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Review

On the existence of cytokines in invertebrates

A. Beschin[a, *], M. Bilej[b], E. Torreele[a], P. De Baetselier[a]

[a] Department of Immunology, Parasitology and Ultrastructure, Flemish Interuniversity Institute for Biotechnology, Free University Brussels (VUB), Paardenstraat 65, 1640 St-Genesius-Rode (Belgium), Fax +32 2 359 0359, e-mail: abeschin@vub.ac.be
[b] Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4 (Czech Republic)

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Abstract. Based on the assumption that invertebrates, like vertebrates, possess factors regulating responses to infection or wounding, studies dealing with the evolution of immunity have focussed on the isolation and characterisation of putative cytokine-related molecules from invertebrates. Until recently, most of our knowledge of cytokine- and cytokine receptor-like molecules in invertebrates relies on functional assays and similarities at the physicochemical level. As such, a phylogenetic relationship between invertebrate cytokine-like molecules and vertebrate counterparts could not be convincingly demonstrated. Recent genomic sequence analyses of interleukin-1-receptor-related molecules, that is Toll-like receptors, and members of the transforming growth factor-β superfamily suggest that the innate immune system of invertebrates and vertebrates evolved independently. In addition, data from protochordates and annelids suggest that invertebrate cytokine-like molecules and vertebrate factors do not have the same evolutionary origin. We propose instead that the convergence of function of invertebrate cytokine-like molecules and vertebrate counterparts involved in innate immune defences may be based on similar lectin-like activities.

Key words. Invertebrate; cytokine; evolution; lectin; inflammation.

Introduction

In the common monophyletic origin of the main animal phyla, the animal kingdom is divided into two groups, the protostomes and the deuterostomes. The two groups differ among each other according to whether in embryonic development the mouth and the anus originate from a single opening (protostomes) or separately (deuterostomes) [1]. In this monophyletic origin model, the animal kingdom bifurcates at the coelenterate level with a separate evolution of the immune system in each group. All protostomes are invertebrates, whereas the deuterostomes include vertebrates and some invertebrates such as echinoderms and protochordates (fig. 1).

From the total number of extant animal species, probably surpassing 2 million, 95% are included in the invertebrate taxa. Invertebrates have evolved for hundreds of millions of years, often surviving very hostile environments. Their successful survival strategies are likely based on short life span combined with numerous offspring. However, all invertebrate species have also evolved a variety of active defence pathways, efficiently recognising and responding to non-self substances.
The parallels between vertebrate and invertebrate cellular host defence have been recognised since the pioneering work of Metchnikoff at the end of the 19th century. Like vertebrates, invertebrates display efficient physicochemical barriers to infection (for example the mucus surrounding the body of many coelenterates, annelids, molluscs and protochordates, or the exoskeleton of coelenterates,

* Corresponding author.

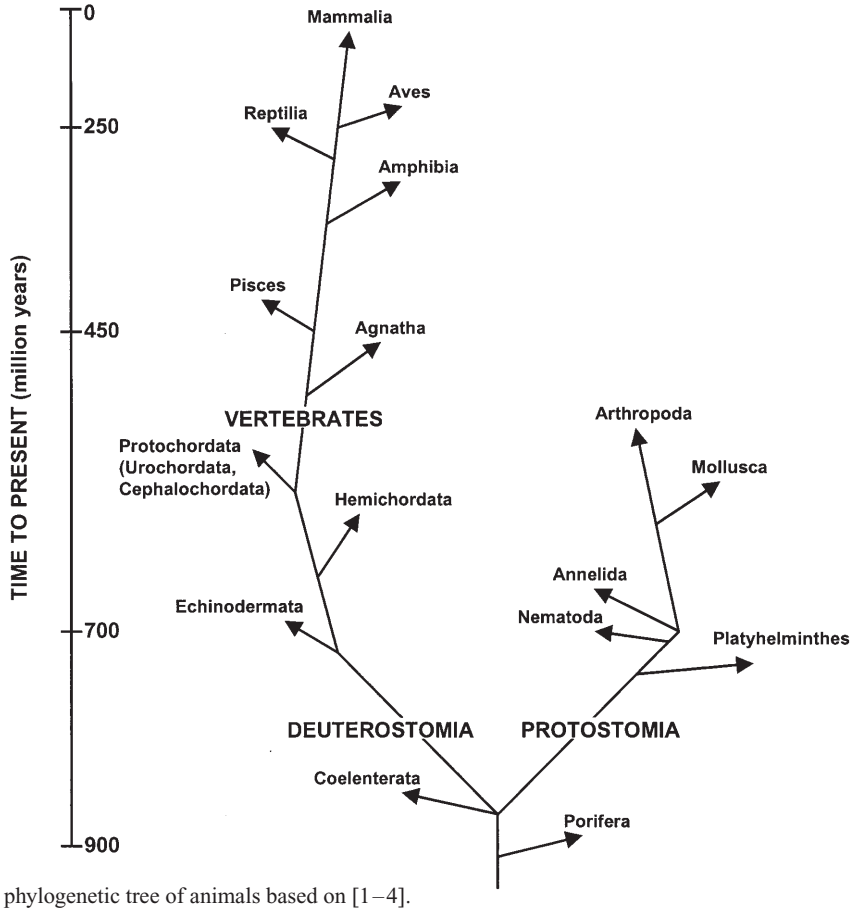

Figure 1. Simplified phylogenetic tree of animals based on [1–4].

molluscs, arthropods, echinoderms and protochordates). Moreover, invertebrates share many of the innate immune mechanisms of vertebrates, but they lack the adaptive immune defences that rely on antibodies or lymphocytes. Cellular defences are an important feature of invertebrate host protection and include wound repair, clotting and coagulation responses, phagocytosis and encapsulation reactions. In many invertebrates, lectin-like molecules [5] and pattern recognition receptors [6] that in most cases do not discriminate between individual pathogens are probably responsible for non-self recognition processes involved in these reactions. Apart from the recognition molecules, the humoral defences also include a range of naturally occurring antimicrobial factors such as lysozyme-like proteins, proteases, cytolysins, antimicrobial peptides, phenoloxidase and metabolites of the prophenoloxidase-activating cascade [7, 8]. Most of the abovementioned immune reactions are nonadaptive, with no accelerated or amplified response following secondary challenge.

