Origins and Evolutionary Relationships Between the Innate and Adaptive Arms of Immune Systems

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SYNOPSIS. Long before vertebrates first appeared, protists, plants and animals had evolved diverse, effective systems of innate immunity. Ancestors of the vertebrates utilized components of the complement system, protease-inhibitors, metal-binding proteins, carbohydrate-binding proteins and other plasma-born molecules as humoral agents of defense. In these same animals, immunocytes endowed with a repertoire of defensive behaviors expressed Toll-like receptors. They made NADPH oxidase, superoxide dismutase and other respiratory burst enzymes to produce toxic oxygen radicals, and nitric oxide synthase to produce nitric oxide. Antimicrobial peptides and lytic enzymes were in their armory. Immune responses were orchestrated by cytokines. Furthermore, genes within the immunoglobulin superfamily were expressed to meet a variety of needs possibly including defense. However, recombination activating genes played no role. With the acquisition of one or more transposases and the resulting capacity to generate diverse receptors from immunoglobulin gene fragments, the adaptive (lymphoid) arm of the immune system was born. This may have coincided with the elaboration of the neural crest. Naturally, the role of the adaptive arm was initially subservient to the defensive functions of the pre-existing innate arm. The strong selective advantages that stemmed from having “sharp-shooters” (cells making antigen-specific receptors) on the defense team ensured their retention. Refined through evolution, adaptive immunity, even in mammals, remains dependent upon cells of the innate series (e.g., dendritic cells) for signals driving their functional maturation. This paper calls for some fresh thinking leading to a clearer vision of the origins and co-evolution of the two arms of modern immune systems, and suggests a possible neural origin for the adaptive immune system.

INTRODUCTION

With few exceptions, the study of immunology has—for the past several decades at least—been predominantly the study of lymphoid biology. And what a fascinating story it has turned out to be: each individual of a species will acquire—de novo—receptors able to recognize with exquisite specificity a huge variety of foreign antigens. For each antigen that is subsequently re-encountered, immunocytes recognizing the antigen will remember the previous encounter and respond more effectively.

In the 1970s, the influential comparative immunologist Bill Hildemann catalyzed a re-examination of the concepts of immuno-phylology (Hildemann et al., 1981). Hildemann appreciated that, in pioneering studies of inflammation and phagocytosis, Eli Metchnikof, the father of immunology, had made use of rose thorns, water fleas and starfish larvae. Attracted by the opportunity to “do immunology” in coral reefs, Hildemann focused the mind of a classical immunogeneticist first on experiments of Nature and later on laboratory-based “grafting” experiments with corals and sponges (Hildemann et al., 1979).

Further illustrating the prodigious fruitfulness that can result when a mind that is well informed in one area engages another, microbiologist Hans Boman in Sweden simultaneously initiated studies of the basis for acquired resistance to bacterial pathogens in insects (Boman, 1998). That work catalyzed the discovery of antimicrobial peptides throughout phylogeny, and eventually the discovery of immune functions for Toll and Toll-like receptors, and the NFκB/Rel system of transcriptional activators (Medzhitov and Janeway, 2000a). Starting in the mid-20th century and continuing to the present, additional free-thinking individualists, more interested in basic biology than in joining the mainstream, have sought to discover non-immunoglobulin based mechanisms of defense. This relatively small cadre of scientists have contributed significantly to the refinement of ideas on the evolution of immunity, through efforts to understand how animals lacking lymphoid systems manage to survive in a world replete with both micro- and macro-pathogens and parasites (see as an example Beck et al., 2001).

As is now generally appreciated, the ability to recombine germline-encoded gene segments and to synthesize numerous unique antigen-recognizing receptor proteins encoded by the novel reading frames was acquired about 430 million years ago in organisms resembling primitive jawed fishes. Genes encoding members of the immunoglobulin superfamily had been expressed for hundreds of millions of years prior to that time, and their functions are thought to have been predominantly though not exclusively non-defensive (e.g., Aurelio et al., 2002). Recent thinking envisages the lateral transfer of a prokaryotic transposase with an aptitude to create recombinants out of pre-existing immunoglobulin gene segments (Agrawal, 2000). This event spawned the now inappropriate term3 “adaptive immune system.”
Seduced by the impressive sophistication (or is it mere complexity?) of lymphoid biology in modern birds and mammals, some immunologists have tended to view all components of innate immunity as subservient to the adaptive arm of the immune system. An influential voice encouraging a change in perspective has been that of Charles Janeway (2001). The present paper contributes to the call for reconsideration by examining the immune landscape around the time when the transposases supposedly invaded and quickly evolved into the recombination activating genes (RAG 1 and 2) in early gnathostomes (Flajnik and Kasahara, 2001). In doing this, it crystallizes the tantalizing notion that the adaptive arm of the immune system may be an evolutionary offshoot of the nervous system.

