia et al. 1999). Active suppression of the immune response, which appears to be the most common mechanism utilized by parasitoids, may be accomplished by substances introduced into the host's body cavity by the female wasp at the time of oviposition to block encapsulation (Beckage 1998). Virus-like particles, polydnaviruses, proteins, and/or venom of maternal origin have been implicated as immune suppressive substances (Beckage 1998; Stoltz 1993; Strand and Noda 1991; Strand and Pech 1995; Vinson 1990, 1993; Webb and Luckhart 1994). In *Drosophila*, parasitoid-derived virus-like particles (Dupas et al. 1996; Rizki and Rizki 1990) appear to be the immune-suppressive substance that targets capsule-forming hemocytes (e.g., lamellocytes) (Fig. 1), changing their morphology from discoidal to bipolar, a modification which presumably diminishes their ability to adhere to form capsules (Rizki and Rizki 1990; Russo et al., 2000). In some insect hosts, the presence of parasitoid-derived virus-like particles has been proposed to block melanization by inhibiting phenol oxidase activity (Stoltz 1993). Avirulent parasitoids, which presumably lack or have incomplete viral-mediated immune-suppressive capabilities, are destroyed by cytotoxic molecules generated during melanotic encapsulation (Nappi et al. 1995).

Genetics of the *Drosophila*-parasitoid interactions

Comparative and comprehensive analyses of the genetics of host resistance and parasite virulence (Carton and Nappi 1997; Dupas and Carton 1999) were made using physiologically different host-parasitoid combinations. These interactions manifest a specific gene-for-gene interaction for each host-parasitoid complex. The outcome of such conflicts would depend in large measure on the interacting genetic systems, which in the *Drosophila*-parasitic wasp associations result in four possible combinations: resistant or susceptible host strains infected with either virulent or avirulent parasitoid strains (Fig. 2).

Host resistance

Crosses made using selected inbred resistant and susceptible selected parental strains (Carton et al. 1992) demonstrated that the capacity of *Drosophila melano-*

		Drosophila melanogaster	
		Resistant strain *Rlb+/ Rlb+*	Susceptible strain *Rlb-/Rlb-*
Leptopilina boulardi	Avirulent strain *Ism-/Ism-*	Encapsulation **(1)**	No capsule **(3)**
	Virulent strain *Ism+/Ism+*	No capsule **(2)**	No capsule **(4)**

Fig. 2 Diagram illustrating the interaction between one locus for resistance in the host (*D. melanogaster*) and one locus for virulence in the parasitoid (*L. boulardi*). The host alleles Rlb^+ and Rlb^- are for resistance and susceptibility, respectively, and the parasitoid alleles Ism^+ and Ism^- for virulence and avirulence, respectively. Resistance (Rlb^+) is dominant to susceptibility (Rlb^-) in the host, and virulence (Ism^+) is codominant to avirulence (Ism^-) in the parasitoid. If a resistance allele and an avirulence allele occur at the matching host and parasitoid loci (combination *1*), then the host successfully encapsulates the parasitoid. In the three other conditions (combinations *2–4*), the parasitoid is not attacked efficiently

gaster to encapsulate *L. boulardi* is due to a Mendelian single-gene system. The results of all crosses suggest a single major segregating locus (*Rlb*, resistance to *L. boulardi*) with two alleles, resistant (Rlb^+) and susceptible (Rlb^-). Differences in the encapsulation capacity of *D. melanogaster* were shown to be inherited autosomally, with the resistant genotype showing complete dominance over the susceptible genotype. Chromosome exchanges (with the balanced-strains technique) between resistant and susceptible strains showed that the gene locus for resistance was on the right arm of the second chromosome (Poirie et al., 2000).