In vertebrates, cytokines are the major regulators of the host defence processes, and as such are involved in responses to exogenous and endogenous insults, repair and restoration of tissue homeostasis. Those soluble mediators of the immune response are secreted mainly by immunocytes and thought to mediate their activity via interaction with specific cytokine receptors. Considerations of the functional parallels between innate (nonadaptive) host defences in invertebrates and vertebrates led to the hypothesis that invertebrates possess soluble cytokine-like mediators that regulate inflammatory responses to infection or wounding [9]. Here, we summarise the studies aiming to demonstrate cytokine-like activities in invertebrates, and critically review the evidence for the existence of common evolutionary origin between invertebrate cytokine-like molecules and related receptors with their mammalian counterparts. We will propose that the functional analogies between invertebrate cytokine-like molecules and vertebrate cytokines result from convergent evolution and are based on similarities in lectin-like activities and domains.

Evidence for the existence of invertebrate cytokine-like factors

The rationale behind the search for inflammatory cytokine-like molecules in invertebrates was based on the assumption that these molecules would perform similar functions in invertebrate and vertebrate hosts. Typical

Table 1. Cytokine-like molecules identified in protostome and deuterostome invertebrates.

Taxa	Cytokine-like molecules	References
Porifera	TNF	10
Nematoda	TGF-β	Yandell et al. (1997)[a]
Annelida	IL-1, TNF	For reviews: 9, 11–14; 15, 16
Mollusca	IL-1, TNF, LT,IL-6, IL-8, TGF-β, IL-2	For reviews: 9, 11–14;17–21
Arthropoda	IL-1, TNF, TGF-β	For reviews: 9, 11–14; 22–28, Van Wormhoudt (1997)[b]
Echino-dermata	IL-1α/β, IL-1 precursor, TNF, IL-6, IL-2, TGF-β	For reviews: 9, 11–14; 29–31
Protochor-data	IL-1, TNF, IL-2, TGF-β	For reviews: 9, 11–14; 32–37

[a] Sequence deposited in GenBank, accession number AF004395, unpublished.
[b] Sequence deposited in GenBank, accession number Y11725, unpublished.

experimental approaches to identify invertebrate cytokine receptor-like or cytokine-like molecules have evaluated the sensitivity of invertebrate immunocytes to vertebrate cytokine action, or vice versa, the responsiveness of vertebrate immune cells to invertebrate factors. Moreover, antibodies neutralising the activity of vertebrate cytokines were used to screen for cross-reactivity with invertebrate factors in hemolymph, immunocytes or neuroendocrine cells. In some studies, similar biochemical characteristics (molecular weight, pI and so on) of vertebrate cytokines and functional invertebrate analogues were considered as evidence for molecular homology. Finally, a few studies attempted to identify homologues of vertebrate cytokine genes or cytokine receptor genes in invertebrates.

Table 1 summarises the studies that led to the identification of several putative functional analogues of inflammatory cytokines. As such, mainly interleukin (IL)-1-, IL-6- and tumor necrosis factor (TNF)-like molecules were detected in a variety of invertebrates such as annelids, molluscs, insects, echinoderms and protochordates [9–37]. In addition, molecules cross-reacting with antibodies elicited against the chemokine IL-8 and against transforming growth factor (TGF)-β1, two other vertebrate cytokines involved in inflammatory processes and produced by macrophages [38, 39] have been identified in molluscs. Furthermore, IL-2-like activity was detected in protochordates and echinoderms, that is in deuterostome invertebrates having a hematopoietic organ and T-like cells [12, 29].

Searches for homologies at the genome level also suggest the existence of cytokine-like molecules in invertebrates. In deuterostomes, Pestarino et al., using equimolar mixture of the exons 5, 6 and 7 (30 bp long each) of the human IL-1β gene, have localised a messenger RNA (mRNA) with homology to IL-1β mRNA in the cerebral ganglion of the protochordate *Stylea plicata* [35].

In protostomes, Beck used primers based on shared vertebrate IL-1 protein sequences to isolate a polymerase chain reaction (PCR) product from *Manduca sexta* mRNA that shows 35% homology to sheep, rat, rabbit, cow, mouse and human IL-1α, IL-1β or IL-1-receptor antagonist (IL-1-ra) [13]. When translated into protein, 22% identity and 31% similarity between the insect PCR product and mammalian IL-1β were observed, whereas similarities to mammalian IL-1α and IL-1-ra were considerably lower.

In *Drosophila*, a 30-nucleotide region in the gene encoding diptericin, an antimicrobial peptide against Gram-negative bacteria, was found to contain multiple functional transcription regulatory sequences homologous to mammalian acute phase response elements (REs), namely nuclear factor kappa B (NF-κB)-RE, IL-6-RE and interferon (IFN)-RE [40, 41].

Collectively, these observations suggest that both deuterostome and protostome invertebrate host defence mechanisms make use of cytokine-related molecules. Interestingly, these cytokine-like products are typically produced by invertebrate immunocytes displaying phagocytic activity, and in invertebrates as in vertebrates, the presence of cytokine-like molecules (IL-1, IL-6, TNF) occurs in neural cells [21, 42–45].

