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MACROKINES: INVERTEBRATE CYTOKINE-LIKE MOLECULES?

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1. ABSTRACT

My laboratory is applying contemporary techniques of biochemistry and molecular biology to an important emerging field in biomedicine: the evolution of the immune system. Our investigations will build upon the discoveries that key immunoregulatory molecules (*i.e.*, cytokines), that function in the mammalian immune response appear to be present in phylogenetically distinct invertebrate species. Herein, we propose the term macrokine to describe invertebrate proteins that have vertebrate cytokine-like activities. The wide distribution of such basic elements of innate host defense responses demonstrates its antiquity in animal evolution. Through these studies we hope to identify the more ancient facets of the vertebrate innate immune response. In turn, these observations may help clarify host defense functions and responses not yet appreciated in the vertebrate immune system.

2. INTRODUCTION

In this article we journey through the animal kingdom to trace the development of inflammatory mediators of the innate immune system. As it turns out, it is also a journey back in time, since most of the animals studied have remained virtually unchanged for millions of years. Imprecise words such as "*primitive* and "*lower* are used deliberately throughout because of our ignorance about the molecular details of invertebrate host defense systems. That invertebrates make up greater than 90% of all species on earth attest to the efficiency of their "primitive" host defense systems (1-4).

3. INVERTEBRATE HOST DEFENSE CONCEPTS

Most immunologists would agree that the mammalian immune system is the most advanced host defense system (1-4). There are two basic mechanisms to this system, natural (innate) and specific (acquired) immunity (2). The innate system is the more ancient mechanism and relies on cells (*i.e.*, phagocytes) and blood-borne molecules (*i.e.*, complement) (1, 2). Acquired immunity relies on specific populations of cells (*i.e.*, lymphocytes) and more advanced blood-based molecules (*i.e.*, antibodies) (1, 2). In general, acquired immunity uses numerous specific cells and molecules that function cooperatively. It uses all aspects of innate immunity but also has two important twists: it "remembers" each encounter with an invader and it focuses the mechanisms of innate immunity to eliminate the invader more efficiently. All animals possess innate mechanisms while elements of acquired immunity can be found scattered through lower vertebrates and higher invertebrate animals (1, 2). It will become apparent that the evolutionary continuity of animal defense systems is quite remarkable.

Host defense mechanisms have been evolving since the first organisms encountered one another hundreds of millions of years ago. The distinction between "self" and "nonself" is achieved by even the most primitive protozoans (1-4). Vertebrates deal with detection and elimination of foreign objects by a multifarious immunoglobulin-based system (2, 3). While the evolutionary precursor of the vertebrate humoral response, immunoglobulin, has not yet been found in invertebrates (3), certain species do have molecules that may represent ancient immunoglobulin (4). Lower animals also lack the cell surface specific immune recognition molecules of vertebrates (membrane Ig, receptors for MHC antigens, and processed antigen receptors) (1, 4). Invertebrates have many of the innate mechanisms vertebrates do, but lack the specificity of the vertebrate immune response (1, 3, 4). These animals are therefore useful in studies of nonspecific host defense mechanisms. In addition, significant contributions have been published on the host defense system of insects and other arthropods, clearly indicating unique features of the insect response (5-9).

An important feature of invertebrate host defenses is the cellular response. The high capacity of invertebrate coelomic fluid or hemolymph cells to phagocytose, entrap and encapsulate invading organisms has long been recognized. Phagocytosis, a major mechanism for nutrient acquisition in lower organisms, is used chiefly to eliminate debris and foreign objects in higher organisms. Invertebrate phagocytes have been found to ingest numerous microorganisms including viruses, bacteria, protozoa, and fungi (7). The detailed mechanisms involved in recognition of foreign objects, chemotaxis towards them, and attachment of phagocytes to them, are yet to be delineated (7, 8). Nodule formation is another cell mediated reaction. The cellular details of this process in insects have been described by several investigators (8-10). It is initiated

by degranulation of granular cells in response to the presence of bacteria in the coelomic fluid or hemolymph. The entrapped bacteria, discharged cellular materials, and disintegrating granular cells are compacted and provoke the cellular encapsulation reactions. Formed capsule consists of a layer of apparently normal hemocytes and a middle layer of extremely flattened cells with clear sites of melanization (7).

In insects the cuticle forms the first line of defense against invasion by foreign objects. In addition, it may be active on other levels in that abrasion of the epicuticle of *Bombyx mori* and *Hyalophora cecropia* in the presence of live bacteria or bacterial cell wall components results in the detection of cecropin mRNA in the underlying epithelial cells. But, fat body cells which are remotely located from the abraded cuticle are activated as well (9). This suggests the action of a soluble mediator(s) [cytokine-like molecules? (see below)].

Finally, humoral-based reactions form an integral part of the host defense response of many invertebrates. Factors such as lectins are normal constituents of invertebrate body fluids, while other mediators are synthesized in response to infection. Lectins have been identified in several genera of invertebrates and play a role in agglutinating invading microorganisms that carry appropriate sugar moieties on their surface (4). The complexes formed are susceptible to either phagocytosis or encapsulation (7).