**Immune Competence**

Being immunocompetent means being able to defend oneself against potentially damaging microbes and parasites. And because neoplasia can be fatal, guarding against it has come to be seen as a second criterion of immunocompetence. This capacity to protect oneself requires both a means of surveillance (detection) and the means to neutralize the threat. Both capabilities appeared very early in the history of the evolution of life. Navigating away from life-threatening conditions and towards nutrients requires discriminating senses and appropriate behaviors that are seen even in prokaryotes. Among unicellular protists (eukaryotes), propagation depends on the ability to discriminate between different mating types, made possible by the presence of specific markers and recognition receptors functionally linked to second messenger systems. Hence the basic components of a surveillance system arose “at the very beginning.”

Elements of the aggressive systems we know best from studies of mammalian macrophages and neutrophils (e.g., lysosomal enzymes, proton pumps, enzyme complexes that produce reactive oxygen radicals, pore-forming molecules, etc.) are also present in unicellular eukaryotes. Studies of their roles in defense are few. However, some studies illustrate offensive use: *Entamoeba histolytica*, for example, rely on pore-forming molecules (“amaebapores,” Bruhn and Leippe, 2001) and cysteine proteinases in the pathogenesis of invasive amebiasis (Que et al., 2002). The capacity to be aggressive has likely been essential for survival since the earliest days of cellular life.

Hundreds of millions of years passed between (i) the appearance of such recognition systems and the means of mounting aggressive responses and (ii) the appearance of RAG 1 and 2. During this time, particularly during the Cambrian explosion and subsequently, evolutionary diversification occurred on an unprecedented scale. The diversifying nature of these events yielded several phyletic lineages. Best known are the protostome and deuterostome lineages, the latter eventually giving rise to the vertebrates. Less widely known is the fact that within the protostomes two major lineages also evolved (Valentine et al., 1999; Fig. 1). The relevance of this dichotomy stems from the fact that a great deal of modern biology is being discovered through detailed, predominantly molecular genetic studies of a small number of model organisms, and the fact that two of these—*Caenorhabditis elegans* and *Drosophila melanogaster*—are members of the Ecdysozoa. The sister lineage (Lophotrochozoa) includes very successful and diverse taxa such as the annelid worms (12,000 described species) and the molluscs (50,000 described species) (World Resources Institute; http://www.wri.org/wri/biodiv/f01-key.html). It is reasonable to predict that immune mechanisms in lophotrochozoan species will be informative as we strive to reconstruct the evolutionary history of immune mechanisms (Loker and Bayne, 2001).

**Immune competence of an immunologically obscure Cambrian taxon, a mollusc**

On account of the facts that (i) planorbid snails are required for humans to become infected with blood flukes (the snails serve as intermediate hosts for these trematode worms), and (ii) both naturally occurring and laboratory-bred snail strains are available that are resistant to the human parasite and therefore able to block transmission (Loker, 1994), a body of knowledge has been accumulating on the immune system of the most important host snail, *Biomphalaria glabrata* (Bayne et al., 2001). Structurally, its immune system...
is comprised of leukocytes that are produced in an hematopoietic tissue within the pericardium and that circulate in the blood, and a plethora of plasma molecules. The variety of leukocytes is restricted to just 2 morphologically distinct types: small, non-granular hyalinocytes that do not spread much on glass or plastic, and larger granulocytes that are phagocytic and have been termed macrophage-like (Bayne et al., 1980; McKerrow et al., 1985). There is a suggestion of subpopulations present within the granulocyte population (Coustau and Yoshino, 1994); this may be indicative of ontogeny or it may have functional relevance.

The quantitatively predominant plasma molecule in planorbid snails is hemoglobin. Much remains to be discovered vis-à-vis additional humoral molecules. However, a tetrameric α-macroglobulin antiprotease (Bender and Bayne, 1996), a family of fibrinogen- and immunoglobulin-related multidomain proteins (Lernard et al., 2001; Zhang et al., 2001) and other molecules that are toxic to trematodes (Sapp and Loker, 2000) are among these. A cecropin-like sequence has been reported (Adema et al., unpublished GenBank #AF134472) but we do not know if this and other putatively defense-related molecules are normally free in the plasma rather than contained intracellularly. As in other spheres of biology, molecular approaches are now accelerating the rate of discovery of immune-relevant plasma molecules in molluscs (Lockyer et al., 2000; Jones et al., 2001; Knight et al., 2000; Schneider and Zelck, 2001).

Clearly, molluscan leukocytes are crucial agents of self-defense and are appropriately thought of as immunocytes. They possess both the equipment and the behavioral repertoire required to mount effective defenses such as parasite encapsulation and killing. The molecular armory that they call upon is diverse and includes pattern recognition molecules (Hahn et al., 2000), lysosomes with lytic enzymes (see Bayne, 1983), an NADPH oxidase enzyme complex producing superoxide anion (Adema et al., 1994; Hahn et al., 2000), nitric oxide synthase (Nappi and Ottaviani, 2000), lysosomes with lytic enzymes (see Bayne, 1983), antiviral peptides (Mitta et al., 2000). The source and nature of the putative anti-trematode molecules (Sapp and Loker, 2000) and of opsonins (Renwrantz and Richards, 1992; Bayne and Fryer, 1994) remain unclear (van der Knaap et al., 1981). The defensive cells also express a behavioral repertoire that enables them to use these mechanisms as effective agents of defense. They are capable of chemotaxis (Kumazawa et al., 1992), degranulation (Bayne, 1983), phagocytosis, and encapsulation of objects too large to engulf.