Evidence for the existence of invertebrate cytokine receptor-like factors

Several groups have tried to demonstrate in invertebrates the presence of membrane molecules related to vertebrate cytokine receptors. In this regard, Legac et al. reported the cross-reaction of antibodies elicited against human IL-1, IL-6, IL-2 and IFN-γ receptors with surface molecules on cells from the echinoderm hematopoietic axial organ [29]. In addition, human IL-1 (huIL-1) was documented to bind specifically to the coelomocyte membrane of echinoderms, whereas huIL-2 was shown to enhance the cell proliferation in protochordate pharyngeal explants [14, 34, 46]. Moreover, Parker and Ourth have detected the specific binding of human IFN-γ to *M. sexta* larvae tissues, indicating the presence of a putative IFN-γ receptor in insects [47].

Gene homology studies also support the existence of cytokine receptor-like molecules in invertebrates. McMahan et al., probing a genomic Southern blot with DNA of different species with complementary (cDNA) probes for type I and IL-1 receptor (IL-1-R), have observed a hybridisation to *Drosophila melanogaster* DNA, suggesting the presence of insect genes with homology to the human IL-1-R gene [48].

In the meanwhile, however, a growing number of mammalian homologues of the type I IL-1-R (IL-1-RI) containing a highly conserved region in their cytosolic domains have been described [49, 50]. Interestingly, these homologous regions are also found in a membrane receptor protein of insects (*Drosophila, Clogmia albipunctata, Tribolium casteneum*) called Toll [50, 51]. This protein was originally described as being important for establishing the dorsoventral polarity in developing fly embryos. However, in adult flies, Toll is involved in antifungal defences [52]. This led to the definition of the IL-1-R/Toll-like receptor (TLR) superfamily, with a conserved 150-amino acid cytosolic region, the Toll/IL-1-R (TIR) domain [50, 53, 54]. The TIR cytoplasmic domain of TLR proteins controls the nuclear localisation of Rel/NF-κB transcription factors in both insects and mammals [49, 55]. The identified sequence similarity between the cytoplasmic portion of Toll and that of the signalling IL-1-RI component (the TIR module) led to the hypothesis that invertebrates may share the machinery for regulation of defence-related proteins and may possess the IL-1/IL-1-RI pathway [13, 14].

Taken together, these studies suggest the presence of functional analogues of cytokines and cytokine receptors in invertebrates, opening the possibility that an invertebrate cytokine network is operative and regulates host defence mechanisms, as in vertebrates. For instance, Hughes et al. have shown that activation of mollusc immunocytes by IL-1 results in part from the synthesis of a TNF-like molecule [56]. However, these studies could not convincingly demonstrate a common evolutionary origin for cytokine-like network interactions. In fact, the gene homology analyses on invertebrate cytokines should be taken with caution in view of the limited length of homologous regions with vertebrate cytokines. Moreover, the mere existence of the TIR cytoplasmic domain in invertebrate TLR proteins does not prove the presence of an IL-1/IL-1-RI homologue pathway. Furthermore, despite the existence of invertebrate homologues of vertebrate TGF-β superfamily members, they may not be involved in immune reactions. The following paragraphs will discuss these two arguments in more detail.

Does the IL-1/IL-1-R pathway exist in invertebrates?

With the growing number of members, the TLR superfamily has been subdivided into two subfamilies depending on whether the extracellular domain of a member displays homology to either the IL-1-RI Ig domain (IL-1-RI-like members) or to the leucine-rich repeat motif of Toll (Toll-like members; for review, (see [49, 50]).

The IL-1-RI-like subfamily includes among others IL-1-RI, IL-18-R and their respective accessory proteins, IL-1-RacP and IL-18-RacP, which are required to associate with the receptor-cytokine ligand for signalling. These molecules are important in inflammatory responses and the regulation of Th1 cells [57]. Interestingly, signalling through IL-1-RI has been implicated in the pathogenesis of two disorders of inappropriate apoptosis (diabetes mellitus and acute neurodegeneration), suggesting a role for IL-1-RI in development [58–60]. Other members of the IL-1-RI-like subfamily such as SIGIRR (single Ig IL-1-R-related molecule) may also be involved in bone metabolism and development [61].

The Toll-like subfamily includes nine *Drosophila* proteins, among which are TLR-5 – involved in antifungal response, like Toll – and 18-wheeler, which participates in antibacterial responses [62–66]. All insect TLRs identified so far are involved in early embryonic development [62]. Several cytosolic proteins from plants involved in disease resistance also belong to the Toll-like subfamily [50, 55]. In mammals, 10 TLRs have been reported to date, and it is estimated that the mammalian genome encodes about 20 different TLRs [50]. Vertebrate TLRs are involved in innate immunity-inducing inflammatory cytokines (TNF, IL-1, IL-6, IL-8) and key costimulatory molecules such as B7 [67]. They are also required for phagocytosis, and for the secretion of radical oxygen intermediates and nitrogen intermediates [68, 69]. Besides playing a crucial immunological role, TLRs and related compounds may also be important in human development, thereby paralleling their proposed developmental role in *Drosophila* [70–72].

In insects, the Toll activation pathway has been amply documented both during development and in the context of responses to microbial infections [52, 63, 65, 70, 73]. In both pathways, the extracellular Spätzle protein first binds to the Toll protein, which in turn through interaction with defined molecules transmits subsequently the signal to a protein complex consisting of Cactus (a homologue to mammalian I-κB) and one of the Rel/NF-κB family of transcription activators (Dorsal, Dif or Relish). The generation of distinct Spätzle fragments by two distinct proteolytic cascades is thought to orientate the Toll pathway towards development or immune regulation. To date, only the proteolytic cascade involved in development has been characterised [50, 52, 64, 74].