Apart from these constitutively present components, a number of factors have been reported to be produced in response to infection. Lectins themselves are known to be inducible by injury and infection (11-13). Boman's group, and more recently Hoffmann's group, has carried out extensive studies on inducible proteins and have characterized a number of these antibacterial proteins (6, 14). These include cecropins as well as attacins. Cecropins are small basic proteins with molecular weights (M_r) of about 4,000 with highly hydrophobic regions, while attacins are relatively larger (M_r 20,000) proteins. Amino acid sequences, as well as DNA sequences, coding for these proteins in *Hyalophora cecropia,* and other species, have been reported (6, 14, 15). The most widespread inducible antibacterial protein isolated from invertebrates (also the first) is lysozyme (15, 16). Invertebrates produce lysozyme upon infection or when exposed to bacterial products (*e.g*., peptidoglycan, LPS) (15, 16). In humans, lysozyme is not inducible, but, is rather an innate defense mechanism. Lysozyme is one of the first defense molecules encountered in the oral cavity where it is a major constituent of saliva. (2, 16).

Consideration of the parallels between nonspecific host defenses in invertebrates and vertebrates led us to test the hypothesis that invertebrates possess similar soluble mediators that regulate responses to infection or wounding. Cytokines are inducible polypeptide mediators released by a variety of activated immune and nonimmune cells in response to pathogens and inflammatory conditions (17-18). These molecules have critical effects on cells of the immune system and they exhibit hormone-like properties that affect numerous organ systems involved in host defense (17). Cytokines include the interferons, the interleukins (to date 19 have been described), and tumor necrosis factor (TNF). Interleukin (IL)-1, IL-6 and TNF are cytokines that are critical immune regulators that participate in every aspect of the vertebrate immune system (18-20).

3.1. Interleukin 1

Biochemical characterization of IL-1 from a number of vertebrate species reveals basic similarities in the structure and properties of this cytokine. Human and murine IL-1 have a M_r of approximately 17,000 and 2 major charged forms, one has a pI of 7.0 (referred to as beta) while the other has a pI of 5.0 (referred to as alpha) (21-23). In humans the predominant form is beta, while in mice it is alpha (24). In other animal species (rabbit, pig, rat, cow) the M_r is usually in the 12,000-18,000 range although large M_r forms of between 35,000-70,000 have been found in all species studied (22, 24). Isoelectric focusing studies show pI values in the 5.0-5.6 and 6.8-7.5 ranges (21-24).

Lomedico *et al.* (25) were the first to clone a vertebrate IL-1 molecule (murine alpha). They found that their cDNA encoded a polypeptide precursor of 270 amino acids that had a M_r of 33,000 (25). It is thought that this precursor was posttranslationally modified either inside or outside the cell to yield the secreted 14,500 M_r form (25). Auron *et al*. isolated a cDNA for human IL-1-beta (26). The cDNA encoded a precursor polypeptide of 269 amino acids that had a M_r of 30,747. This precursor was also processed into an active, secreted 17,500 M_r protein (26). Soon thereafter, March *et al.* reported of the isolation of cDNAs for human IL-1-beta, as well as for the alpha form (27). Their cDNA for the beta protein was essentially the one described by Auron. The other cDNA, which was 271 amino acids long, encoded the alpha protein. The cDNA's for rabbit alpha and murine and bovine alpha and beta have been described (28-30). When the amino acid sequences were analyzed for similarity, the results were puzzling. Bovine IL-1-alpha was 73%, 62%, and 71% homologous with human, murine, and rabbit IL-1-alpha sequences, respectively (30). Bovine IL-1-beta was 62% homologous with human beta and 59% with murine beta (30). But, when the sequences of human alpha and beta were compared they shared only 23% homology (27). The same small degree of homology was true for murine (22%) (31) and bovine (23%) (30) sequences. What was most confusing is that both molecules mediate the same biological functions and bind with equal affinity to the IL-1 receptors (32, 33). These results imply that there are basic structural similarities between the proteins not immediately revealed by their amino acid sequences (34). Another member of the family is the IL-1 receptor antagonist (ra). The IL-1-ra binds to the IL-1 receptor and blocks the binding of both IL-1-alpha and beta without inducing a signal of its own (35). The ra has significant sequence homology to beta but even less with alpha (36). The human IL-1 genes have been localized to chromosome 2 (37). No signal peptide has been found or encoded in the DNA sequence of the alpha or beta protein (38). Recently a new member of the IL-1 family, IL-1-gamma, has been proposed (39).

