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Is there any kind of adaptive immunity in invertebrates?

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Abstract

The possible existence of a peculiar form of adaptive immunity in invertebrates is important for a better understanding of immunological evolution and for the development of vaccination strategies. These may be relevant in the control of infectious diseases, common under intensive farming of economically important crustaceans. Adaptive immunity has been assumed to be absent from invertebrates because they lack the immunoglobulin (Ig), T cell receptor (TCR) and Major histocompatibility complex (Mhc) high diversity molecules. Since adhesion Ig super family (SF) molecules, which in mammals are known to be involved in adaptive immune response, are present in invertebrates, it can be postulated that they may also be responsible for invertebrate adaptive immunity. However, because invertebrate IgSF molecules are not phylogenetically homologous to those of vertebrates, the existence of an anticipatory immunity has not been accepted in invertebrates. It has also been postulated that the antigen receptors in invertebrates have a low range of diversity leading to similar responses to disparate immunostimulants. We have observed that the hemocyte proliferation rate (HPR) of *Penaeus japonicus* was increased by a similar extent after stimulation with different mitogens, although at a lower magnitude than after fungal infections. Besides, *Drosophila* responses discriminate between fungus and bacteria. Furthermore, upon comparison of the HPR after a single and a second challenge with fungal antigens, we observed that after a second challenge there was an increased HPR that correlated with cell activation. This increase was, however, much smaller than that observed in lymphocyte proliferation between a vertebrate primary and secondary immune response. This observation is suggestive

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of a peculiar form of adaptive immunity in invertebrates that can constitute, nevertheless, the basic tool for vaccination strategies.

Keywords: Invertebrates; Adaptive immunity; Vaccination; Adhesion molecules

1. Attempts at immunoprotection against crustacean infectious diseases

Crustacean farming, mainly of shrimp, is not an insignificant source of world income. Since crustacean microbial infections are rather frequent under intensive aquaculture, it is important to determine if some kind of adaptive immune response does exist in invertebrates, particularly in crustaceans. This could allow development of possible strategies of protective immunointervention. Several empirical shrimp and lobster anti-microbial vaccinations have been attempted. It has been reported that treatment of *Penaeus monodon* with β-1-3-glucan alone (Kenkyu, 1994), killed Vibrios alone (Teunissen et al., 1998) and killed Vibria and β-1-3-glucan (Teunissen et al., 1998) significantly enhanced the resistance to shrimp vibriosis. Interestingly, it seems that treatment with dead Vibria and β-1-3-glucan is more effective in the protection against vibriosis than treatment with dead Vibria alone. Similarly, in the case of gaffkemia infections in lobsters, efficient vaccination in laboratory and field trials has been achieved by treatment of the crustaceans with inactivated bacteria (Keith et al., 1992). This prophylactic immunopotentiation is accompanied by immune cell activation. It was observed that treatment with β-glucan induced a higher percentage of hemocytes with superoxide anions than with other immunostimulants in *P. monodon* (Song and Hsieh, 1994). It also stimulated various defense systems in crustaceans (Takahashi et al., 1995a,b). Moreover, treatment of *P. japonicus* with peptidoglycan was shown to enhance the phagocytic activity of granulocytes and increase shrimp resistance to *Vibrio penaeicida* (Itami et al., 1989). Nevertheless, it remains to be clarified if these results can be explained by the existence of an adaptive secondary immune response in invertebrates homologous to that observed in vertebrates or to a distinct type of immunoprotective pathway.

1.1. Is there an adaptive secondary immune response in invertebrates homologuous to that of vertebrates?

Adaptive immune responses in invertebrates have been investigated for a long time (Paris, 1961). Short-term specific memory has been documented or postulated to be present in invertebrates such as sponges (Hildemann et al., 1980a; Bigger et al., 1983), cinidarians (Hildemann et al., 1980b; Salter-Cid and Bigger, 1991), insects (Hartman and Karp, 1989) or urochordates (Raftos, 1991, 1994). Still, conflicting reports have claimed that immunological memory is not a general property of such invertebrates (Sauer et al., 1986; Rinkevich and Weissman, 1990; Shenk and Buss, 1991). Most of these studies have focused on morphologically detectable graft rejection (Rinkevich, 1996) because criteria for rejections are closely similar in vertebrates (Hildemann, 1984). However, these approaches also have some disadvantages because a limiting spectrum of animals is usually investigated and evaluation of rejection is often complex and subjective (Rinkevich, 1996).

