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VISIONS AND PERSPECTIVES

Specificity, learning and memory in the innate immune response

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Summary

Immunity in invertebrates was for long analyzed in terms of the overall response; this resulted in misunderstandings concerning specificity and memory. Recent reports of maternal transmission of immunity, and the discovery of the high diversity of receptors-effectors, have required the status of innate immunity to be reconsidered. There are few examples of obvious specificity towards some pathogens, but this cannot be generalized to all invertebrate species. The existence of memory is even more controversial. Here, we suggest looking for immune memory by quantifying key molecular effectors (i) within single individuals following first and second exposures to a pathogen and (ii) in primed mother and her offspring.

Key words: innate immunity; invertebrates; vertebrates; *Drosophila*

Introduction

Although invertebrates are able to develop immune reactions, it has long been assumed that their immune systems are non adaptive (not anticipatory) and respond identically to multiple challenges. In other words, it was believed that they lack specificity and memory, and this conviction was strongly supported by the fact that, unquestionably, invertebrates do not possess antibodies, or T or B cells. However, this dogma that adaptive (anticipatory) immunity is absent from invertebrates is now cracking, not because the long search for antibodies has succeeded, but because at least some invertebrates possess functional equivalents of the acquired responses of vertebrates (see Kvell *et al.*, 2007 for a review).

Since the work of Carton *et al.* (1992), diverse examples of strongly specific immune responses in invertebrates against potential parasites or pathogens have been reported (Little *et al.*, 2003; Pham *et al.*, 2007). In addition, the wide diversity of receptors and effectors, including fibrinogen-related proteins (FREP) in snail (Adema *et al.*, 1997),Toll-like receptors (TLR) in *Drosophila* (Tauszig *et al.*, 2000) and in sea urchin (Hibino *et al.*, 2006), the gene family 185/333 in sea urchin (Buckley and Smith, 2007), antimicrobial peptide (AMP) in shrimps (Padhi *et al.*, 2007) and in mussels (Padhi and Verghese, 2008; Pallavicini *et al.*, 2008), the latest presumably generated by gene duplication and positive Darwinian selection, argues in favour of the existence of a sharp specificity in some of the invertebrate immune responses.

In most studies on invertebrates, and in the present paper as well, what was called “specificity” referred to the overall result of the immune reaction, whereas in mammal immunity studies, “specificity” referred to the process of antigen recognition. Concerning memory, various studies concluded that previous experience of a pathogen can provide an individual invertebrate, or its descendant, with enhanced immunity (Cooper and Roch, 1986; Kurtz and Franz, 2003; Little *et al.*, 2003). This process of enhanced immunity following previous encounter, can be divided in two steps. In the first step (referring to "learning"), the host has met the pathogen and has learnt something from this meeting: this is a behavioural or dynamic feature. In the second step (referring to "memory") the host remembered what he has learnt: this is a physiological or static feature. Learning can happen without memory (the lesson was lost) but memory cannot exist without previous learning. In other words, it is evident that memory is inseparable from

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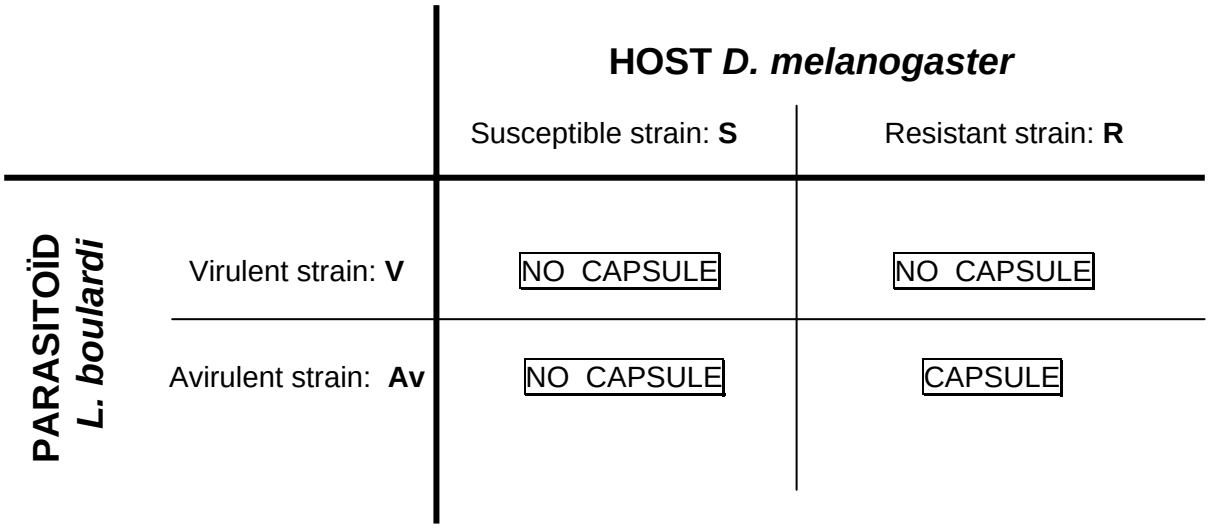