To investigate the specificity of resistance, a second species of parasitic wasp, *Asobara tabida*, was used to infect the host strains that were characterized as resistant or susceptible based on their interactions with *L. boulardi*. These investigations were the first to provide evidence that, in *Drosophila*, separate genes were involved in the recognition of different parasitoids. Both Rlb^+ and Rlb^- host strains were found to be highly immune reactive against *A. tabida*, exhibiting encapsulation rates of approximately 90 and 95%, respectively. Thus, the strain of *Drosophila* that was susceptible to *L. boulardi* was highly immune-reactive against *A. tabida*, employing cellular and biochemical killing mechanisms identical to those used (by Rlb^+ larvae) to kill *L. boulardi* (Vass et al. 1993). The genetic basis of the capacity to encapsulate *A. tabida* was confirmed by analyzing reciprocal crosses made using inbred resistant and susceptible parental host strains (Benassi et al. 1998; Orr and Irving 1997). Differences in encapsulation capacity are inherited autosomally with one locus (*Rat*, resistance to *A. tabida*) and two alleles (Rat^+ and Rat^-), with the reactive phenotype showing complete dominance over the nonreactive one. This *Rat* gene is also located on the second chromosome, 35.4 cM from the *Rlb* gene (Poirie et al., 2000). These data suggest the existence of two independent immune gene systems, *Rlb* and *Rat*, each concerned with the recognition of a different species of parasitoid.

Parasitoid virulence

The genetic mechanisms underlying parasitoid success were analyzed with Mendelian crosses between inbreed lines of *Leptopilina* that were totally avirulent or virulent (Carton et al. 1992). Comparative data from reciprocal F1 crosses indicated that the two lines of *Leptopilina* possessing opposite capacities to evade host encapsulation differed only at a single locus. The allele for virulence, designated Ism^+ (i.e., immune suppressive against *D. melanogaster*), was co-dominant to the allele for avirulence (Ism^-). The minimum number of segregating factors for the trait was estimated as 1.34 (Dupas et al. 1998), suggesting integration of viral DNA into the genome of the parasitoid (Stoltz 1990).

The genetic factors contributing to the virulence of *L. boulardi* against *D. melanogaster* were examined among different species of *Drosophila* and were found to be host specific (Dupas and Boscaro 1999). In the case of *D. yakuba*, immune suppression was found to be due to a separate gene designated *Isy* (for immune suppression against *yakuba*), with two alleles, Isy^+ and Isy^-. Similar specific immune-suppressive systems, each at different loci, are certainly present in the genome of *L. boulardi* against *D. simulans* and *D. tessieri* (Dupas and Boscaro 1999; Dupas and Carton 1999).

Thus, the outcomes of the conflicts among resistant or susceptible host strains of *D. melanogaster* infected with either virulent or avirulent parasitoid strains of *L. boulardi* are based on monogenic factors, and result from a gene-for-gene interaction for each host-parasitoid system, a situation somewhat similar to the immune complex described for plant-pathogens interactions (Briggs and Johal 1994). Application of the model of gene-for-gene interactions suggests that in *D. melanogaster*, the product(s) of the resistance gene could function as a specific receptor for a given parasitoid epitope. The receptors may be considered as pattern recognition receptors (Franc and White 2000; Yoshino et al. 1998). A variety of lectins have previously been demonstrated to bind to *Drosophila* hemocytes, suggesting the existence of lectin receptors on those cells (Nappi and Silvers 1984; Theopold et al. 1999) expressed during the cellular response.

Interacting immunogenetic systems: a two-component model

The fate of a host-parasitoid encounter depends in large measure on the opposing genetic systems, both of which are continually evolving so that varying frequencies and degrees of expression of the genes for resistance and virulence are to be expected in different geographic populations and at different times (Fellowes and Godfray 2000; Kraaijeveld et al. 1998). The designation of a host as susceptible or a parasitoid as avirulent refers only to the organism's developmental fate in a given relationship, and does not imply immunologically compromised organisms. A strain of *D. melanogaster* susceptible against *L. boulardi* can be highly resistant against an avirulent strain of *A. tabida*, employing the same cellular and biochemical killing mechanism as those used by hosts resistant to *L. boulardi* (Vass and Nappi, 2000; Vass et al. 1993).

In the *Drosophila*-parasitic wasp associations, comparative physiological and biochemical studies made during the early stages of infection in both resistant and susceptible hosts infected with either virulent or avirulent parasitoids provide a comprehensive basis for interpreting the interactions of the opposing genomes involved in host nonself recognition and parasitoid virulence. An interpretation of the interacting

genetic systems in each of the four possible host-parasitoid combinations is presented in Fig. 3. Only when resistant *D. melanogaster* larvae are infected with the avirulent strain of *L. boulardi* is the parasitoid destroyed by melanotic encapsulation. In these resistant hosts, there is an increase in the number of circulating hemocytes, many of which undergo a precocious developmental transformation to become discoidal lamellocytes (Nappi and Streams 1969; Russo et al., 2000; Vass and Nappi, 2000; Walker 1959). Also detected in the hemolymph of resistant hosts during the early stages of infection are eumelanotic precursors, such as L-dopa and 5,6-dihydroxyindole (Fig. 1), which constitute a part of the melanogenic cascade that terminates with the formation of pigmented capsules (Carton and Nappi 1997; Nappi and Vass 1993; Nappi et al. 1992b).

