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REVIEW

New immune systems: pathogen-specific host defence, life history strategies and hypervariable immune-response genes of invertebrates

L Bowden, NM Dheilly, DA Raftos, SV Nair

Department of Biological Sciences, Macquarie University, North Ryde, New South Wales, 2109, Australia

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Abstract

Our understanding of invertebrate immune systems is undergoing a paradigm shift. Until recently, the host defence responses of invertebrates were thought to rely on limited molecular diversity that could not tailor reactions toward specific microbes. This view is now being challenged. Highly discriminatory defence responses, and hypervariable gene systems with the potential to drive them, have been identified in a number of invertebrate groups. These systems seem to be quite distinct, suggesting that pathogen-specific responses might have evolved on numerous occasions. Here, we review evidence that inducible, disease-specific immunity might be commonplace in the animal kingdom.

Key words: adaptive immunity; host defence; immunization; invertebrate immunology; hypervariability

Introduction

The continuing growth of invertebrate aquaculture worldwide, and its seemingly inherent problems with catastrophic disease outbreaks, is driving a renewed demand for research into invertebrate immune systems. Much of this research revolves around two unresolved questions - *can invertebrate immune systems be fine-tuned to fight specific infections,* and, *can that fine-tuning provide protective immunity to re-infection?* For the first time, powerful new molecular technologies are parallelling classical immunization experiments to provide evidence for disease-specific immunity among invertebrates. However, these two experimental approaches have rarely been unified to elucidate the mechanisms underpinning pathogen-specific defense. Providing this synthesis will continue to drive the paradigm shift in invertebrate immunology. It could lay the foundation for the use of immunization as a powerful new method for disease control in invertebrate aquaculture, and provide fundamental insights into the adaptive selection pressures that drive the evolution of immune systems. In this article , we explore evidence for pathogen-specific immunity among invertebrates, and the role that hypervariable gene families play in those responses.

Corresponding author:
David A Raftos
Department of Biological Sciences,
Macquarie University, North Ryde,
New South Wales, 2109, Australia
E-mail: draftos@rna.bio.mq.edu.au

Traditional views of invertebrate immune systems

Until recently, highly variable immune-response molecules were thought to be confined to the jawed vertebrates (gnathostomes) (Raftos and Raison, 1992; Raftos, 1993; Litman *et al.*, 2005). Adaptive immune responses among gnathostomes revolve around hypervariable antibodies and T-cell receptors (TCRs), which act as pathogen-specific recognition proteins. The enormous diversity of antigen-specific antibodies and TCRs is generated using enzymes encoded by somatic recombination activating (RAG) genes. Production of each unique antibody or TCR isotype is confined to individual lymphocyte clones, which are responsible for providing lasting immunity against specific pathogens via the process of clonal selection.

Since Burnet (1959) put forward his theory of clonal selection, it has been assumed that invertebrates lacked highly specific immune responses based on pathogen-specific immunorecognition (Raftos, 1993). This presumption was based, almost exclusively, on the failure to find hypervariable antibodies or TCRs amongst invertebrates (Raftos and Raison, 1992). Remarkably, there is only a limited history of appropriately controlled immunization experiments

being used to test whether antibody/TCR-independent pathogen-specific immune responses occur among invertebrates.

The purported lack of pathogen-specific immunity among invertebrates has often been explained by the differential use of r- and K-selection strategies by invertebrates and vertebrates (Klein, 1989, 1989; Rinkevich, 1999). The terms, r and K, come from ecological algebra defined by Verhulst's equation describing population dynamics (Verhulst, 1838).

$$dN/dt = rN (1-N/K),$$

where "r" is the growth rate of a population, "K" is the carrying capacity of the environment and "N" is population size. The r/K-selection hypothesis has been used to distinguish invertebrates most often as r-selected, indicating that they are smaller, have higher fecundity, shorter lifespans and less stable populations than most vertebrates (Pianka, 1970). This is seen as a high risk reproductive strategy that is more susceptible to chance events. It explains why organisms that are r-selected display higher fecundity. Vertebrates, on the other hand, were seen to have lower reproductive outputs, longer lifespans and generally smaller, more stable populations that could be described as K-selected.

The r/K-selection argument with respect to the evolution of pathogen-specific immune systems is simple - *invertebrates don't live as long as vertebrates, and they don't invest as much in each of their offspring. Therefore, they do not require sophisticated immune responses that rely on pathogen-specific immunorecognition to maintain population stability*. This is a simplistic argument that has always been flawed (Stearns, 1977; Getz, 1993). In terms of life history strategies, it is difficult to imagine that some form of adaptive immunity would not have evolved among invertebrates. Based on the r/K selection paradigm, it has been taken for granted that vertebrates, with their presumed long life cycles and relatively lower reproductive outputs, had a greater evolutionary "need" for adaptive immunity than invertebrates (Klein, 1989; Medzhitov and Janeway, 1997). However, r/K-selection does not neatly distinguish vertebrates from invertebrates (Stearns, 1977; Getz, 1993). Both r- and K-selection strategies can be observed in both taxa, including important stem groups. Some invertebrates, such as bivalves, gastropods, echinoids and decapods, can live for many decades (up to 100 years in some cases), and clearly express features of K-selected strategies, including large body size and relatively low effective fecundity (Powell and Cummings, 1985). Conversely, some gnathostomes, including fish, in which adaptive immunity appears to have first evolved, have short lifespans and high fecundity. For instance, coral fish (*Eviota sigillata*) reach sexual maturity within weeks of birth and have a maximum lifespan of 59 days (Depczynski and Bellwood, 2005). So, using the r/K model to determine whether a broad taxonomic group "requires" highly specific responses to particular pathogens has never been well supported by the available evidence.