3.2. Interleukin 6

Interleukin 6 (IL-6) is another macrophage derived cytokine that is responsible for mediation of nonspecific host defenses. IL-6 exhibits multiple biologic activities on different target cells. Activation of T cells and thymocytes, induction of acute phase proteins, stimulation of hematopoietic precursor cell growth and differentiation, pyrogenic activity, and antiviral activity, are only a few of its host defense related activities (40, 41). The calculated M_r of the mature IL-6 polypeptide is 20,781 (42). The intracellular protein has a 28 amino acid signal peptide that is required for secretion. Two potential *N*-glycosylation sites are present on the molecule (43). Comparison of the cDNA sequence of human and murine IL-6 shows a homology of 65% at the DNA level and 42% at the protein level (44). The positions of four cysteine residues are completely conserved, suggesting that the cysteine-rich middle region of the protein plays a critical role in IL-6 activity (42). Comparison of the sequence with G-CSF

reveals a significant homology in this limited region (42). The positions of the four cysteine residues are precisely matched. In addition, there is similarity in genomic organization. This suggests to some investigators that these cytokines may be derived from a common ancestral gene (42). The human IL-6 gene maps to chromosome 7 (44), while murine IL-6 is found on chromosome 5 (45).

The receptor for IL-6 is a member of the Ig superfamily, as is the receptor for IL-1 (46, 47). The receptors for these molecules will undoubtedly also be present in invertebrates and their study may shed light on vertebrate cytokine-receptor interactions. Other members of this superfamily have been identified in the invertebrates (48), but as yet no rearranging Ig-like molecules have been found (4).

Many of the activities of IL-1 and IL-6, overlap. These include pyrogenicity, tumor cell cytotoxicity, acute phase protein release, and protection of mice against lethal irradiation (49, 50). All these properties are characteristic of nonspecific resistance to infection. Another interesting property they share is the ability of IL-1 or TNF to induce the production of each other as well as IL-6 (49, 50) and thus, to participation in a cytokine network. IL-6 cannot induce IL-1 or TNF production (50).

3.3. Our Previous Studies of Invertebrate Cytokine-like Molecules

Studies from our laboratory, dealing with the evolution of the immune response, have focused on the isolation and characterization of putative "cytokine-like" molecules from invertebrates (19, 20, 51, 52). We have hypothesized that invertebrates possess cytokine-like molecules that regulate responses to infection or wounding.

To stress this hypothesis we decided to employ mammalian IL-1 bioassays to detect invertebrate IL-1-like molecules. We reasoned that IL-1-like molecules or similar ancestral cytokine-like molecules are likely to be present in invertebrates for several reasons. First, IL-1, and its companion inflammatory cytokines IL-6 and TNF, are molecules that regulate specific, and more important, nonspecific aspects of the vertebrate response (*e.g.*, acute phase protein synthesis, antiviral activity, neutrophil recruitment, *etc.*). Second, the molecular conservation of IL-1 and its host defense functions have been preserved in vertebrates. Finally, those cells that produce IL-1, *i.e.*, macrophages, are present throughout the animal kingdom. We chose echinoderms and urochordates for our studies because they are the most advanced invertebrates and they belong to the deuterostome group of animals, the ancestral branch for the vertebrates.

We began our search for an IL-1-like molecule by testing coelomic fluid from the common Atlantic starfish *Asterias forbesi* for IL-1-like bioactivity. We isolated coelomic fluid, fractionated it and isolated a protein with IL-1-like bioactivity (51, 52). The echinoderm IL-1-like molecule was active in stimulating several vertebrate IL-1-responsive cells. The physicochemical and biological activities of the echinoderm IL-1-like molecule are the same as those of mammalian IL-1 as measured in mammalian systems. Specifically, the invertebrate IL-1-like molecule has a M_r of 18,000 daltons and two major charged forms (pI 7.2 and pI 5.0) (52), similar to vertebrate IL-1. In addition, a polyclonal antiserum specific for human IL-1 was capable of neutralizing the activities of the echinoderm IL-1-like molecule.

We have shown that tunicates also possess IL-1-like bioactivity (53). Blood or whole organism extracts from 8 different species contained IL-1-like activity (with similar M_r and pI to vertebrate IL-1 and echinoderm IL-1-like molecules) and an antibody to human IL-1 inhibited tunicate IL-1-like activity just as effectively as human IL-1. We have found IL-1-like activity in protostome invertebrates (insects) as well. The insect IL-1-like molecule is similar to vertebrate IL-1, and the tunicate, and echinoderm IL-1-like molecules.

A significant aspect of our rationale for searching for inflammatory cytokines in invertebrates is the assumption that these molecules will subserve similar functions in an invertebrate host. To test this idea we have investigated the biological activities of the invertebrate IL-1-like molecule from both starfish and tunicates. We envision a central role for cytokine-like molecules in orchestrating the host defenses of invertebrates.

Coelomocytes are the invertebrate correlates of vertebrate leukocytes, specifically the macrophage and are, therefore, likely to be a major source of the echinoderm IL-1-like molecule. Echinoderm coelomocytes can be stimulated with bacterial products (*e.g.*, LPS) and other substances (*e.g.*, silica) to produce the IL-1-like molecule as has been shown with mammalian macrophages (24). The ability to stimulate secretion is consistent with the adaptive functions of the invertebrate IL-1-like molecule including regulation of inflammatory reactions in echinoderms and tunicates.