In vertebrates, at least two modes of TLR activation leading to nuclear localisation of NF-κB have been described. These are not mutually exclusive. Distinct Toll-like molecules may be directly involved in recognising of different classes of pathogens [63, 65, 75, 76]. For example, TLR4 binds LPS and as such acts as a pattern recognition molecule for Gram-negative bacteria, whereas TLR2 binds peptidoglycan and lipoteichoic acid of Gram-positive bacteria, and mannans of fungi. On the other hand, regulation of immunity by TLRs may rely on a proteolytic cascade activated upon pathogen recognition by pattern recognition molecules, generating a TLR-

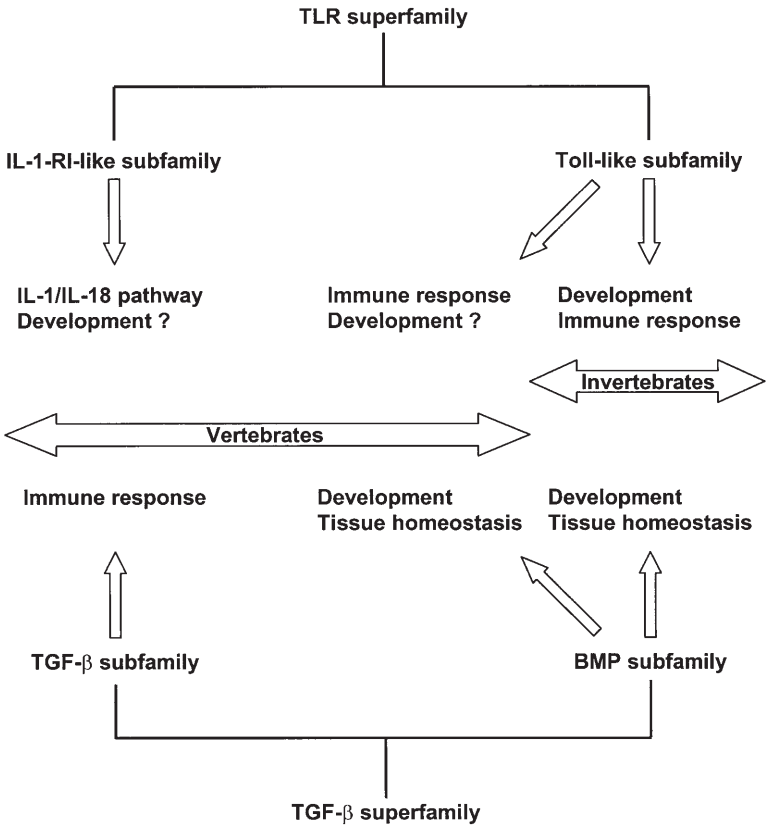

Figure 2. Compared putative involvement of TLR and TGF-β superfamily members in the development and immune response of invertebrates and vertebrates.

specific endogenous Spätzle-like ligand [52, 77, 78]. Interestingly, upon triggering by a pathogen-associated molecular pattern, TLR2 and TLR4 may also mediate apoptosis [60]. This was suggested to limit the life span of inflammatory cells, and thus to control the duration of the acute response to microbes. In vertebrates, an increased expression of TLRs also occurs during tissue injury [71]. In view of the ability of human hsp60 to act as an endogenous ligand of the TLR4 complex [72], these data indicate that the TLR activation pathway may also facilitate the engulfment of apoptotic injured cells in the absence of infection.

Hence, invertebrates and vertebrates share an evolutionarily conserved pathway based on NF-κB for the expression and regulation of host defence genes that involves the cytoplasmic domain of transmembrane proteins belonging to the TLR superfamily. However, the extracellular portions of TLRs as well as their specific ligands (Spätzle and IL-1) do not show any significant similarity [69]. Moreover, although Spätzle could be considered as an invertebrate cytokine [62], no Spätzle homologue has been yet identified in vertebrates [53, 69]. Furthermore, no vertebrate cytokine has so far been shown to bind or activate vertebrate Toll-like members of the TLR superfamily [69]. Finally, analysis of the completed genome sequences of *Drosophila* and *Caenorhabditis elegans* did not reveal any IL-1-RI homologues [62]. Therefore, despite the conservation of the cytoplasmic TIR domain, the rapid divergence of the extracellular domains of TLRs, even between homologous genes [49, 50, 55, 63, 69], may have allowed them to function in a variety of contexts, from development to immune responses (fig. 2). Hence, the existence of an invertebrate IL-1/IL-1-R pathway homologous to the vertebrate one remains hypothetical.

Are invertebrate TGF-β superfamily members involved in immune reactions?

The TGF-β superfamily is a group of multifunctional cytokines that includes bone morphogenetic proteins (BMPs), TGF-βs and others [79–82]. These secreted signalling molecules regulate many aspects of cellular responses, including cell proliferation, differentiation, migration and apoptosis. BMP subfamily members have a critical role in embryogenic patterning and in maintaining tissue homeostasis in adult life, whereas TGF-β subfamily members mainly modulate the immune response, for example in inflammatory and infectious processes [39, 83, 84]. In mammals, TGF-β locally produced within a site of inflammation, infection or injury acts, in the early stages, as a proinflammatory

agent by recruiting and activating resting monocytes [85, 86]. As the recruited monocytes are activated and differentiated, they lose responsiveness to TGF-β, levels of autocrine-stimulated TGF-β decline and specific immunosuppressive actions of TGF-β predominate. These include the reduction of cytokine production and the suppression of macrophage and lymphocyte activation, contributing to the resolution of inflammation.