Factors other than r/K life history strategies are also likely to provide significant selection pressures for the evolution of pathogen-specific immune systems. Many invertebrates occupy niches that place them in close contact with a broad range of pathogens. Others establish high density populations that are prone to epizootic disease outbreaks (Stow *et al.*, 2007). Many flies and other arthropods feed and/or breed in faeces or decomposing plant material, whilst some other insects, such as ants, bees and termites, live in highly social colonies that facilitate the transmission of virulent pathogens and parasites.

Eusociality, or the production of sterile workers to care for reproductive members of the population, is perhaps the most extreme example of a life history strategy that might necessitate pathogen-specific immunity. Eusociality often involves asexual reproduction that limits genetic variability within populations, combined with high population densities in confined environments (hives or nests). According to Van Valen's (1973) Red Queen theory, which suggests that genetic diversity within populations is required to counter rapidly evolving pathogens, the genetic and social strictures of eusociality should make eusocial populations more susceptible to epizootic disease. As a result, there should be a strong link between eusociality and enhanced defensive capacity. This prediction fits well with recent data indicating that, among social insects, immunological capacity increases proportionately with the degree of sociality. Increased eusociality and genetic relatedness among bees is strongly correlated with an increase in the production of antimicrobial compounds (Stow *et al.*, 2007). Some eusocial species also show strong evidence for pathogen specific immunity (see below).

New evidence for pathogen-specific immunity among invertebrates

Even though the r/K selection argument has been used since Burnet's time to predict that invertebrates lack pathogen-specific recognition of the type provided by vertebrate antibodies and TCRs (Klein, 1989), new data question this assumption. Recent work indicates that arthropods and molluscs can generate fine-tuned responses against specific pathogens. These studies have used classical immunization experiments to test for pathogen specificity; usually by measuring host survival after inoculation and by comparing responses between primary and secondary inoculations using different pathogens (Fig. 1). In these experiments, more rapid and powerful secondary immune responses against the original, inoculating pathogen are presumed to indicate that the host has developed an induced response targeting that pathogen (Little *et al.*, 2005). Pathogen specificity is further implied if enhanced responses do not extend to microbes other than the one used in the initial, priming inoculation.

This approach is not new. In the past it has been used to show that *Drosophila melanogaster* can distinguish between broad groups of pathogens, such as fungi and bacteria (Lemaitre *et al.*, 1997). These broad responses are often mediated by the