Fig. 1 The cellular immune reaction of *D. melanogaster* against the parasitoïd *L. boulardi* is called a capsule. It is a multilayer sheath built by hemocytes around the parasitoïd egg which is killed. Depending on the combination between susceptible (S) or resistant (R) strains of *D. melanogaster* and virulent (V) or avirulent (Av) strains of *L. boulardi*, the capsule is built or not. The result of the interaction is predictable [adapted from Carton and Nappi (2001)].

learning and that "learning-memory" is different from specificity. Curiously, this obviousness has been forgotten in some recently published papers (Kurtz and Franz, 2003).

Although the specificity of some immune responses in invertebrates has been reported as being much stronger than that generally allocated to innate immunity, there is still no direct evidence for memory in invertebrates. To this end, we suggest quantifying key molecular effectors to discriminate between what is related to transfer of effectors (see below) and what is indeed memory.

What was tested when looking for specificity?

Studies with populations of *Drosophila melanogaster* parasitized by the parasitoïd *Leptopilina boulardi* demonstrated that the immune response was strain specific: some strains of the host fly can kill (encapsulate) some but not all strains of this parasitoïd species (Fig. 1) (Carton and Nappi, 2001). Similarly, different *Daphnia* clones have been reported to be differently protected against diverse strains of the same pathogenic bacteria (Little *et al.*, 2003); thus, different individuals within the same species seemed to respond differently, and this corresponds to a level of specificity not far from that observed in vertebrate adaptive immunity.

Specificity is generally understood to be the recognition of a foreign body by the host (Kurtz, 2005). For instance in *D. melanogaster*, both alternative splicing of Peptidoglycan Recognition Protein (PGRP) can play a role in the immune response (Werner *et al.*, 2003) and also as many as 18,000 isoforms of the receptor Down Syndrome Cell Adhesion Molecule (Dscam) can be generated (Watson *et al.*, 2005). Consequently, the observed specificity of *D. melanogaster* immune responses may be related to the selection of specific isoforms of receptors (Agaisse, 2007), as also suggested for molluscs (Zhang *et al.*, 2004), or to synergism between receptors (Schulenburg *et al.*, 2007).

In the *D. melanogaster-L. boulardi* model described by Carton and Nappi (2001) (Fig. 1), the different steps of the defence reaction can be dissected because this system predicted the outcome of the immune response of selected host strains to particular parasitoïd strains. The immune reaction triggered in *D. melanogaster* larvae against parasitoïd eggs is called encapsulation and involves four main steps (Russo *et al.*, 1996, 2001): (i) an increase in the number of circulating hemocytes, (ii) differentiation of a particular hemocyte type, the lamellocytes, (iii) the activation of the phenol-oxidase cascade and (iv) formation of the capsule by accumulation of lamellocytes. The egg must first be recognized as foreign for the reaction to occur and, as stated by several authors (Carton *et al.*, 1992; Poirie *et al.*, 2000; Schmidt *et al.*, 2001), the host-parasitoïd relationships in the *D. melanogaster-L. boulardi* system resembles the "gene-for-gene" recognition model of plants. Possibly, the absence of capsule formation results from non recognition of the parasitoïd egg by the host; however, various observations are inconsistent with this possibility. For instance, whether or not the capsule is formed (step iv), the first two steps, involving modifications of blood constituent, are in all cases completed (Russo *et al.*, 2001). In addition, when the same larvae of resistant *D. melanogaster* strain are parasitized both by a virulent *L. boulardi* strain (no capsule formation) and by an avirulent strain (capsule formation), both types of egg were protected from encapsulation (this reaction was termed "cross protection") (Labrosse *et al.*, 2003). If

only the avirulent strain were recognised, capsule formation would have been triggered; however, there was no capsule formation. Finally, active inhibition of the *Drosophila* defence response by the parasitoïd has been described (Labrosse *et al.*, 2003). All these data clearly demonstrated that in the four possible combinations of host-parasitoïd strains (Fig. 1), the immune response was always triggered, but then actively inhibited by the parasitoïd in three of the combinations. Whatever the combination, *i.e*. whatever the reaction outcome, parasitoïd eggs were always recognized as foreign bodies. Therefore, the specificity of the reaction does not depend only on the recognition process, but also, and in this case mainly, on a putative depressive effect of the parasitoïd.