Serious efforts have been devoted to cloning TGF-β superfamily members in invertebrates, including protostomes (the nematodes *C. elegans*[1], *Brugia malayi* and *B. phangi*, and the insect *Drosophila*) and deuterostomes (the sea urchin *Strongylocentrotus purpuratus* and the tunicate *Halocynthia roretzi*) [24–28, 30, 31, 36, 37, 87, 88]. None of the probes used in any of the extant studies has revealed members of the TGF-β subfamily [30]. Rather, the identified invertebrate TGF-β superfamily members are closer to the BMP subfamily. Similarly, members of the TGF-β-receptor superfamily cloned in insects and *C. elegans* act as receptors for their respective BMP-like protein and closely resemble the mammalian BMP receptors [82, 87, 89–95]. Moreover, *Drosophila* and *C. elegans* TGF-β receptors can bind human BMPs, allowing larval development. On the other hand, recombinant insect BMP-like protein can induce bone formation in mammals [82, 90, 91, 94, 96–99]. Finally, the TGF-β signalling pathway through activation of specific Smad proteins seems conserved in diverse metazoan phyla in evolution [82, 95, 100–102]. Since the identified invertebrate TGF-β- and TGF-β-receptor-like proteins are closer to mammalian BMP subfamily than to TGF-β subfamily members, they may not be involved in immune responses in invertebrates (fig. 2).

Invertebrate cytokine- and cytokine-receptor-like molecules: a critical assessment?

Our current knowledge of invertebrate cytokine- and cytokine-receptor-like molecules is based mainly on immunocytochemical methods and functional assays. Cross-reactivity based on mammalian monoclonal and polyclonal antibodies to mammalian cytokines or receptors does not prove that the antigen in question is present in invertebrates. Indeed, monoclonal antibodies may cross-react with unrelated proteins, providing ambiguous evidence [103, 104]. Therefore, the observed cross-reactivity of invertebrate cytokine-like molecules with antibodies elicited against mammalian cytokines shows only that molecules from different phyla share epitopes recognised by the antibody used. In the absence of a further molecular characterisation of these molecules, conclusions regarding homology or common evolutionary origin remain presumptive.

Investigations aiming to sequence cDNA coding for invertebrate cytokine-like molecules were initiated more than 10 years ago [11]. However, despite continuing efforts [13, 46], the success of such studies remains limited. Actually, with the exception of TGF-β-like molecules, amino acid or gene sequences of the putative invertebrate cytokine or cytokine receptor molecules are too scarce to claim unequivocally a phylogenetic relation with their vertebrate counterparts. This lack of molecular data is at least surprising in view of the present state of the art techniques allowing cloning and sequencing of genes and proteins. Similarly, despite completion of the *Drosophila* genome sequence, the presence of cytokine receptor or cytokine homologues has not yet been reported.

Although the lack of molecular evidence does not preclude the existence of true homologues of vertebrate cytokines and their receptors, the question of whether the first cytokine arose in invertebrates still remains open. In view of the importance of this question for the scientific community interested in comparative immunology, we want to insist that protein or gene sequences of invertebrate cytokine or cytokine-like molecules be released when available, even if partial and/or unexpected in terms of lack of homology.

Interestingly, up to now the antibodies used for cross-reactivity studies have mainly been antibodies that neutralise the activity of mammalian cytokines by impairing their binding to specific cytokine receptors. This approach is based on the commonly held view that mammalian cytokines exert their activity (uniquely) through interaction with a specific cytokine receptor. As will be discussed in the next paragraphs, evidence is accumulating that this assumption underscores possible modes of actions of cytokines. Indeed, cytokines also exert biological functions in a cytokine-receptor-independent way, which may shed new light on the molecular basis of the functional analogies between vertebrate and invertebrate defence molecules.

Cytokine activities mediated by lectin-like interactions

Mammalian cytokines are usually produced and secreted by various cell types and often exert multiple and sometimes synergistic or antagonistic functions on different target cells (pleiotropy). A particular cytokine exerts its autocrine, paracrine or endocrine activities through binding to specific cell surface receptors. The coordinated regulation of expression of cytokines, as well as their corresponding receptors, underlies the concept of cytokine networks [105, 106].

[1] Yandell M. D., Ross R. M., Suzuki Y. and Wood, W. B. (1997) Characteristics of dbl-1, a *C. elegans* decapentaplegic homologue, support a conserved role for BMP-family signaling in bilaterian development. Unpublished, sequence deposited in GenBank (AF004395).

Many cytokines can also be considered as mammalian lectins. In this regard, IL-1α/β, IL-2 to IL-8, IL-10, IL-12, IFN-γ, TNF, TGF-β1, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to interact with soluble glycosaminoglycans (GAGs) or GAG side chains of proteoglycans expressed on the cell surface of mammalian cells, thereby resulting in modulation of their biological activities [107–115]. For example, binding of cytokines to cell-surface proteoglycans or to the extracellular matrix proteoglycans may provide a local tissue-bound reservoir of cytokines, facilitating their interaction with high-affinity receptors and/or triggering receptor dimerization and biological responses [116]. In turn, soluble GAGs such as heparin or heparan sulfate can modulate the activity of cytokines by competing with membrane receptors or by protecting the cytokines from proteolytic inactivation in the circulation [113–115, 117–126].

Certain cytokines, in particular IL-1, IL-2 and TNF, have also been shown to directly interact with various pathogens through *N,N′*-diacetylchitobiose and Man5/6GlcNac2-R lectin-like interactions [127, 128]. IL-1 binds virulent *Escherichia coli*, acting as a growth factor for the bacteria. Similarly, IL-2 binds *Candida albicans*, and acts as a growth factor for virulent *E. coli* and *Leishmamia mexicana*. In addition, TNF was shown to interact through lectin-like interactions with Gram-negative bacteria or fungal β-1,3 glucans [129, 130]. TNF also exerts a direct lytic activity on bloodstream forms of the protozoan parasite *Trypanosoma brucei* by binding *N,N′*-diacetylchitobiose moieties on parasite surface components [131, 132].