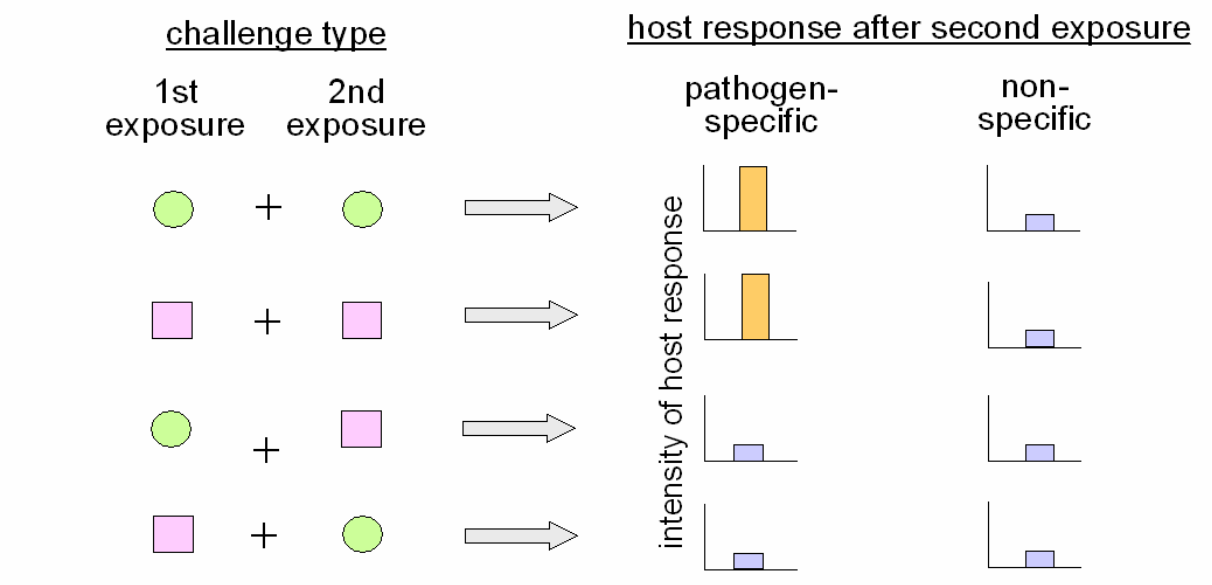

Fig. 1 A generic protocol for immunization experiments. Host organisms are inoculated with one of two different pathogens (circles and squares). Some time later, they are re-challenged either with the same pathogen used for the initial inoculation, or with the other pathogen. The intensity of host responses to the secondary challenge is then measured, often in terms of host mortality over time or the clearance rate of the pathogen (modified from Little *et al.*, 2005).

Toll and IMD/Relish signalling pathways and probably differentiate between highly conserved molecular structures on pathogens, such as bacterial lipopolysaccharides (LPS) and fungal β-glucans (Leclerc and Reichhart, 2004; Beutler *et al.*, 2006). Janeway (1989, 1992) has designated these types of highly conserved target molecules as "pathogen-associated molecular patterns (PAMPs)". PAMPs define broad groups of pathogens for detection by generic "pattern recognition" receptors (PRRs), such as Toll-like receptors and lectins. Pattern recognition, which lacks fine-scale differentiation between closely related microbes, is common throughout the animal kingdom and was thought to be the only molecular recognition paradigm available to invertebrates (Medzhitov and Janeway, 1997, 2002; Janeway and Medzhitov, 2002).

However, more recent immunization experiments have shown that *D. melanogaster* is capable of much finer scale discrimination between microbes. Pham *et al.* (2007) found that immune responses by *D. melonogaster* against *Streptococcus pneumoniae* could be enhanced by inoculation with heat-killed *S. pneumoniae*. Primed responses against *S. pneumoniae* did not provide protection against other pathogens, even closely related bacterial species. And prior exposure to a variety of other bacteria did not elicit protection against *S. pneumoniae*. Specific protection could also be stimulated by injecting *Beauveria bass*iana, a natural pathogen of *D. melanogaster*. However, inoculation with *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium marinum*, did not induce pathogen-specific protective responses. The enhanced responses to *S. pneumoniae* and *B. bassiana* in *Drosophila* clearly have features in common with gnathostome adaptive immunity, yet they appear to be mediated by the same Toll-like pathways and phagocytic responses that are associated with simple pattern recognition (Pham *et al.*, 2007).

Even more compelling evidence for pathogen-specific immunity among insects comes from studies of bumblebees (*Bombus terrestris*). Sadd and Schmid-Hempel (2006) immunized bees with a defined isolate of the gram-negative bacterium, *Pseudomonas fluorescens*, and two closely-related gram-positive bacteria (*Paenibacillus alvei* and *Paenibacillus larvae*). Bees were re-challenged either 8 or 22 days after the initial, priming injection, and their relative survival was recorded. The data showed that *B. terrestris* were capable of mounting highly specific immune responses that could differentiate even between the congeneric bacteria (Sadd and Schmid-Hempel, 2006). Such targeted responses only became evident when bees were left for 22 days before being re-challenged. Pathogen-specific responses were masked by less specific reactions when bees were re-challenged within 8 days of the initial inoculation.

These examples of pathogen-specific immunity in *Drosophila* and *B. terrestris* are among a number of highly targeted immune responses so far identified among invertebrates. In 2006, Kurtz cited five studies of either insects, crustaceans or molluscs, in which there is strong evidence for the induction of pathogen specific immunity by inoculation with trematodes, bacteria, tapeworms or trypanosomes (Webster and Woodhouse, 1998; Schmid-Hempel *et al.*, 1999; Carius *et al.*, 2001; Little *et al.*, 2003; Kurtz and Franz, 2003). Among these studies, Kurtz and Franz (2003) showed that infections of the copepod crustacean,

Macrocyclops albidus, with the tapeworm, *Schistocephalus solidus*, were far less severe if the hosts had been primed with siblings of the worms used for the subsequent infections. This acquired protection was not evident if the tapeworms used in the initial and subsequent challenges were genetically distinct. Similar immunization experiments in the prawn, *Penaeus monodon*, identified discriminatory responses to virulent white spot syndrome virus (WSSV) (Witteveldt *et al.*, 2004). Injecting the WSSV envelope protein, VP28, provided substantial protection against white spot infection, whereas a closely related antigen, VP19, did not. In a another series of immunization experiments, it was shown that induced, specific protection against pathogens in the water flea, *Daphnia magna*, can be transmitted from mother to offspring (Little *et al.*, 2005).