Of considerable interest are the observations that elevated numbers of hemocytes, and their differentiation into lamellocytes, occur in both resistant and susceptible hosts, regardless of whether they are infected with virulent or avirulent strains of *L. boulardi* (Russo et al., 2000). However, only in resistant hosts infected with avirulent parasitoids does melanogenesis and cytotoxic activity occur (Vass and Nappi, 2000). The activation of melanogenic enzymes and the synthesis of cytotoxic molecules appear to be critical stages in the nonself recognition process, as these cellular and biochemical responses are manifested only in resistant hosts that are confronted with avirulent strains of the

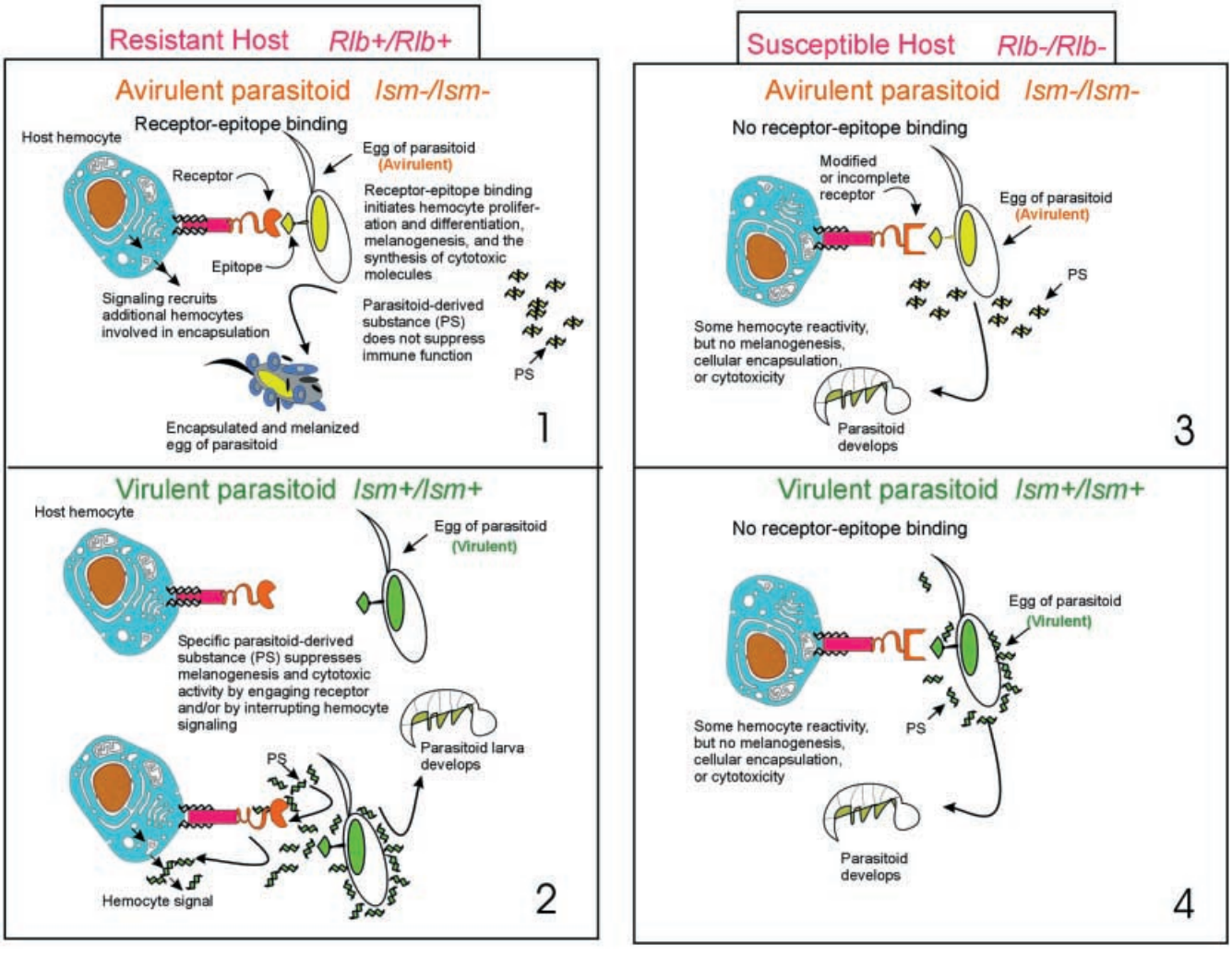

Fig. 3 Diagram depicting four scenarios proposed to account for the observed cellular and biochemical responses made by resistant (*Rlb*$^{+}$/*Rlb*$^{+}$) and susceptible (*Rlb*$^{-}$/*Rlb*$^{-}$) *D. melanogaster* hosts against eggs of virulent (*Ism*$^{+}$/*Ism*$^{+}$) and avirulent (*Ism*$^{-}$/*Ism*$^{-}$) *L. boulardi*. Assuming the gene for resistance against *L. boulardi* (*Rlb*) codes for a cell surface receptor, resistant hosts would possess at least one dominant allele (*Rlb*$^{+}$) for the functioning receptor, whereas susceptible hosts would be homozygous recessive for a nonfunctioning receptor (*Rlb*$^{-}$). Recognition of the specific foreign epitope and generation of cytotoxic cascades is manifested only by *Rlb*$^{+}$ individuals against avirulent parasitoids (scheme *1*). The virulent parasitoid strain succeeds against both host genotypes because it can actively suppress the encapsulation response. Active suppression by a parasitoid-derived substance (*PS*) may target receptor-epitope binding, or some downstream signaling cascade that potentiates melanogenesis and the synthesis of cytotoxic molecules. An immune-suppressive substance, if produced by the avirulent parasitoid strain, is ineffective against the resistant host strain (scheme *1*)

parasitoid. Thus, there appear to be two components to the successful cellular immune response of *D. melanogaster* against *L. boulardi*: a general, nonspecific hemocytic response involving an increase in the number of circulating cells and their differentiation into lamellocytes, and the activation of melanogenic and cytotoxic cascades. Virulent parasitoids possess effective immune-suppressive capabilities that likely target specific host cell surface receptors, and/or