The carbohydrate binding domains of cytokines are spatially distinct from the cytokine-receptor binding sites [133–135]. In the case of TNF, antibodies specific to classical mammalian cytokine receptors do not inhibit the interaction of cytokines with pathogens [127, 131, 132]. Moreover, the binding of many cytokines to GAGs does not interfere with cytokine-cytokine receptor interactions [136, 137]. It has been suggested that the lectin-like domains of cytokines represent pathogen-specific recognition sites that can contribute to the elimination of pathogens via opsonisation and/or leukocyte activation [127, 129, 131, 138, 139].

Recent evidence suggests that cytokines may also contribute to inflammatory reactions in vertebrates through their lectin-like activity. In this regard, TNF was reported to increase the membrane conductance in endothelial cells and peritoneal macrophages [140, 141]. This effect is independent of the TNF receptor since it also occurs in cells isolated from mice deficient in both types of TNF receptors. In contrast, the ion-channel-gating activity of TNF is mediated by the *N,N′*-diacetylchitobiose lectin-like domain of molecule (the TIP domain) located at the tip of the trimeric TNF molecule and spatially distinct from the TNF-receptor binding site [127, 131, 140, 141]. The increased ion permeability can be inhibited by amiloride, an inhibitor of sodium transport, suggesting that the increase in whole-cell current across the plasma membrane of mammalian cells results from the binding of TNF to an endogenous ion-channel transporter or to a protein coupled to ion channels. The TNF-induced change in ion permeability was suggested to be important in the resorption of edema during acute inflammatory responses [142] [R. Lucas et al., personal communication]. Thus, at least in the case of TNF, the lectin-like activity/domain of the cytokine may represent a functionally conserved primitive recognition mechanism, contributing to innate defence mechanisms and inflammatory reactions in vertebrates.

Invertebrate cytokine-like molecules as lectins

Studies from the group of Raftos and our group suggest that cytokine-like molecules in deuterostome and protostome invertebrates and mammalian inflammatory cytokines share similar lectin-like activities. This saccharide-recognition activity may be responsible for the functional analogies evidenced between invertebrate cytokine-like factors and vertebrate counterparts, as discussed below.

Protochordate IL-1 as a functional analogue of IL-1

Raftos [34] showed that tunicate protochordate IL-1 (tunIL-1) and huIL-1 trigger murine fibroblast proliferation, production of IL-2 by human mononuclear cells and expression of IL-2-R on murine EL-4 lymphoma cells. However, tunIL-1 cannot block the binding of anti-IL-1-R (type I and II IL-1-R, CDw 121b) antibodies to EL-4. Moreover, an anti-IL-1-R antibody does not inhibit the capacity of tunIL-1 to stimulate thymocyte proliferation. Therefore, Raftos concluded that tunIL-1 does not exert its IL-1-like activity via a structural homology to mammalian IL-1 and does not stimulate mammalian cells by interacting with the IL-1-R. As an alternative mechanism, the action of tunIL-1 may be related to its galactosyl-specific lectin-like property [143], which that may be responsible for the IL-1-like effect of tunIL-1 on mammalian cells. Mammalian IL-1 has been shown to bind derivatives of β-D-glucose and of $\alpha\beta$-6-deoxy D-galactose (D-fucose) [135]. Hence, tunIL-1 and huIL-1 may not be evolutionarily related, though they share similar biological activities. Instead, the functional analogy between invertebrate and vertebrate IL-1-like molecules may be related to a similar lectin-like activity. It would be interesting to know whether the interaction between huIL-1 and invertebrate immunocytes observed by Beck et al. [46] is also based on saccharide recognition.

Coelomic cytolytic factor as a functional analogue of TNF

For more than 30 years earthworms (*Annelida, Oligochaeta*) have provided a useful model for comparative immunology. Their coelomic fluid exerts a large variety of biological effects, including bacteriostatic, hemolytic, proteolytic and cytolytic activities that are involved in effective defence mechanisms against invaders [144, 145].

We have identified a 42-kDa protein named coelomic cytolytic factor (CCF)[2] from the coelomic fluid of the earthworm *Eisenia foetida* [146]. Upon interaction with cell wall components of Gram-negative bacteria or yeast (the O-antigen of LPS, β-1,3-glucan or *N,N′*-diacetylchitobiose), CCF triggers the activation of the prophenoloxidase (proPO) cascade, an important invertebrate defence mechanism [8]. Moreover, CCF agglutinates smooth but not rough Gram-negative bacteria or Gram-positive bacteria and contributes to the opsonising properties of the coelomic fluid, thereby providing an efficient mechanism for phagocytosis in earthworm defence reactions [16, 146]. CCF is also involved in the cell-mediated cytotoxic reaction in earthworms [147]. More recently, CCF was also shown to potentiate the lytic activity of coelomic fluid on rat, mice and guinea pig red blood cells by interacting with H3 hemolysin [148, 149]. As far as this interaction is concerned, we hypothesise that by binding to saccharide moieties on red blood cells, CCF favours the interaction of hemolysins with phospholipids. Collectively, the pleiotropic activities of CCF indicate that this pattern recognition molecule plays a key role in innate defence mechanisms of *E. foetida* earthworms.