The interpretation of these data is extremely controversial (Hauton and Smith, 2007). It does suggest that some protostomes can mount defensive responses that are fine-tuned toward some invading pathogens (Schmid-Hempel *et al.*, 1999; Kurtz and Franz, 2003; Schmid-Hempel and Ebert, 2003; Little and Kraaijeveld, 2004; Kurtz, 2005; Little *et al.*, 2005; Kurtz and Armitage, 2006). However, there is still insufficient evidence to tell whether these systems are widespread throughout the metazoa. It is also unclear if they can generate targeted protection against many different pathogens, or whether they have been selected only to provide protection against pathogens that are the most relevant to the host species.

Hypervariable immune-response gene families of invertebrates

At roughly the same time that traditional immunization experiments were revealing the existence of pathogen-specific responses in some invertebrates, new molecular technologies were being used to identify hypervariable immune response molecules that might underpin pathogen-specificity (Flajnik, 2004; Flajnik and Du Pasquier, 2004; Loker *et al.*, 2004; Litman *et al.*, 2005; Du Pasquier, 2006). It is now evident that a range of highly variable immune-response gene families are expressed among invertebrates and jawless vertebrates. Some of these families have already been found to play key roles in immunological functions, such as self/non-self recognition and differentiation between broadly different pathogen types (i.e. fungi vs. bacteria).

From the existing data it is clear that the gene families encoding highly variable immune-response proteins have arisen independently on numerous occasions in higher plants and in animals (Flajnik, 2004; Flajnik and Du Pasquier, 2004). None of the families that have been identified to date are closely related to each other, even though many incorporate immunoglobulin-superfamily (IgSF) domains (Flajnik, 2004; Flajnik and Du Pasquier, 2004; Litman *et al*., 2005). This suggests that there has been strong selection pressure within individual taxa to develop mechanisms that promote survival in the presence of rapidly evolving pathogens.

A number of the highly variable immune-response families identified among invertebrates and "lower" vertebrates are described in more detail below.

Variable lymphocyte receptors in agnathans

The two extant groups of agnathans (jawless fish; hagfish and lampreys) do not express highly diverse antibodies or TCR (Raftos and Raison, 1992). However, expressed sequence tag (EST) analyses of lamprey lymphocytes revealed immune-response genes that encode highly variable lymphocyte receptors, or VLRs. These molecules contain numerous leucine-rich-repeat (LRR) modules, and may act as cell surface receptors implicated in immunological defence (Flajnik, 2004; Pancer *et al.*, 2004; Litman *et al.*, 2005). VLRs have non-variable glycosyl-phosphatidyl-inositol (GPI) lipid anchors for attachment to the plasma membranes lymphocytes. These GPI anchors may become detached to free humoral forms of VLR molecules (Litman *et al.*, 2005). The high level of diversity displayed among VLRs arises from variation in their nucleotide sequences and in the number of LRR modules that they incorporate (Pancer *et al.*, 2004). There is some evidence that different lamprey lymphocytes express different and unique VLRs, implying a clonal expression system akin to that of gnathostome antibodies (Flajnik, 2004).

V region-containing chitin binding proteins in cephalochordates

Cannon *et al.* (2002) identified five families of highly variable immune-response molecules in the protochordate, amphioxus (*Branchiostoma floridae*), that incorporate two N-terminal IgSF V-region domains and one chitin-binding domain at the carboxy-terminus. These molecules have been designated V region-containing chitin binding proteins (VCBPs). Their V regions show high levels of diversification and might function as specific immune-recognition domains akin to the adaptive immunological receptors of gnathostomes. The five distinct families of VCBP transcripts identified by Cannon *et al.* (2002) are highly diversified, showing only 27 % - 41 % sequence identity among families. The chitin-binding region incorporates a diagnostic sequence of spaced cysteine residues that is characteristic of chitin-binding proteins in other species, and recombinant VCBPs can bind chitin. *In situ* hybridisation revealed that VCBPs are expressed exclusively in the intestine. Given the filter feeding life history of amphioxus, this distribution fits well with a potential role in the control of pathogens in the gut. Cannon *et al.* (2002) concluded that VCBPs may have a dual function, combining pattern recognition by the chitin-binding domain with diversified specific immunorecognition afforded by the highly variable IgSF V-regions.

185/333 proteins in sea urchins

The *185/333* gene family from the purple sea urchin, *Strongylocentrotus purpuratus*, has high levels

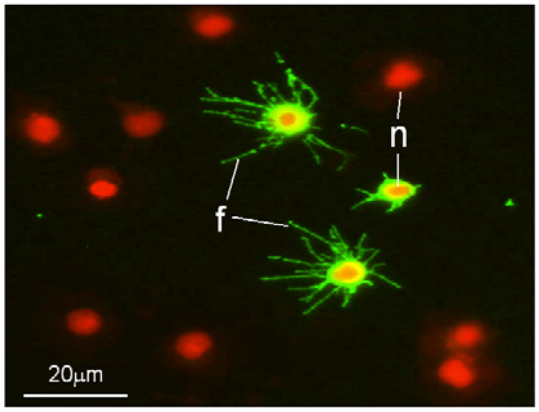

Fig. 2 185/333 proteins are expressed primarily on the surface of one sea urchin (*S. purpuratus*) coelomocyte type, small phagocytes. In this image, 185/333 staining is shown in green and nuclei are counterstained red. Note the expression of 185/333 on the extensive filopodia of small phagocytes. f, filopodia; n, nuclei.