We have tested the purified echinoderm IL-1-like molecule for its ability to stimulate proliferation of coelomocytes. We incubated coelomocytes with the echinoderm IL-1-like molecule under conditions of temperature and osmolarity approximating those encountered in the ocean and found that it stimulated coelomocyte proliferation over the course of a week (54). That phagocytes proliferate in response to the invertebrate IL-1-like molecule is consistent with its role as a major regulatory molecule active in host defense in these animals.

Phagocytosis is for now the most convincing and apparently predominant host defense mechanism in invertebrates ranging from single celled ameba to highly complex tunicates (1-4). Homologies between vertebrate and invertebrate host defenses extend to the recognition events that precede phagocytosis of foreign particles or microorganisms (1-4). Invertebrate phagocytes may rely on a variety of lectins to help in eliminating any invading component. The invertebrate IL-1-like molecule was tested for its ability to opsonize foreign particles. We found that pretreatment of yeast cell wall products (but not latex beads) with the tunicate IL-1-like molecule stimulated phagocytosis by amebocytes (55). Similarly, the echinoderm IL-1-like molecule acted as an opsonin for phagocytosis by starfish coelomocytes. We tested vertebrate IL-1 for opsonic activity but were unable to detect any (55).

Opsonization is, however, not the only activity of IL-1 related to phagocytosis. Phagocytosis by both echinoderm and tunicate amebocytes is stimulated by invertebrate IL-1-like molecules (55). This activation is distinct from opsonization. When the tunicate IL-1-like molecule is incubated directly with amebocytes, phagocytic activity toward both yeast and latex is enhanced. Since latex cannot be opsonized by the tunicate IL-1-like molecule, its enhanced ingestion most probably results from a general activation of phagocytic cells. Moreover, enhanced phagocytosis of unopsonized yeast or latex is maintained after the removal of the tunicate IL-1-like molecule from activated phagocytes, so that opsonization could not have occurred before phagocytic ingestion.

Table 1. Several names, any of which could be used, to describe invertebrate cytokine-like molecules

CELL TYPE	DESCRIBED IN INVERTEBRATE	NAME BASED ON CELL TYPE
Phagocyte	All	Phagokine
Amebocyte	All	Amebokine
Coelomocyte	All	Coelomokine
Leukocyte	All	Leukokine
Macrophage*	All	Macrokine
Immunocyte	All	Immunokine

*cell as described by Metchnikoff (59, and see text)

The human IL-1 receptor is a member of the Ig-superfamily (as is the receptor for IL-6) (46). Since members of this family have been found in invertebrates and comparison of the vertebrate and invertebrate receptors would be invaluable in the study of the evolution of IL-1, we searched for an invertebrate IL-1-like molecule receptor. We recently identified a high affinity binding protein on the surface of echinoderm coelomocytes. Each cell had approximately 6,000 binding sites. Binding was inhibited by recombinant human IL-1 and purified echinoderm IL-1-like molecules, but not by unrelated vertebrate recombinant cytokines thus demonstrating the specificity of the receptor.

A TNF-like molecule exists in invertebrates that is active in a cytotoxicity assay for mammalian TNF (19, 20). Most recently, we have shown that echinoderm coelomic fluids contain an IL-6-like protein (56). This activity was present in a protein with physicochemical properties similar to those of vertebrate IL-6 and was neutralized by a polyclonal antiserum directed against recombinant human IL-6. Thus, invertebrates possess correlates of the three major vertebrate inflammatory cytokines.

As of now there are no sequences of the invertebrate cytokine-like molecules, or their genes, and all that is known about them is based on functional assays and similarities at the physicochemical level (57). This may change based on some of our most recent data (see below). Until they have been sequenced fully, characterized at the molecular level, and compared to vertebrate cytokines the question arises as to what to call these "putative" invertebrate cytokine-like molecules. Originally the vertebrate molecules (antigen-nonspecific T cell proliferation and helper factors) were called monokines and lymphokines, denoting activity isolated from either monocytes or lymphocytes, respectively (21-24). Chadwick and Aston (58) have proposed the term hemokine based on cytokine-like molecules produced by insect hemocytes. This would be a suitable name if these molecules were only found in insects. Since there are numerous cell types in invertebrates, a more generalized term based on any invertebrate phagocytic cell, we feel, would offer a more universal appeal. We suggest the phagocyte since it is similar to the vertebrate monocyte/macrophage (the cells from which cytokines were first characterized) and it has been implicated as the cell(s) responsible for the release of these mediators in all the studies reported so far. Several alternatives are suggested in table 1. We are particularly fond of macrokine since it would relate to the original identification of macrophages [as large phagocytic cells (as opposed to the smaller, microphage) found in invertebrates] as observed and described first by Metchnikoff (59). We feel, in describing specific activities similar to the vertebrate molecules, IL-X-like or TNF-like could still be used. It will be up to the scientific community if macrokine would be a useful word for describing inflammatory mediator activities in invertebrates, similar to the activities of cytokines in the vertebrate innate host defense system.