CCF was originally identified in experiments aimed at characterising a novel cytolytic factor from the coelomic fluid of *E. foetida*. We indeed observed that the coelomic fluid is capable of lysing TNF-sensitive tumour L929 cells in a protease-independent way, and subsequent isolation of the lytic proteins led to the identification of a coelomic cytolytic factor, that is CCF [16]. The activity of CCF is not inhibited by anti-TNF neutralising monoclonal antibodies, suggesting that the mechanisms of TNF- and CCF-mediated lysis differ. In addition to TNF-like lytic activity, CCF manifests other similarities with this mammalian cytokine. For instance, CCF is secreted upon LPS stimulation by macrophage-like coelomocytes while TNF is produced by LPS-activated macrophages [147, 150]. Moreover, as mentioned above, CCF contributes to the opsonising properties of earthworm coelomic fluid [16], and TNF has been reported to provide opsonin-like signals that mediate the attachment of bacteria to macrophages [129]. Furthermore, CCF and TNF proteins bind β-1,3-glucans via lectin-like interactions [130, 146]. Finally, it was found that CCF, as the TNF protein, binds immobilized *N,N′*-diacetylchitobiose (β-1,4-*N*-acetylglucosidic link) [15]. The binding of CCF or TNF to β-1,3 glucan is inhibited by *N,N′*-diacetylchitobiose. Conversely the binding of CCF or TNF to *N,N′*-diacetylchitobiose is impaired by β-1,3-glucan. In addition, murine monoclonal antibodies elicited against the TIP lectin-like domain of TNF [132] cross-react with CCF [15]. Finally, a monoclonal antibody elicited against CCF reacts with TNF, without impairing the interaction of TNF with its specific receptor [16, 131, 132]. Together, these data suggest that CCF and TNF protein share similar β-1,3-glucan and *N,N′*-diacetylchitobiose lectin-like activities and domains.

The lectin-like domain of TNF was shown to be involved in the killing of African and American trypanosomes [131, 132, 151]. Hence, in view of the similar lectin-like activity of CCF and TNF, the possible trypanolytic activity of CCF was investigated [15]. We observed that the coelomic fluid of *E. foetida* as well as purified CCF display potent trypanolytic activity that can be inhibited not only by anti-CCF monoclonal antibodies but also by *N,N′*-diacetylchitobiose and anti-TIP TNF antibodies. Conversely, anti-CCF antibodies neutralise TNF-mediated trypanolysis. In addition, using the ability of CCF to trigger the prophenoloxidase cascade upon saccharide recognition in *E. foetida* coelomic fluid, the N-linked *N,N′*-diacetylchitobiose core of the variant-specific glycoprotein (VSG) that acts as a protective coat on bloodstream forms of *T. brucei* was identified as a possible target for CCF on the trypanosome surface during trypanolytic events [15]. Moreover, preincubating VSG with TNF impairs the activation of the proPO cascade, confirming that the interaction with a *N,N′*-diacetylchitobiose carbohydrate moiety on VSG lies at the basis of CCF and TNF trypanolytic activity.

As mentioned above, TNF increases the membrane conductance in mammalian cells, interacting with ion-channels or ion-channel-coupled molecules through its TIP *N,N′*-diacetylchitobiose lectin-like domain [140, 141]. Similarly, when murine endothelial cells or macrophages were activated with CCF, an increase in membrane conductance occurs [A. Bloc et al., in preparation]. As observed with TNF, the ion-gating effect of CCF does not occur in cells from TNF-receptor I and II knockout mice, but is blocked by *N,N′*-diacetylchitobiose or amiloride. Hence, CCF, like TNF, may interact via its *N,N′*-diacetylchitobiose lectin-like domain with ion-channel or ion-channel-coupled molecules on mammalian cells. These results also suggest that intracellular signalling may be conserved within vertebrate and invertebrate cells, in view of the similar activities triggered by CCF and TNF.

Taken together, our data suggest that invertebrate CCF and mammalian TNF share β-1,3-glucan/*N,N′*-diacetylchitobiose lectin-like activities that may have been

[2] Originally described as CCF-1.