of both cDNA and genomic DNA sequence diversity (Nair *et al.*, 2005; Terwilliger *et al.*, 2006, 2007; Buckley and Smith, 2007). About 50-60 distinct genomic *185/333* loci have been estimated and almost 700 unique mRNA transcripts have been sequenced to date (Terwilliger et al., 2007; K Buckley, George Washington University, personal communication). There is also evidence for *185/333* gene sequences and gene expression in other sea urchin species (M Roth, Macquarie University, personal communication). Unlike the highly variable gene families of other invertebrates, sequence comparisons have failed to reveal any similarities between *185/333* genes and those from other organisms (Nair *et al.*, 2005). However, based on their expression patterns, 185/333 proteins appear to be involved in immune responses. Titres of both *185/333* mRNA transcripts and proteins increase after immunological challenge (Brockton *et al.*, 2007; NM Dheilly, unpublished data). The frequency of *185/333* mRNAs increases 70-fold during bacterial infections, and *185/333* transcripts can comprise more than 60 % of the mRNA specifically induced by immune responses (Nair *et al.*, 2005). There is also some evidence that different PAMPs elicit the expression of different *185/333* variants (NM Dheilly, unpublished data; Terwilliger *et al.*, 2007).

Proteomics and transcriptome analysis have shown that 185/333 molecules have high levels of molecular diversity within and between sea urchins. Individual sea urchins can express in excess of 200 distinct 185/333 proteins, and each animal has a distinctive suite of the proteins that differs from all other individuals (NM Dheilly, unpublished data; Terwilliger *et al.*, 2007). 185/333 proteins are localized to the surface of a distinct class of coelomocytes, the frequency of which is substantially enhanced in coelomic fluid after immunological challenge (Fig. 2) (Brockton *et al.*, 2007). The observed molecular weights of 185/333 proteins are much higher than those predicted from mRNAs, suggesting that 185/333 proteins form strong associations with other molecules, or with each other (Brockton *et al.*, 2007; NM Dheilly, unpublished data).

The variability of *185/333* genes can be explained by combinatorial diversity, single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels). *185/333* mRNA transcripts are comprised of 25 different blocks of sequence, or “elements”, that are either present or absent in many different combinations (Nair *et al.*, 2005; Terwilliger *et al.*, 2006; Buckley and Smith, 2007). SNPs and indels also occur frequently in all *185/333* transcripts, magnifying their diversity. Nucleotide substitutions between *185/333* variants are often non-conservative, resulting in amino acid changes. This suggests that *185/333* diversity has been driven by positive selection of the type associated with pathogen-driven adaptation (Nair *et al.*, 2005; Terwilliger *et al.*, 2006). The processes that generate *185/333* diversity remain unclear, but they could include genomic or somatic recombination, alternative splicing, RNA editing and post translational modifications (Buckley and Smith, 2007).

The biological activities of 185/333 proteins have not yet been defined and their lack of homology with other molecules makes it difficult to predict structure/function relationships. However, evidence from sequence analyses is shedding more light on the structure of these proteins. *185/333* mRNAs encode polypeptides with a hydrophobic leader, separate glycine- and histidine- rich regions, numerous N-linked and O-linked glycosylation sites, acidic sequence patches, and an arginine-glycine-aspartic acid (RGD) motif (Terwilliger *et al.*, 2006). Predicted polypeptides do not contain cysteines, transmembrane regions, GPI linkage sites or identifiable domains. The only regions with at least some similarity to other molecules are the RGD motif and one of the histidine-rich domains, which is comparable to histatins, a group of mammalian salivary proteins with powerful antifungal activities (Xu *et al.*, 1990).

More detailed analysis of *185/333* sequences reveals many short amino acid sequence repeats that are found at numerous positions in the predicted proteins (Nair et al., 2005; Buckley and Smith, 2007) (Fig. 3). Distinctive types of repeats are found in different regions of predicted polypeptides, most notably in the N-terminal glycine-rich region and a C-terminal region that incorporates numerous potential N-glycosylation sites. In many cases, different types of short repeats are clustered together, and these clusters are duplicated throughout the polypeptide. Distinct combinatorial patterns of repeats define eight distinct *185/333* sub-families, which phylogenetic analysis suggests arose by progressive recombination events (NM Dheilly, unpublished data; K Buckley, George Washington University, personal communication).