Note that in numerous studies performed on invertebrates, with the exception of some works in *Drosophila* (Lemaitre and Hoffmann, 2007) and in molluscs (Zhang *et al.*, 2004), specificity refers to the overall result of the response and not only to the process that triggered the reaction. For instance, Carton and Nappi (2001) looked for the formation of a capsule around the parasitoïd egg, Little *et al.* (2003) analyzed the fitness of *Daphnia* after exposure to pathogen, Kurtz and Franz (2003) counted the percentages of copepods infected, Moret and Siva-Jothy (2003) assessed the survival and global antibacterial activity of *Tenebrio*, Pham *et al.* (2007) tested for the protection of *Drosophila* against pathogen; in all these studies what was called "immune specificity" was in fact the product of both (i) recognition (reviewed in Du Pasquier, 2005) and (ii) complex interactions between the host and the pathogen/parasite.

Putative supports of specificity

There are clearly multiple targets for inhibiting factors in the *D. melanogaster-L. boulardi* system. Once the potential parasite/pathogen has been recognized, signal pathways are triggered. These signal pathways involve various enzymes, especially kinases, phosphatases, hydrolases and GTPases. Each one of these molecules is a potential target for immunosuppressive factors. For example, the entomopathogenic bacterium *Photorhabdus luminescens* can prevent its phagocytosis by insect macrophages by inhibiting the activity of the Rho and Rac GTPases in these cells (Brugirard-Ricaud *et al.*, 2005). Indeed, there are tens or even hundreds of possible targets that could be exploited to abort an elicited defence reaction. Each of the inhibitor/target complexes could display a very high specificity, as is characteristic of enzyme-substrate or enzyme-inhibitor relationships. In addition, the putative inhibitor from *L. boulardi* and its putative target in *D. melanogaster* may be subject to mutations, which could affect the binding affinity. Also, alternative splicing or other genetic diversification mechanisms (Schulenburg *et al.*, 2007) could greatly increase the efficiency of the combinations. Specificity could also be improved by the combination of different inhibitors with different targets in the same host-parasite model. Therefore, active inhibition could result in an overall immune response with stronger specificity than that expected from the recognition mechanisms alone. As a consequence, the dogma of the weak specificity of innate immunity led to misinterpretation of those invertebrate immune responses that display strong specificity.

Is there a need for immune memory in invertebrates?

The first line of evidence involved graft transplantation assays and concluded that there was recognition of foreign antigens in annelids (Cooper, 1969; Valembois, 1963). It was suggested that so-called “anti-graft immunity” was mediated by particular leukocytes which are stimulated during rejection (Cooper and Roch, 1984; Valembois and Roch, 1977). Anti-graft immunity can be transferred to naïve earthworms by transferring stem cells from a grafted individual, suggesting the existence of memory (Roch, 1973). The earthworm’s anti-graft immunity was described as having three characteristics: accelerated rejection, weak specificity and short-term memory (less than 10 days). The same grafting technology applied to scleratinian coral (Hildemann *et al.*, 1977), the sea urchin *Lytechinus pictus* (Coffaro and Hinegardner, 1977), and the marine sponge *Callyspongia diffusa* (Bigger *et al.*, 1982) lead to similar conclusions. Curiously, and despite repeated efforts in insects, a specific short-term memory has only been found in the American cockroach, *Periplaneta Americana* (Karp and Rheins, 1980; Rheins *et al.*, 1980; Hartman and Karp, 1989). In our example of the *D. melanogaster-L. boulardi* system (see above), the capsule formation process did not show any memory as the specific interaction is dependent on genetic and not phenotypic adaptation. However, there are an increasing number of reports of the existence of memory in invertebrate immune responses. Even if the term memory was used by the authors, its short-term duration was destroying their efforts to demonstrate the existence of a true immune memory.

Immune memory is advantageous only if there is a chance of being exposed to a previously encountered pathogen; there is therefore a direct relationship with the lifespan of the species. Most invertebrates will have died before a secondary exposure is likely, and long-lived invertebrates, such as the cephalopod mollusc *Nautilus*, the horseshoe crab *Limulus polyphemus*, and some insect species (up to 17 years for a North American Cicada), seem more likely than for instance does a rotifer as candidates for immunological learning-memory. Indeed, Little and Kraaijeveld (2004) suggested that the lifespan relative to the delay between exposures is probably important. Even in short-lived invertebrates, several cycles of infections may occur according to the life cycle of the pathogen. Additionally, the same individual may be repeatedly or persistently exposed to similar types of pathogen in their natural environment. Consequently, specific immune memory might exist in short-lived as well as in long-lived invertebrates.