An adaptive secondary memory immune response has been described in vertebrates with respect to immunoglobulins (Igs), T cell receptors (TCRs), the Major histocompatibility complex (Mhc), and memory T cells (Klein, 1989). Based on the assumed lack of antigen receptor diversity in invertebrates, Klein postulated that anticipatory (memory) and non-anticipatory immune responses were observed in vertebrates, whereas only non-anticipatory responses could be seen in invertebrates (Klein, 1989, 1997). Thus, the impossibility of vaccine development in invertebrates has been a paradigm for those that assume the non-existence of an adaptive immune response in these animals. As pointed out by Kaattari (1994), the arbitrary use of semantic definitions of adaptive immune response leads to potential great errors in immunointervention. This is particularly worrying when accelerated responses from a first to a second antigen challenge do not constitute enough evidence for considering an adaptive immune response (Klein, 1997). Moreover, besides memory T cells, Igs, TCR, and Mhc molecules, adhesion molecules belonging to the super family of the Igs (IgSF) are also important for the development of immune memory in vertebrates, and invertebrates have the latter kind of molecules.

1.2. Adhesion molecules and immune responses in vertebrates

The role of adhesion molecules in the immune memory of vertebrates becomes evident by the existence of higher numbers of these molecules in memory cells than in naive T cells (Amhed and Gray, 1996). Due to the presence of these molecules in the memory immunocompetent cells, these cells have the capacity to be activated faster and respond to smaller amounts of antigens than naive cells (Swain et al., 1991; Vitetta et al., 1991; Gray, 1993; Mackay, 1993; Kearney et al., 1994; Sprent, 1994; Sprent and Tough, 1994). Among these molecules, the B cell marker, CD40, is particularly important. The interaction of this marker with its ligand expressed in activated T cells is crucial for the generation and maintenance of the immune response. This response involves cell proliferation, Ig production and switch of class, cytokine production, and generation of memory B cells (Banchereau et al., 1994; Holder et al., 1993; Parry et al., 1994; Van Kooten and Banchereau, 1996). Moreover, it has been observed that CD40 is involved in the adaptive immune response because unsaturated bonds between CD40 and the T cell ligand induce B cell proliferation and Ig production (Holder et al., 1993; Parry et al., 1994), whereas saturated bonds lead to apoptosis and decrease the Ig production (Inui et al., 1990; Fluckiger et al., 1992; Bergman et al., 1996; Goldstein and Watts, 1996; Hess and Engelmann, 1996; Lens et al., 1996).

1.3. Adhesion molecules and immune responses in invertebrates

Although selectins have not been identified in invertebrates, other adhesion molecules such as catherins, Ig-like proteins, integrins, collagens, and laminins have been found in several invertebrates (Johansson, 1999). However, some invertebrate adhesion proteins such as extracellular matrix protein and tiggrin do not appear to have any counterparts in vertebrates (Johansson, 1999). In this review, we will focus on the invertebrate adhesion molecules that seem to be more important and better studied.

Peroxinectin shares both peroxidase and cell adhesion properties (Johansson and Soderhall, 1988, 1989; Johansson et al., 1995). However, these functions appear to be

distinct and due to different amino acid residues (Johansson, 1999). Peroxinectin, which has been mainly described in crayfish but also in *P. monodon*, cockroach, *Blaberus craniifer*, and *Drosophila*, is produced by hemocytes. It supports cell adhesion and stimulates phagocytosis and encapsulation (Kobayashi et al., 1990; Rantamaki et al., 1991; Ng et al., 1992; Thornqvist et al., 1994). It was recently reported that the human peroxidase homologue of peroxinectin also has microbicidal activity and mediates cellular adhesion through the integrin $\alpha M\beta 2$ largely expressed in neutrophils, monocytes, macrophages, and NK cells (Johansson et al., 1997).