FREPs in molluscs

Highly variable fibrinogen-related proteins (FREPs) in the snail, *Biomphalaria glabrata,* and in at least five other genera of gastropods, consist of

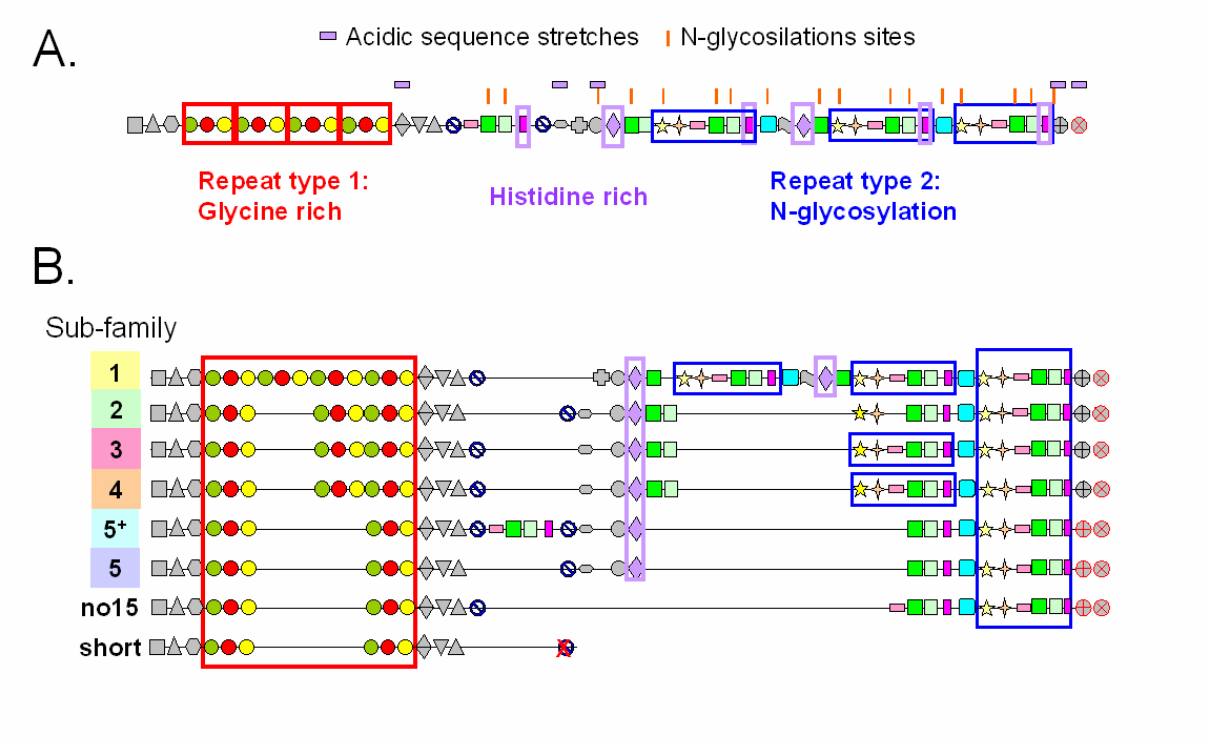

Fig. 3 A) The pattern of amino acid repeats evident in a model 185/333 protein from the sea urchin, *S. purpuratus*. Each colored shape represents a unique amino acid repeat. Boxed areas show where different clusters of repeats are duplicated throughout the predicted polypeptide. Distinct repeat clusters are found in the glycine-rich and N-glycosylated regions of the polypeptide. **B)** Amino acid repeat patterns found in 8 distinct *185/333* sub-families. Each family is distinguished by a unique combinatorial pattern of sequence repeats.

either two or three domains. One is a C-terminal fibrinogen domain, while the other one or two domains are members of the IgSF (Fig. 4A) (Zhang *et al.*, 2004). FREPs are reported to have lectin-like (carbohydrate-binding) activity and their expression increases in response to challenge with the trematode parasites, *Echinostoma paraensei* and *Schistosoma mansoni* (Adema *et al.*, 1997). One strain of *B. glabrata* that is resistant to *S. mansoni* increases FREP expression by up to 57-fold after exposure to the parasite, whilst FREP expression in *S. mansoni*-susceptible snails is unaltered by immunological challenge (Hertel *et al.*, 2005). FREPs can precipitate antigens that are secreted by *S. mansoni* and can bind to the surface of *E. paraensei*. All of these observations suggest that FREPs are involved in host/parasite interactions and may act as highly diversified recognition and/or effector proteins (Loker *et al.*, 2004; Hertel *et al.*, 2005).

Substantial diversity is evident in the IgSF domain(s) of FREPs. Expressed sequence tag (EST) analysis of transcripts enriched for the FREPs of *S. mansoni*-resistant snails by suppression subtractive hybridisation (SSH) identified 88 unique SSH-ESTs among the 112 clones sequenced (Nowak *et al.*, 2004). Sequence diversity incorporates a number of distinct FREP sub-families, and additional variability is evident in the form of SNPs within each of these sub-families. Diversity seems to be generated through a combination of alternative exon splicing, gene conversion and somatic hypermutation, meaning that different individuals express different suites of FREPs (Fig. 4B) (Zhang and Loker, 2003; Zhang *et al.*, 2004). FREP genes appear have diversified faster at the genomic level than other genes, and all of the variants identified to date seem to be derived from just nine ancestral or "mother" sequences (Zhang *et al.*, 2004).