interrupt the ensuing signaling responses that are vital to effector responses involving the recruitment of additional cells and the synthesis of cytotoxic molecules. The virulent strain of *L. boulardi* has been shown to modify the normal differentiation of spherical plasmatocytes to lamellocytes, a response that perhaps also interferes with the ability of these cells to adhere to foreign surfaces (Rizki and Rizki 1994; Rizki et al. 1990; Russo et al., 2000). A factor, perhaps derived from the parasitoid or associated with cuticular injury during infection, possibly triggers an initial, nonspecific proliferation of hemocytes that is seen in both resistant and susceptible hosts, and only by preventing the ensuing differentiation of hemocytes and activation of the melanogenic cascade do virulent parasitoids circumvent encapsulation.

It is of considerable interest to note that a dual signal for triggering immune reactivity also appears to be involved in the antimicrobial responses of *Drosophila* (Basset et al. 2000). In mutants of *Drosophila* that possess reduced numbers of hemocytes, antibacterial peptide gene expression by cells of the fat body is significantly diminished, implicating the involvement of a hemocyte-derived signal in the induction of antimicrobial peptide gene expression (Basset et al. 2000). Clearly, the interactions of both parasitoids and pathogens with their *Drosophila* hosts provide resolute models for dissecting the specificity of co-evolved adaptations. Essential elements in the development of host innate immunity are the hemocytes, which in *Drosophila* now appear to play critical roles both in the cellular melanotic encapsulation of parasitoids, and in the initiation and regulation of antimicrobial responses. The acquisition by *Drosophila* parasitoids of immune-suppressive viruses or virus-like molecules represents an extremely effective approach to circumvent host defenses, one that is not unfamiliar to researchers investigating vertebrate immunity. We anticipate that *Drosophila*-parasitoid relationships will continue to be extremely useful models for genetically dissecting the mechanisms of invertebrate innate immunity, and for providing new directions for investigating the unresolved questions concerning immune recognition in insects. Of considerable interest are the mechanisms of immune gene activation, and the signaling pathways involved in effector responses.

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