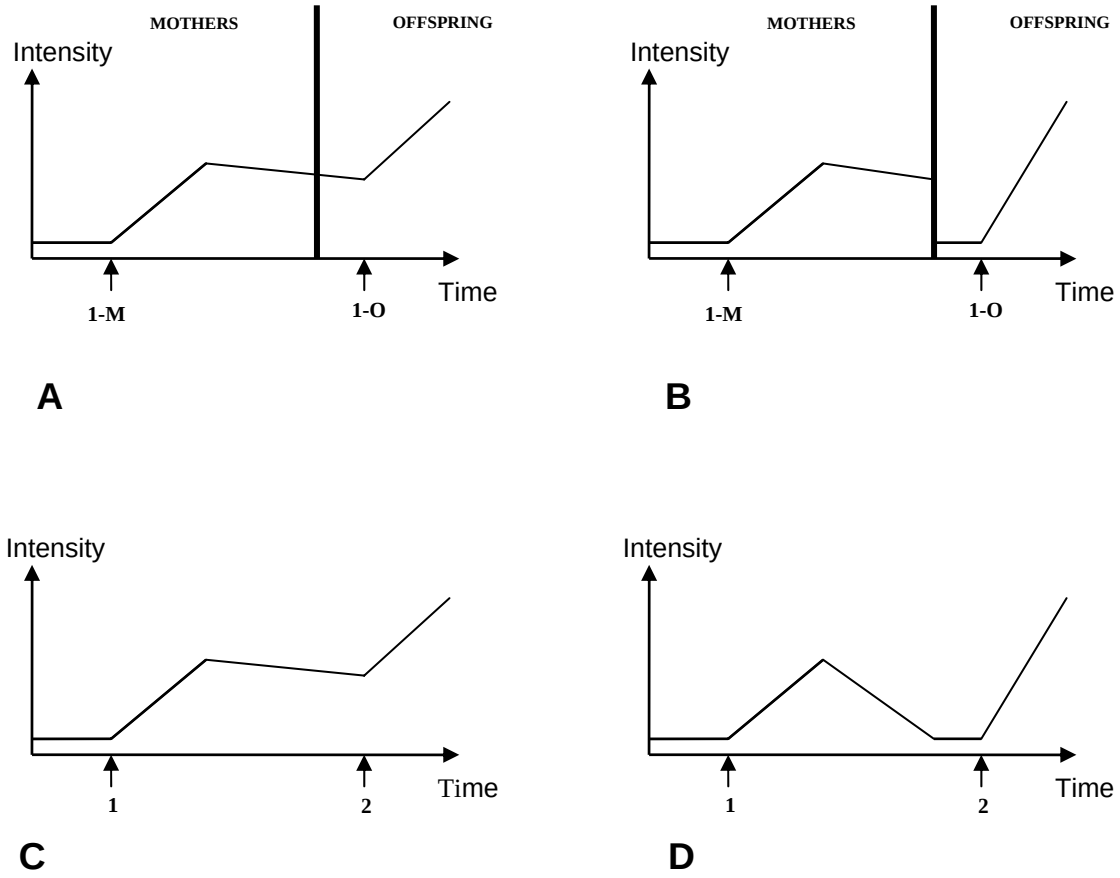

Fig. 2 How to test the hypothesis of learning through transmission of memory from mothers to offspring (A-B), or within the same individual subjected to two different exposures to the same pathogen (C-D).
Evolutions of the intensity of the immune reaction according to time and reproduction (vertical bar) following a first exposure of the mothers to the pathogen (1-M), and a first exposure of offspring issued from the challenged mothers to the same pathogen (1-O). A: hypothesis of transmission of immunity by transfer of effectors leading to an increased response, i.e. no learning. B: hypothesis of learning after exposure of the mothers and transmission of the memory to offspring leading to an accelerated immune response.
Evolutions of the intensity of the immune reaction according to time in the same individual following a first exposure to the pathogen (1), and a second exposure to the same pathogen (2). C: hypothesis of the persistence of high concentration of immune effectors leading to an increased response after the second exposure, i.e. no learning. D: after the first challenge, the immune response return to baseline and the accelerated immune response observed after the second exposure is due to learning-memory resulting from the first exposure.