Another IgSF adhesion molecule present in invertebrates is hemolin (Sun et al., 1990; Ladenhorff and Kanost, 1991; Bettencourt et al., 1997). Hemolin does not show a direct microbicidal activity but it is the main inducible protein in insect hemolymph after bacterial stimulation (Rasmuson and Boman, 1979; Anderson and Steiner, 1987; Ladenhorff and Kanost, 1990). It was also observed that adhesion of hemolin to the hemocytes inhibits hemocyte aggregation (Ladenhorff and Kanost, 1991) and facilitates phagocytosis. This effect is potentiated by LPS and promotes an alteration of the phosphorylation pattern of the hemocyte proteins and subsequent increase in protein kinase (Lanz-Mendonza et al., 1996). Hemolin is expressed in fat body cells, a functional analogue of the mammalian liver (Abel et al., 1992; Falb and Maniatis, 1992). It also is expressed in hemocytes, in insect nodular bacterial infected formations (Trenczek and Faye, 1988) and in embryonic tissue, suggesting that hemolin has a function in signal transduction during immune responses and embryogenesis (Bettencourt et al., 1997).

Besides these two important and well-studied adhesion molecules, others have been described such as Limulus agglutination–aggregation factor (LAF) (Fujii et al., 1992), hemocytine (Kotani et al., 1995), A74 protein (Takahashi et al., 1997), croquemort (Franc et al., 1996), and the plasmatocyte-spreading peptide (PSP1) (Clark et al., 1997, 1998). LAF, which is produced by horseshoe crab hemocytes and shows amino acid sequence homology to mammalian dermatoponfin, is an hemagglutinin that triggers cell aggregation. Hemocytine is produced by silkworm hemocytes and it is also an hemagglutinin with domains similar to those of mammalian von Willebrand factor. The A74 protein is a membrane hemocyte antigen from the ascidian *Halocynthia roretzi* that seems to be involved in cell aggregation. Croquemort, present in hemocytes of Drosophila, seems to facilitate the phagocytosis of apoptotic cells. It is somewhat similar to mammalian CD36, which recognizes apoptotic cells when associated with integrin. PSP1 is expressed primarily in the fat body of the moth, *Pseudoplusia includes*, and triggers cell spreading.

1.4. Similarity between toll cascade in Drosophila and in humans

The *Drosophila* Toll/Cactus/Dorsal (TCD) molecules are probably the most interesting adhesion molecules developed in invertebrates. As shown in Fig. 1, the Toll cascade is very similar in *Drosophila* and humans. The *Drosophila* TCD molecules were initially described as promoters of the development of the embryonic dorsoventral axis (Rushlow and Arora, 1990). *Drosophila* Toll is a transmembrane protein with an extracellular leucine-rich domain and cytoplasmic domain (Medzhitov et al., 1997). The ventral activation of Toll is transduced by the cytoplasmic proteins Tube and Pelle. Tube