Dscams in insects

The Down's syndrome cell adhesion (*Dscam*) molecules of *D. melanogaster* and other insects are members of the IgSF. *Dscam* genes comprise clusters of Ig-like exons with high levels of sequence diversity that are flanked by relatively conserved regions (Fig. 5) (Schmucker *et al.*, 2000). Alternative splicing recombines exons by mutually exclusive excision, potentially generating more than 38,000 distinct extracellular domains. Individual hemocytes can generate between 14-50 different isoforms of *Dscam* and there are a total of about 19,000 hemocyte-specific isoforms (Watson *et al.*, 2005). Hemocytes from mutant flies with impaired *Dscam* expression, and hemocytes in which *Dscam* expression had been specifically knocked down using interference RNA (RNAi), have significantly lowered phagocytic activities than hemocytes from normal flies. Antibodies that specifically bind to the extracellular Ig domains of *Dscam*s can also inhibit phagocytosis and *Dscam* isoforms can bind onto pathogen surfaces (Watson *et al.*, 2005). This suggests

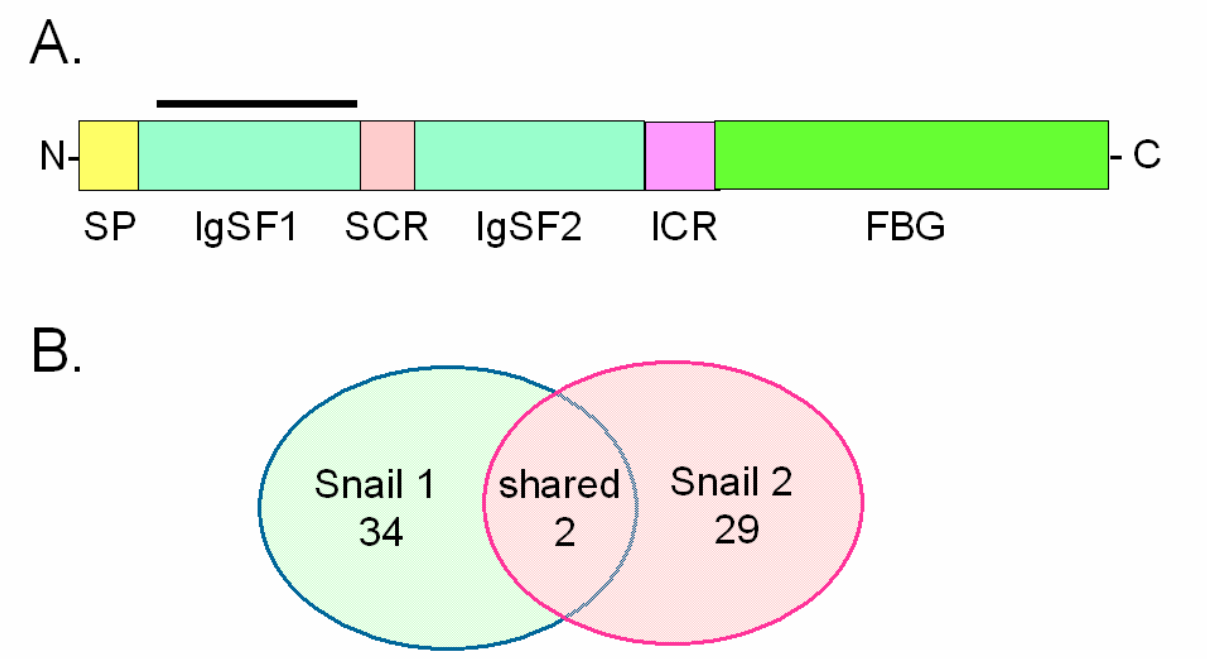

Fig. 4 A) Schematic polypeptide structure of the FREP3 sub-family showing different domains (N-terminus on left). SP, signal peptide; IgSF1 and 2, immunoglobulin superfamily domains; SCR, small connecting region; ICR, interceding region; FBG, fibrinogen domain. **B)** Comparison of FREPs expressed by two different individuals of the same snail species (*B. glabrata*). The region of IgSF1 shown by the black bar in A. was amplified from transcripts isolated from the two snails and sequenced. The numbers shown in the figure are the number of clones that were unique to each snail, and the number shared by the two snails (modified from Zhang *et al.*, 2004).

that *Dscam*s are involved in the phagocytic uptake of pathogens, possibly acting as pathogen-specific recognition proteins (Watson *et al.*, 2005). Interestingly, *Dscam*s also act as axon guidance receptors during neuronal development, with different *Dscam* isoforms being expressed in the brain compared to hemocytes (Watson *et al.*, 2005).