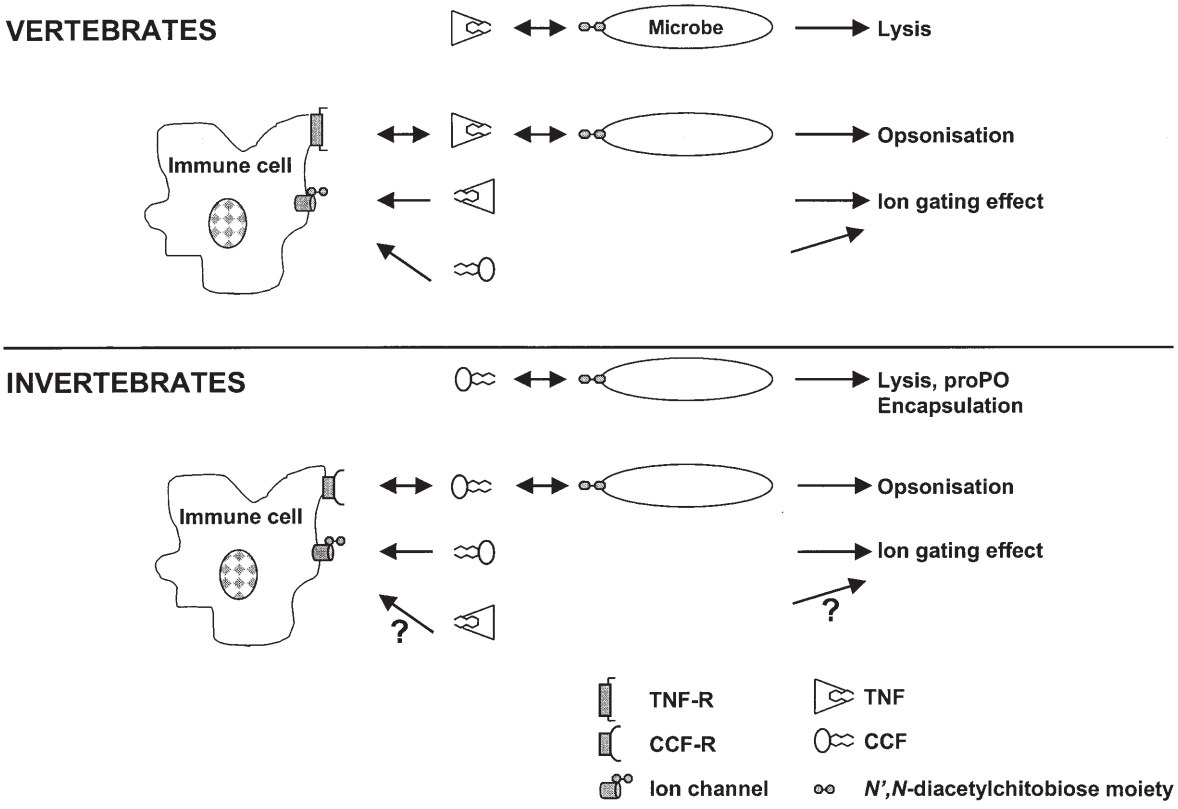

Figure 3. Putative involvement of TNF and CCF in vertebrate and invertebrate innate defence mechanisms.

functionally conserved as a recognition mechanism in innate defence reactions in invertebrates and vertebrates, respectively (fig. 3). Importantly, despite their functional analogies, CCF and TNF do not show gene homology, indicating a lack of common evolutionary origin [15].

Conclusion

One of the challenges in comparative biology is to ascertain to what extent animals have homologous structures that derive from a common ancestor or whether any similarities are due to convergent evolution resulting from the need to perform similar biological functions. The striking superficial similarities between processes taking place in the invertebrates and vertebrates represent a great temptation to consider these two to be homologous, that is to have the same evolutionary origin. Accordingly, investigators studying invertebrate immune response borrowed most if not all terms from vertebrate immunology. In many of these instances, the application of this terminology (and hypotheses about mechanisms of action) for invertebrates is based on functional similarities with vertebrates, mainly mammals and particularly human and mice. However, homology must not be based on functional similarities but on gene or protein sequence similarities. Moreover, to prove homology between a protein in two taxa, the existence of an ancestor gene in the common ancestral group must be proposed [152].

Hughes has compared the gene sequence of nine proteins thought to be involved in innate immunity in both protostomes and deuterostomes [153]. Of the nine, only the phylogeny of lysozymes shows evidence of conservation, but the phylogenies of the eight other proteins (Natural Resistance-Associated Macrophage, Nitric Oxide Synthase, serine protease, serpin, chitinase, hemolin, C-Reactive Protein and Toll) do not. Similarly, phylogenetic analyses of TLR-related genes revealed a separate clustering of Toll and related proteins from insects and mammals, although they share a common ancestor [62, 154]. The closer homology of the invertebrate TGF-β superfamily to the BMP subfamily, as well as the involvement of TLRs such as Toll in invertebrate embryogenesis, suggest in fact that molecules involved in the development of invertebrates have been diversified and recruited by the immune system with the emergence of vertebrates.

Although the comparative analysis of the TLRs and TGF-β-related proteins suggests that intracellular mechanisms based on NF-κB signalling [52, 63, 65, 70, 73] or Smad signalling have been conserved [102, 155], invertebrate and vertebrate immune cells may have adopted them

independently. The suggested independent evolution of proteins of the TLR and TGF-β superfamilies in invertebrates and vertebrates is also consistent with the conclusion of discontinuous evolution of immunity between invertebrate and vertebrates [30, 62, 153, 154]. Families of proteins have thus apparently evolved immune system functions in the vertebrate and invertebrate phyla. Interestingly, the discontinuity between invertebrate and vertebrate immune system involves several proteins expressed in macrophages. Since phagocytic cells have long been thought to provide a probable evolutionary link between the vertebrate and invertebrate immune systems [14, 21], the possibility that many mechanisms of macrophages have evolved independently in different animal phyla cannot be excluded.

In conclusion, in view of the little amino acid or gene sequence information available, it remains premature to propose that the invertebrate cytokine or cytokine-receptor analogues described share a phylogenetic relation with their vertebrate counterparts. Hence, the question whether the genes coding for the first cytokines and cytokine receptors originated in invertebrates remains. On the other hand, cumulative evidence suggests that cells of the immune system may also be regulated through lectin-carbohydrate interactions (selectins, pattern recognition molecules and so on). In fact, the functional analogies between cytokines and their presumed invertebrate counterparts may actually not reflect homology, but rather result from molecular convergence [156] based on three-dimensional structural similarity of the lectin-like recognition domain. This is at least suggested by the work of Raftos on the protochordate IL-1-like molecule and our comparative analysis of CCF and TNF [15, 16, 34]. Thus, invertebrate defence molecules and mammalian cytokines may display a similar lectin-like activity that has been functionally conserved as a recognition mechanism in innate immunity in invertebrates and vertebrates, respectively [15]. In fact, various cytokines possess lectin-like properties that may be involved in the regulation of innate immune responses in vertebrates (recognition of microbes, antiinflammatory processes, others) independent of binding to their cytokine-specific receptor [127, 129, 131]. This may indicate that we are currently underscoring the biological relevance of lectin-like interactions in the activity of vertebrate cytokines.

Despite all the progress, no definitive proof of any homology between invertebrate and vertebrate immunity has been reported. In fact, it may very well be that invertebrates use different molecules for the same purpose as vertebrates. This does not mean that homology between the vertebrate and invertebrate immune systems does not exist. The question whether the immune system arises by analogy from various origins, by homology through diversification from a common ancestor or by a combination of both remains open.

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