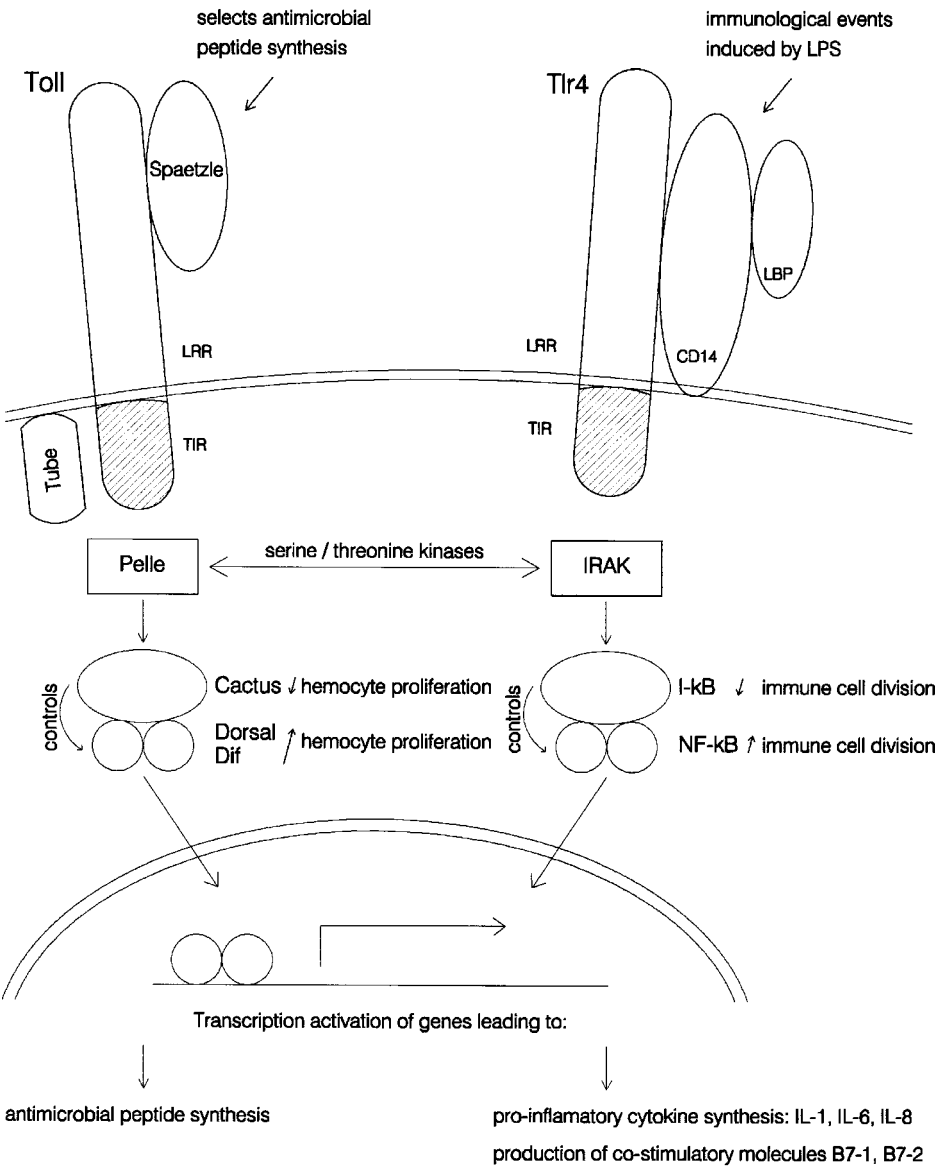

Fig. 1. Comparison between *Drosophila* and human Toll cascades.

is a unique protein (Letsou et al., 1991) and Pelle is a serine/threonine kinase (Shelton and Wasserman, 1993). The Toll/Tube/Pelle complex acts on Dorsal/Cactus proteins. Dorsal is a member of the Rel family of rapid inducible transactivators (Rushlow and Arora, 1990). Cactus causes the retention of Dorsal in the cytoplasm (Kidd, 1992; Whalen and Steward, 1993). Thus, Cactus is considered to be an inhibitor of Dorsal. The complex Toll/Tube/Pelle mediates phosphorylation and degradation of Cactus and the