What role do highly variable gene families play in pathogen-specific immunity?

The data on *185/333* genes, VCBPs, FREPs and *Dscam*s imply that all of these gene families contribute to novel forms of inducible, pathogen-specific immune systems (Flajnik, 2004; Flajnik and du Pasquier, 2004; Litman *et al.*, 2005). However, there is little direct evidence to support this conclusion. The molecular approaches that have been used to identify these genes have often lacked accompanying information about their physiological functions. In hindsight, resolving the biological activities of hypervariable proteins, particularly their contribution to pathogen-specific immune responses, is proving to be problematic and time consuming. In most cases, we still do not understand the biological relevance of these gene systems, or whether they help to combat infection.

Traditional immunization experiments have suffered from the opposite problem compared to molecular biological approaches. Whilst immunization studies have been very useful in demonstrating the existence of acquired, pathogen-specific reactions, they have not provided information about the molecular mechanisms underlying induced responses (Little and Kraaijeveld, 2004; Little *et al.*, 2005). A new goal of invertebrate immunology will be to address this gap between molecular immunology and immunization experiments (Hauton and Smith, 2007). New work needs to combine classical immunization protocols with comprehensive molecular analyses to simultaneously identify pathogen-specific immune responses and the genes that control them.

To date there are only a few convincing studies that have implemented this approach. Dong *et al.* (2006) showed that particular *Dscam* splice-variants in the mosquito, *Anopheles gambiae*, are associated with resistance to malarial (Plasmodium) and bacterial infections. RNAi silencing of *Dscam* expression significantly reduced survival rates from bacterial infections and increased Plasmodium ooycst development in the midgut of mosquitoes. Most significantly, the *A. gambiae Dscam* (*AgDscam*) variants produced in response to inoculation with particular bacteria, (*Escherichia coli* and *Pseudomonas veronii*) could bind onto these microbes with higher affinity than the *AgDscam*s produced in response to challenge with Gram-positive bacteria (*Staphylococcus aureus*) or non-challenged cell lines. Silencing specific forms of *AgDscam*s associated with responses to different bacteria made mosquitoes more sensitive to infections by the target bacterial species, but not

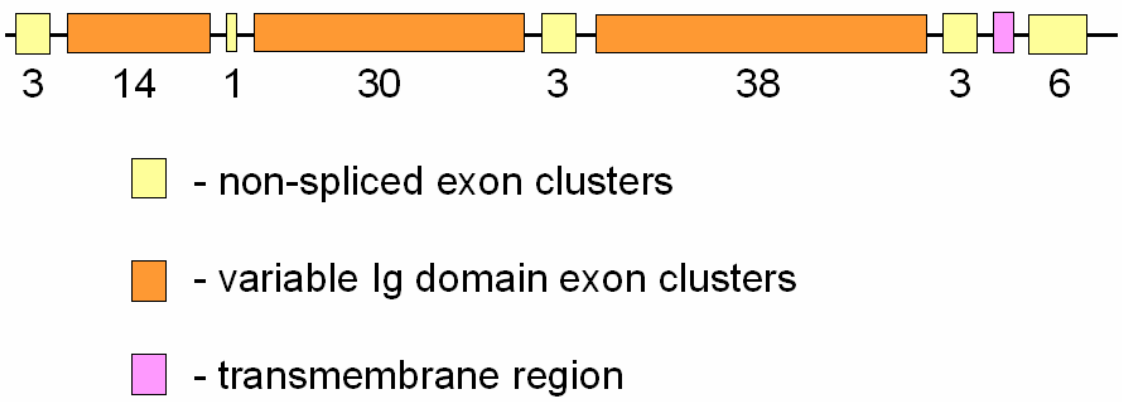

Fig. 5 Gene organization of *Dscams* from the mosquito *Anopheles gambiae* showing clusters of highly variable IgSF domains and constant domains. The numbers below the figure show the number of Ig exons per cluster (modified from Dong *et al.*, 2006).

others. These data imply a strong association between *Dscam*s and pathogen-specific immunity in insects (Dong *et al.*, 2006).

Similarly, Robalino *et al.* (2005) have demonstrated a clear link between pathogen-specific systems in shrimp and protective immunity to WSSV. They found that protection against WSSV in *Litopenaeus vannamei* could be elicited by injecting double-stranded RNA (dsRNA) (Robalino *et al.*, 2005). Even though randomly generated dsRNAs had some effect, much higher pathogen-specific protection against white spot disease was induced when dsRNAs based on WSSV sequences were used, indicating that shrimp can use pathogen-specific RNAi systems to generate highly targeted protection against viral diseases.

Conclusions

Studies that combine immunization experiments with manipulative molecular analyses represent a new emphasis for invertebrate immunology. The data presented in this article suggest two things - that pathogen-specific, inducible immune systems might be common among invertebrates, and that these systems have evolved independently on numerous occasions. This implies that many more pathogen-specific immune systems, and their associated hypervariable recognition molecules, await discovery.

Acknowledgments

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