3.4. Macrokine molecules and receptors in other lower species

Molecules with macrokine properties have been described from a variety of invertebrate species (reviewed in 19 and 20). In general they are released by phagocytes and act on various aspects of the inflammatory response. Chain and Anderson isolated a factor from the waxmoth, *Galleria mellonella* which they call "plasmatocyte depletion factor." This protein, released by hemocytes after activation, induced the disappearance of plasmatocytes from circulation (60). Cherbas has described another mediator called haemokinin, a protein isolated from larvae of several moths. Haemokinin acts on hemocytes to cause them to become activated (61). Ratner and Vinson have characterized a protein, "encapsulation-promoting factor" (EPF), also from the hemolymph of insects (*Heliothis* sp.). EPF is released by hemocytes and appeared to be involved in stimulating these cells to start or join the encapsulation reaction (62). "Phagocytosis-stimulating mediator" has been isolated from the hemolymph of *G. mellonella* by Mohrig and Schittek. As its name implies this protein stimulated phagocytosis within minutes of exposure (63). Sigel and colleagues have isolated a factor from the tunicate *Ecteinascidia turbinata* that was found to act on mammalian macrophages and lymphocytes (64). Prendergast and colleagues isolated a protein from starfish (sea star factor) that inhibited vertebrate immune responses to T-dependent antigens and inhibited macrophage migration among other effects (65).

Evidence for IL-1-like molecules in invertebrates have been reported by several laboratories. A snail (*Biomphalaria glabrata*) IL-1-like activity has been reported by Granath and coworkers (66). Recently, the same group (67) reported that human IL-1 stimulates phagocytosis and superoxide production by *B. glabrata* hemocytes. Ottaviani and coworkers (68) have demonstrated the presence of several macrokine molecules (IL-1, IL-2, IL-6 and TNF) in several freshwater snails (*Planorbarius corneus* and *Viviparus ater*). Burke and Watkins (69) showed that human IL-1 stimulates starfish (*Pisaster ochraceus*) coelomocyte phagocytosis and that an IL-1-like factor could be detected in coelomic fluids. Iizuka *et al.* (70) have characterized several tunicate (*Halocynthia rortezi*) growth factors that are capable of stimulating the proliferation of mouse thymocytes. Pestarino *et al.* (71) have localized IL-1-beta mRNA in the cerebral ganglion of the tunicate, *Styela plicata*.

Several groups have identified a TNF-like activity in invertebrates. Müller and colleagues (72) have shown that xenografts between two sponge species (*Geodia cydonium* and *G. rovinjensis*) contain a TNF-like molecule. While, Dissous and colleagues (73) have characterized an immunoreactive TNF-like molecule in *B. glabrata*. Stefano and colleagues have detected, by immunoreactivity, several macrokines (TNF, IL-1, and IL-6) in a mollusk (*Mytilus edulis*) (74, 75). Sawada and colleagues have shown that *Aplysia kurodai* neurons respond to human TNF and IL-1 (76). Bilej *et al.* (77) have identified an earthworm (*Eisenia foetida*) cytolytic protein that may be a primitive cytokinelike molecule. Chadwick and Aston (58) have isolated a lepidopteran (*G. mellonella*) cytotoxic molecule (*Gal*lysin 2) that may be an analogue of TNF.

Studies by Hoffmann and colleagues (78, 79) have shown a sequence homologous to an interferon consensus response element in the diptericin promoter of *Drosophila*.

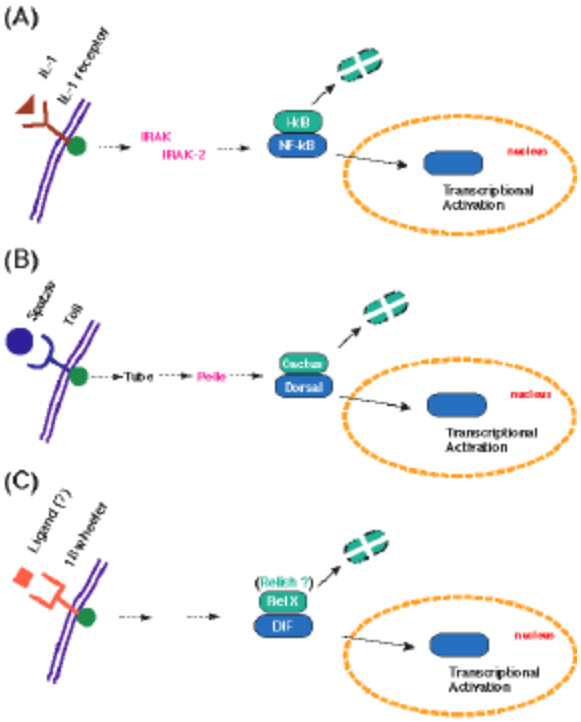

Figure 1. Model of three signalling pathways leading to the translocation of Rel proteins to the nucleus. The three systems share several common components. The cytoplasmic domain of the receptors (IL-1 receptor, Toll, 18-wheeler;,) are highly conserved. The receptor-associated kinases (IRAK, IRAK-2, and pelle;) are highly conserved, as are the Rel proteins [IkB, cactus, and relish; and NFkB, dorsal, and DIF (and CIF)]; , respectively). (A), mammalian immune response; (B and C), *Drosophila* activation of nonspecific host defense responses. Dashed arrows indicate unknowns. Similar signalling pathways have been described in plants as well (93, 95). (Adapted from references 84, 111 and 112 and see text for further details and abbreviations).