concomitant nuclear translocation of Dorsal (Belvin et al., 1995). Since the process of nuclear localization is graded, Cactus can be visualized after signalling in a dorsal-to-ventral cytoplasmic gradient, which inversely correlates with the ventral-to-dorsal gradient of Dorsal in the nucleus (Bergmann et al., 1996; Reach et al., 1996). The Toll protein has proven also to be responsible for the generation of microbicidal peptides (Kylsten et al., 1990; Wicker et al., 1990; Bulet et al., 1993; Dimarcq et al., 1994; Fehlbaum et al., 1994; Hultmark, 1994; Morisato and Anderson, 1994; Asling et al., 1995). The microbicidal peptide responses are controlled by spaetzle the extracellular Toll ligand (Morisato and Anderson, 1994), involving the gene cassette spaetzle/Toll/cactus (Lemaitre et al., 1996). However, instead of Dorsal, the Dorsal-related immunity factor (Dif) seems to be involved in this signal transduction pathway (Ip et al., 1993; Hultmark, 1994; Qiu et al., 1998). In addition, it was suggested that the Toll/Cactus pathway controls hemocyte viability whereas Cactus controls hemocyte proliferation (Qiu et al., 1998). The TCD cassette proteins are primarily produced by the *Drosophila* fat body. More recently, the *Drosophila* TDC proteins were reported to be expressed also in the *Drosophila* nascent hemocytes of the larval lymph gland (Qiu et al., 1998).

Interestingly, five Toll-like receptors (Tlrs) were cloned in humans (Rock et al. 1998) with intra- and extra-cellular structures similar to the Drosophila Toll receptor. The cognate Tlr genes reside on chromosomes 4 (Tlr 1, 2 and 3), 9 (Tlr 4) and 1 (Tlr 5) (Rock et al., 1998). Tlr2 and Tlr4 have been the most well-studied so far. Tlr2 was originally described on the basis of studies performed in embryonic cell lines as the co-receptor for LPS, the other co-receptor being CD14 (Kirschning et al., 1998). However, in studies performed in C3H/Hej, C57BL/10ScCr mice that do not respond to LPS, and in Tlr4 mutant mice it was concluded that Tlr4 was indeed the co-receptor for LPS (Poltorak et al., 1998; Quereshi et al., 1999). In any case, since the cytoplasmic domain of human Toll receptors of IL-1R are homologous, it is not surprising that both Tlr2 and Tlr4 are involved in the IL-1R cascade leading to the expression of the pro-inflammatory cytokines, IL-1, IL-6 and IL-8 as well as the co-stimulatory B7-1 molecule which is required for the activation of naive T cells (Medzhitov et al., 1997). The IL-1 receptor associated kinase (IRAK) which, like Pelle, is a serine/threonine kinase (Cao et al., 1996), is involved in signal transduction from the IL-1R. This pathway is transcribed by the nuclear factor (NF)-kB, a mammalian member of the Rel family of transcription factors. It is controlled by I-kB, which binds to NF-kB, as Cactus binds to Dorsal (Hultmark, 1994) (Fig. 1).

As depicted in Fig. 1, the *Drosophila* Toll/Dorsal signalling pathway parallels the mammalian Toll/NF-kB pathway, with Dorsal and its inhibitor Cactus being homologous to NF-kB and I-kB proteins, respectively (Hultmark, 1994). First, *Drosophila* and human Toll receptors are remarkably well-conserved (Medzhitov et al., 1997). Second, the cytoplasmic domains of both the *Drosophila* and human Toll proteins have high homology to the cytoplasmic domain of IL-1R (Gay and Keith, 1991; Schneider et al., 1991). Third, signalling through Toll and Cactus proteins and activation of Dorsal morphogen or Dif parallels signalling induced by Toll/IL-1R and I-kB and activation of NF-kB (Hultmark, 1994; Medzhitov et al., 1997). Fourth, many of the insect genes for the inducible antibacterial proteins share a common motif similar to the binding site for

NF-kB (Sun et al., 1990). Fifth, LPS activates an insect NF that binds to the conserved kB-like motif, and this factor cross-reacts with antibodies against NF-kB (Sun and Faye, 1992). Sixth, paralleling the control of hemocyte viability by Cactus, hyperstimulation of NF-kB in mice results in potentiation of the apoptotic effects of TNF- (Qiu et al., 1998). Finally, hyperstimulation of NF-kB or lack of effective I-kB in Rel mutant mice results in increased immune cell division, which is similar to what was observed in *Drosophila* with a Cactus mutation (Qiu et al., 1998).

2. Can some anticipatory-like immune response, although markedly different from the one seen in vertebrates, be uncovered in invertebrates?