The importance of the concept of immune learning

The existence of long-lasting cells, known to be the support of memory in vertebrates, has never been fully demonstrated in invertebrates. However, in addition to graft rejection assays, there are several examples of a second response being modified by a previous encounter, suggesting the existence of memory derived from learning. In particular, experiments in shrimp, (Huang and Song, 1999) and *Daphnia* (Little *et al.*, 2003) provide strong arguments in favour of immune learning and memory: In these studies, larvae were specifically protected against one pathogen after "vaccination" of their mothers. To explain their results, the authors suggested that mothers impregnated their eggs with immune peptides. It is also possible that there was a kind of learning and memory that allowed better protection. This is exactly what differentiates between innate and adaptive immunity and has led some authors to speculate on adaptive aspects of immune responses in invertebrates (Flajnik and Du Pasquier, 2004; Agaisse, 2007; Kurtz, 2005; Kvell *et al.*, 2007; Pham *et al.*, 2007). Invertebrates have no antibodies, or T and B cells, so there must be a

completely different system that supports learning and memory. Before dissecting the molecular basis of such a putative system, we must be sure that the shrimp or *Daphnia* (or other invertebrates) are indeed capable of immune learning.

To achieve this goal, we suggested continuously measuring protection between two exposures to the same pathogen; surprisingly, reports of such studies are rare, if any. If the protection (expressed as the level of one particular effector, for instance) remained almost the same between the two exposures, the hypothesis of a transmission of immunity from mothers to eggs (by transfer of the effector) remains valid (Fig. 2A). However, in contrast to the persistence of specific B cells in vertebrates, this cannot be called memory but only transfer of molecular effectors, as observed for antibodies between mothers and offspring through the placenta in mammals, for instance. In contrast, if there was a decrease in protection between mothers and offspring, followed by a large increase after exposure of the offspring (Fig. 2B), this would argue for existence of immune learning and transmission of memory from mothers to offspring. However, this phenomenon has not been found in the adaptive immunity of vertebrates.

The situation is somewhat different in the case of multiple exposures of one individual to different strains of a pathogen species. Exposure of the copepod *Macrocyclops albidus* to its tapeworm parasite, *Schistocephalus solidus*, reduces the chances of re-infection of the same host by siblings of the infecting tapeworm but not by unrelated parasites (Kurtz and Franz, 2003); this clearly evidences immune specificity at the level of the strain, as observed in the *D. melanogaster-L. boulardi* system or in *Daphnia*. But can we call this phenomenon memory? Two different models may apply to these observations. If the protection (expressed as previously as the level of one particular effector) remained almost the same between the two exposures, the persistence of the molecular effector in itself could account for the improved protection at the second exposure (Fig. 2C). However, if protection decreased after the first exposure, then was substantially accelerated on the second exposure, it would be good evidence for learning and memory induced by the first exposure (Fig. 2D). To confirm that true memory is established, referring to a kind of “vaccination”, it would be relatively simple to test whether the level of protection just before the second challenge was returning to baseline. To do that, several prerequisites included at least established optimum breeding conditions and minimum knowledge of immune capacities of the species under investigation.

Existence of memory remains to be demonstrated

When considering the global immune response generally (and not only the recognition process), it is evident that specificity is very accurate, at least in some invertebrates (Carton *et al.*, 1992; Kurtz and Franz, 2003; Little *et al.*, 2003; Pham *et al*., 2007). The specificity of some innate immune responses is very probably also present in vertebrates where it might have been obscured by the strength of adaptive immunity. It seems likely, given the extreme diversity of invertebrate (and of vertebrate) species, that there is not one single universal system underlying specificity. We have shown that the inhibiting effect triggered by the parasitoïd could explain the strong specificity of *D. melanogaster-L. boulardi* relationships. The recently discovered extended genomic diversity of immune effectors (Imler and Bulet, 2005; Padhi *et al.*, 2007), and receptor PGRPs (Royet *et al.*, 2005), Dscam (Watson *et al.*, 2005) and TLRs (Takeda *et al.*, 2003), is still not understood but may also account for the specificity of innate immune responses.

Finally, the existence of a true memory remains to be clearly demonstrated in invertebrates. We agree with Hauton and Smith (2007) that before there can be a "theory", facts about invertebrate immune responses must be established for a wide range of taxa. But unlike these authors, we recommend establishing the existence of memory in an invertebrate species before looking for its biochemical and molecular supports, also suggested by Little *et al.* (2005).

We proposed a change in the paradigm of a weak specificity for innate immunity and, considering the overall response, to allow for the possibility of sharp specificity to some of the innate immune responses, both in invertebrates and in vertebrates. Meanwhile, on the basis of currently available facts and referring to the learning-memory concept, the dogma that adaptive immunity is absent from invertebrates must be maintained.

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