This suggests that interferon-related molecules may be present in insects and are part of an insect host defense response. They have demonstrated that the gene for the *Drosophila* defense peptide, diptericin, has upstream sequences with putative transcription regulatory sequences identical or similar to cytokine consensus motifs present in promoters of genes encoding acute phase proteins in mammals.

Transcription factors that were first discovered in vertebrate immune cells have been found to be part of insect host defense responses as well (80, 81). The *Drosophila* transmembrane protein Toll is involved in determining the asymmetry of the dorsal–ventral pattern of the *Drosophila* embryo (82). The Toll protein is related to the human IL-1 receptor (83) as is another *Drosophila* protein that seems to be involved in insect host defenses, 18 wheeler (84). Hultmark and colleagues (85) have recently shown that Toll can activate a *Cecropin A1-lacZ* reporter gene construct in a *Drosophila* hemocyte cell line, thereby mimicking an immune response. It is likely that Toll, 18 wheeler, and the IL-1 receptor transmit their signals by similar mechanisms because both Toll and the IL-1 receptor are involved in controlling nuclear localization of transcription factors, the dorsal protein in the case of Toll and NF-kB in the case of the IL-1 receptor (86, 87). Both dorsal and NF-kB are members of the Rel family of transcription factors (88). Furthermore, both inactive dorsal and NF-kB are bound to cytoplasmic regulators of Rel transcription factors (cactus and I-kB, respectively) (88, 89). In mammals these factors play a central role in the transcriptional regulation of immune-related genes. A new dorsal related transcription factor, Dif (Dorsal-related immunity factor) has been isolated from *Drosophila* (81). Dorsal-related immunity factor is a nuclear transcription factor that is induced and accumulates rapidly in the nucleus during infection or wounding (81). It is a sequence-specific *trans*-activator of *Drosophila Cecropin* gene expression (91). Dorsal-related immunity factor is related to another insect transcription factor, Cecropia immunoresponsive factor (CIF) (92). These data are summarized in figure 1. A report by Whitman and colleagues (93) suggests that plants may use a Toll-IL-1-like pathway for host defense responses as well. They found that the tobacco mosaic virus (TMV) resistance gene *N* is similar to Toll and the IL-1 receptor, and can confer resistance to TMV susceptible tobacco. A human homolog of the *Drosophila* Toll protein has been identified (94). These results suggest that not only do vertebrates and invertebrates share common host defense related proteins, but the machinery of gene expression and regulation may be shared by both animals and plants (95)! This would suggest that these proteins have a longer evolutionary history than previously thought.

There have been several studies demonstrating cytokine-like molecule receptors in invertebrates. A genomic southern blot with DNA of different species (zoo blot) probed at reduced stringency with cDNA probes of both the type I and type II IL-1 receptors showed strong hybridization to both *C. elegans* and *D. melanogaster* DNA (96). These results suggest that other invertebrates possess molecules similar to the human IL-1 receptors. Other growth factor receptors have been demonstrated in invertebrates as well. A fibroblast growth factor binding protein has been characterized in *Drosophila melanogaster* (97), and a heparin-binding growth factor receptor was identified in *Nereis diversicolor* (98).

3.5. Molecular evolution studies of vertebrate cytokines and their possible relationship to identification of macrokines

The only true way to identify invertebrate cytokine-like molecules is through their isolation and characterization at a molecular level. Studies using vertebrate molecules should help to identify the putative invertebrate molecules. Although having very low sequence homology it appears that vertebrate IL-1-alpha, beta, and the ra evolved from a common ancestral molecule (35). They all share similar exon-intron junction number and position. IL-1-alpha and beta both consist of 7 exons coding for similar regions of the molecules (35). IL-1-ra's second, third and fourth exons align with alpha and beta's fifth, sixth and seventh exons (35, 99-101). The crystal structure for human IL-1-alpha (99), beta (100), and ra (101) have been determined. In general, the overall structure is composed of 12 beta strands. Six of these form a barrel which is closed at one end by the other 6 strands. The structure can best be thought of as a tetrahedron with 3 antiparallel beta-strands forming each triangular face [figure 2 and (100)]. Although they are structurally similar, only alpha and beta are biologically active. Indeed, despite the similarity in size, biological characteristics, and receptor binding IL-1-alpha and beta possess only 23% amino acid sequence identity (26, 27).