It was postulated by Janeway (1989) that invertebrates have a low range of pattern diversity of receptors for immunostimulants, resulting in similar intense responses to disparate antigens. In agreement with Janeway's postulate, we have found that the shrimp's constitutive hemocyte proliferation rate (HPR) was increased (from 1%) after stimulation with different mammalian B cell mitogens or with combinations of two of these mitogens (Sequeira et al., 1996). However, in contrast to the Janeway postulate, we have also observed that the HPR of *P. japonicus* was considerably higher in *Fusarium* infected shrimp than in the mitogen treated animals.

Undoubtedly, vertebrate and invertebrate IgSF adhesion molecules are functionally comparable, and humans and *Drosophila* share very similar Toll cascade regulatory pathways. The high diversity of *Drosophila* Toll receptors and possible different conformations of Spaetzle Toll ligands are accepted to provide some selectivity to the cascade transduction of different microbicidal insect peptides (Lemaitre et al., 1996; Qiu et al., 1998). However, the non-existence of an adaptive immune response in invertebrates is taken for granted because there is an absence of direct homology between these molecules and those of vertebrates (Klein, 1997; Hughes, 1998, 1999). On the other hand, it is generally accepted that both vertebrates and invertebrates have in common the existence of innate immunity, which must have been conserved from primitive life forms to humans (Ottaviani et al., 1998; Hoffmann et al., 1999), and that their expression is up-regulated by immune challenge (Lemaitre et al., 1996). This up-regulation can be considered a peculiar form of adaptive immune response since this response is increased upon challenging with immune stimulants. In addition, it is postulated that invertebrates have an immune-neuroendocrine recognizing system that is optimized for a gross but efficient discrimination between self and non-self and which is only slightly less complex than the vertebrate immune system (Ottaviani et al., 1998). We were able to observe very significantly higher HPRs in *P. japonicus* challenged twice with the same fungal constitutive antigens than in *P. japonicus* challenged only once (Sequeira et al., 1999). Interestingly, the 5% increase in HPR observed after a second fungal antigenic challenge is a percentage identical to the constitutive HPR observed in cactus mutant *Drosophila* compared to 1% in wild-type flies (Qiu et al., 1998). The higher HPR observed in shrimp stimulated twice with fungal antigens also correlated with cell activation detected by transmission microscopy and by increased "cytokine-like determinations" (Sequeira et al., 1999). However, a secondary HPR increase was not observed

Table 1
Comparison of postulated secondary adaptive immune responses in vertebrates and invertebrates

Cellular events	Vertebrates	Invertebrates
Proliferation	+++[a]	+
Morphological activation	+++	++
Memory cells	+++	– (?)
Molecular expression		
Igs	+++	–
TCRs	+++	–
MHC	+++	–
Adhesion IgSF molecules	+++	+++
Cytokines	+++	++ (?)[b]

[a]Intensity of observation.
[b]Evidences obtained by fluorescent staining but never cloned so far.

after challenge with all antigens and when observed after fungal antigen stimulation was reduced in intensity compared to a secondary immune response seen in vertebrates (Sequeira et al., 1999).

3. Conclusions

We conclude that: (1) Although invertebrates have a lower diversity of receptors for immunostimulants than vertebrates, this limited diversity does allow immune responses of different intensity, at least to some antigens. (2) An adaptive immune response can be detected in invertebrates after challenge with certain antigens but not with others. This secondary response is markedly different from the one observed in vertebrates. In addition, the cell proliferative levels observed in a secondary immune response are markedly smaller in invertebrates than in vertebrates.

It should be noted that the secondary responses that we have observed (Sequeira et al., 1999) fit with the designation of immune memory stated by Hildemann (1984) as follows, "A common denominator of all memory responses is selectively altered reactivity following repeated antigen stimulation", or with the concept of Kaattari (1994) indicating that "a memory response is one which is distinctive in its form and function from that of a primary response". Thus, it seems reasonable to assume that a peculiar form of adaptive immune response, quantitatively and qualitatively different from that of vertebrates, probably exists in invertebrates (Table 1).

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