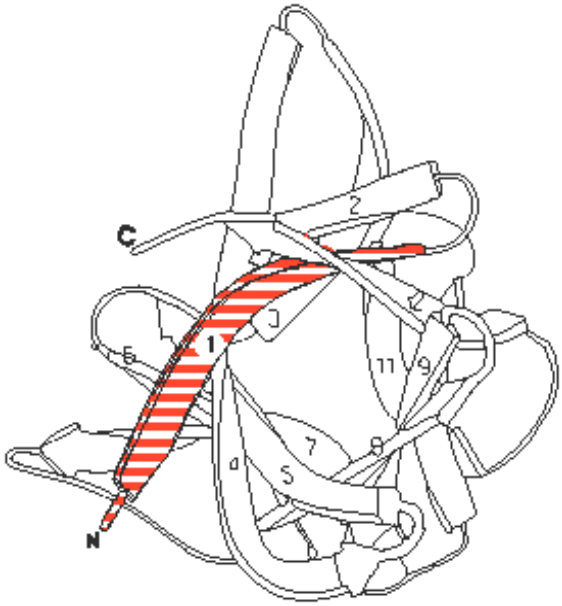

Figure 2. Stereo cartoon of human IL-1-beta. The twisted arrows represent β-strands. The view is down the barrel formed by 6 of the beta-strands (100). In lower vertebrates (trout), beta strand 1 (shaded) appears to be missing (108). (Adapted from references 100 and 108 and see text for further details).

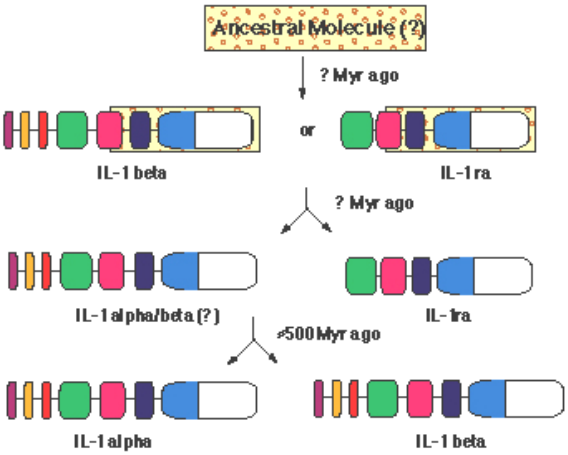

Figure 3. Possible evolution of the vertebrate IL-1 gene family. The hatched area is the hypothesized original ancestral gene segment. The estimation of time of divergence is based on biochemical data (identification of a beta- and alpha-like molecule), functional assays, and appearance of echinoderms in the fossil record not by the mutation rates of the genes (19, 20, 69-71). The colored blocks correspond to exons, while the white blocks are untranslated regions. (Adapted from references 35 and 36 and see text for further details).

The molecular data of Eisenberg *et al*. clearly indicate that the IL-1-ra and IL-1-beta are more homologous to each other than either of them is to IL-1-alpha (35). They also reported that based on their comparison of the human and mouse genes, IL-1-alpha is evolving over twice as fast as IL-1-beta and the IL-1-ra. They attribute this difference to a difference in the mutation rate. They gave two hypotheses for the origin of the IL-1 family: the IL-1-ra gene evolved from an ancestral IL-1-alpha/beta gene, or the ancestral IL-1-alpha/beta genes evolved from an IL-1-ra-like gene. These data are pictured in figure 3. Other studies have shown that the IL-1-alpha family appears to be evolving faster than the IL-1-beta family, which implies that IL-1-beta is closer to the common ancestral protein and perhaps retains more of its properties (102). It has also been suggested that IL-1-ra evolved from a duplicated IL-1-beta gene (36).

Several groups have mapped IL-1 to determine functional areas. To localize the active regions of IL-1, Mosley *et al.* (103) constructed a series of truncated forms of human IL-1 by removing amino acids from both the amino-terminal and carboxy-terminal ends. They demonstrated that core sequences of 147 amino acids for IL-1-beta (120-266) and 140 amino acids for IL-1-alpha (128-267) must be left intact to retain full biological activity and that the biological activities of the IL-1 polypeptides parallel their receptor binding capabilities. Auron *et al.* (36) showed regional homology between human IL-1-beta and murine IL-1-alpha. This homology is strongest within the carboxy halves of the molecules [in regions they labeled as C (amino acids 150-162), D (165-185), and E (219-240)]. Using surface plasmon resonance mapping, D'Ettorree *et al.* (104), characterized the IL-1-beta molecular domains relevant to receptor binding and biological activity. They found several discrete areas of the molecule that were necessary for full biological activity. For our purposes two areas are important: The amino acid stretches 177-186 and 218-273 are critical for binding to the IL-1 receptor. These regions correspond to beta strands 5 and 6 and beta strands 8 and 9, respectively.

Comparative molecular studies based on vertebrate IL-6 sequence homologies should aid in the identification of an invertebrate IL-6-like molecule as well. The crystal structure for IL-6 has only recently been solved (105). It is a member of the 4 helix bundle cytokine superfamily (105). This crystal data and site-directed mutagenesis studies mapping biologic activity and receptor binding domains of several vertebrate molecules will help as well. Ehlers, *et al.* (106) have identified a conserved region (region 2) of IL-6 that is important for receptor binding and biologic activity. Several subregions, important for full biologic activity of the molecule, have also been mapped. Region 2a is near the end of the A helix, region 2b is a central part of small helix E, and region 2c is in the A/B helix joining loop. In addition, a third area, site 3 (107) is a highly conserved region that is critical for receptor binding.

More recently, studies of lower vertebrate cytokines have resulted in some unexpected findings. Secombes' group (108 and personal communication) has found that the trout IL-1-beta molecule is missing the first beta strand (see figure 2). This implies that the molecule could be ra-like and not an agonist, but this can not be since the molecule is biologically active (108). What it does suggest is that PCR studies using primers based on nucleotides located in the N-terminal region may be ineffective in amplifying any IL-1 sequences from lower vertebrates.

Other odd findings concerning lower vertebrate cytokines have been reported. Several groups have attempted to clone chicken IL-2 for over 7 years. Sundick and Gill-Dixon (109) finally had to rely on an expression vector system and a complex biological activity assay to succeed. When they finally cloned the molecule the reason for the difficulty was revealed. The chicken IL-2 protein is very similar to both mammalian IL-2 and IL-15. Similarly,

Table 2. Percentage nucleotide identities calculated using pair-wise alignment of a PCR amplicon and several vertebrate IL-1-beta, -alpha, and -ra molecules

IL-1 b/a/ra	Sheep	Rat	Rabbit	Mouse	Human	Cow	Manduca sexta (?)
Sheep	100%						
Rat	64*/34/–	100%					
Rabbit	69/38/–	62/67/–	100%				
Mouse	65/35/–	87/88/–	60/66/57	100%			
Human	70/38/–	56/66/–	60/74/68	57/67/53	100%		
Cow	90/87/–	90/37/–	49/39/–	48/37/–	62/37/–	100%	
M. sexta (?)	40/25/–	38/24/–	36/26/39	37/23/41	39/26/39	38/25/–	100%

*Values are based on sequences presented in 25-29, and 34-36

Dixon *et al*. (110) were forced to pick random sequences from a trout cDNA library in order to find a beta chemokine-like cDNA. These studies, as well as our own, attest to the time consuming and difficult nature of these vertebrate-invertebrate cloning strategies.

3.6. Our most recent molecular data

Based on information obtained by the structural/molecular data reported above we feel confident in our endeavors to clone macrokine molecules. In fact, we recently sequenced a PCR amplicon that we isolated using mRNA from the insect *Manduca sexta* and primers based on shared vertebrate IL-1 protein sequences (as discussed above). We aligned the *Manduca* nucleotide sequence with several vertebrate IL-1 nucleotide sequences using ClustalW (table 2).

While we realize these are DNA sequences and only 35% homologous we are very encouraged. When translated, the optimal amino acid sequence of the *Manduca* PCR product is most similar to mammalian IL-1-beta and ra family members. For example, it is 22.3% identical and 31.3% similar to rabbit IL-1-beta, and 19.5% identical and 28.6% similar to rabbit IL-1-ra. The similarities to vertebrate IL-1 alpha are considerably less. We are continuing to use a variety of PCR techniques to characterize invertebrate IL-1- and IL-6-like molecules. In addition, we ligated this amplicon into a suitable vector in preparation for screening our cDNA libraries for a full length clone.

4. PERSPECTIVE

Studies of cytokines have concentrated on their role as mediators of the vertebrate immune system. Biochemical characterization and studies of the molecular biology of cytokines from a number of vertebrate species have revealed basic similarities in the structure and biological properties of these important host defense molecules. That activities of macrokine molecules are easily assayed in vertebrate systems suggests that the structure-function relationships of these molecules have been conserved. It appears that cytokine-like molecules have been present for millions of years in animals and as such are important, ancient, and functionally conserved host defense molecules. In general terms, our database of cytokine sequences at present is limited and entirely confined to human cytokines, laboratory model species, and economically significant domestic animals. The accumulation of primary sequence data on a wider range of species will be a valuable extension of our knowledge of this medically and scientifically important group of molecules.

Apart from the intrinsic significance of such basic biological research into the mechanisms by which diverse animals defend themselves from pathogens, comparative immunology will be expected to make important contributions to human biology and medicine. This prediction is based on experience from other fundamental areas of comparative physiology, biochemistry and molecular biology, such fields, for instance as genetics and neurobiology. Lower organisms often provide less complex model experimental systems. Moreover, an evolutionary perspective by providing an understanding of how a more complex system emerged step by step can yield unique insights into the function of such complexity. The study of invertebrate host defense systems can also be expected to lead directly to useful applications, such as the discovery of unique molecules of pharmacological importance and the development of strategies for control of harmful insects and parasites.

In addition, increasing public awareness is focused on the use of mammals for routine investigations. The high price of feeding and housing vertebrates renders the use of invertebrate model systems an attractive, and perhaps necessary, alternative. Invertebrates are noncontroversial, inexpensive and economical to maintain as experimental animals. Invertebrate cell culture systems are as easily maintained and are as practical as vertebrate systems.

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