Components of the armory of a molluscan immunocyte

Pattern recognition receptors. In animals, recognition of pathogen-associated molecular patterns is often achieved by multi-valent lectins with high affinities for complex carbohydrate structures (Janeway, 1989; Medzhitov and Janeway, 2000b). Consistent with this, leukocytes of the escargot (Helix pomatia) express receptors for mannose-6-phosphate (Renwrantz and Richards, 1992). Evidence that leukocytes of B. glabrata express pattern recognition lectins was obtained first by competitive inhibition of yeast phagocytosis using laminarin, a poly-glucose (Fryer et al., 1989). Using a fluorescence in-plate assay for the respiratory burst and, as stimulants, neo-glycoproteins (ngp; bovine-serum albumin complexed with sugars), galactose-, mannose- and fucose- ngp elicited the burst, whereas glucose-, lactose-, melibiose-, n-acetyl-D-glucosamine- and n-acetyl-D-galactosamine- ngp did not (Hahn et al., 2000) (Fig. 2). These data implicate hemocyte surface receptors that, on binding their ligands, stimulate cell production of reactive oxygen species.

The respiratory burst. The NADPH oxidase enzyme complex is responsible for producing superoxide anion, the initial reactant in the respiratory or oxidative burst—a cascade capable of yielding additional damaging radicals that can kill microbes (Babior, 1999). This enzyme activity was first detected for molluscs in several snail species (Dikkeboom et al., 1989), and it was suspected to play a role in the killing of schistosome blood flukes by Lymnaea stagnalis, a species that is naturally resistant to Schistosoma mansoni (Dikkeboom et al., 1988a). Subsequently it was demonstrated that B. glabrata uses the enzyme in its killing response to S. mansoni (Adema et al., 1994; Hahn et al., 2001a; Fig. 3). As the bivalves Mytilus edulis (Noel et al., 1993), M. galloprovincialis (Arunugam et al., 2000) and the oyster Crassostrea gigas (Takahashi et al., 2000) also contain this system, it is likely present generally in the Mollusca.

The fates of reactive oxygen species generated fol-
Fig. 3. The ability of Biomphalaria glabrata hemocytes to kill schistosome sporocysts is compromised by the NADPH oxidase inhibitor protocatechuic acid (PCA; adapted from Hahn et al., 2001a). Killing of parasite larvae by hemocytes from parasite-resistant snails was scored in real time by the appearance of fluorescence due to the incorporation of propidium iodide in nuclei of the parasite. Sporocyst mortality was evident whenever hemocytes were present, but PCA significantly reduced the killing.

Fig. 4. When hemocytes were allowed to attack S. mansoni sporocysts in vitro, L-NAME (an inhibitor of nitric oxide synthase) reduced the killing (adapted from Hahn et al., 2001b). Killing assays resembled those used for Figure 3, with the exception that the nitric oxide inhibitor L-NAME or its inactive isomer D-NAME was present. The inhibitor significantly reduced the killing.

Following superoxide anion depend largely on the nature of enzymes present at or near the sites of production. In mammalian neutrophils, myeloperoxidase is a key player, converting hydrogen peroxide to hypochlorous acid. In strains of B. glabrata that differ in their ability to kill S. mansoni sporocysts, a putative myeloperoxidase appears to be differentially expressed (Schneider and Zelck, 2001), and this may be important in determining susceptibility or resistance of the snail to the parasite (Bayne et al., 2001).

Nitric oxide synthase (NOS). In its high output form, this enzyme serves the defensive needs of animals, since nitric oxide and the product of its reaction with superoxide (peroxynitrite) will damage proteins (Reiter et al., 2000). The mRNA for NOS has been identified in snail neurons but not (yet) in leukocytes (Moroz, 2000). We obtained indirect evidence of a defensive role for NO in the leukocyte-mediated attack of B. glabrata on the human blood fluke, Schistosoma mansoni. The arginine analog L-N-arginine methyl ester (L-NAME) but not the D isomer inhibits NOS. Consistent with the involvement of this enzyme in the leukocyte-mediated attack of B. glabrata on S. mansoni, we observed a reduction of killing when leukocytes were allowed to attack S. mansoni sporocysts in vitro in the presence of L-NAME (Hahn et al., 2001b) (Fig. 4).

Antimicrobial peptides. The widespread occurrence of antimicrobial peptides (Ganz, 2003) and their earlier report in various nudibranch molluscs (sea slugs; Yamazaki et al., 1990) make it unsurprising though interesting that they have been reported to occur in leukocytes of the bivalve M. galloprovincialis (Mitta et al., 2000). Sequencing of ESTs from leukocytes of B. glabrata (Schneider and Zelck, 2001) has yielded evidence of a putative defense. Furthermore, a cecropin-like peptide has been reported in this species (Adema et al., unpublished GenBank #AF134472). Functional data are yet to be reported for either.

Anti-trematode molecules. With genome sequencing projects being completed and new ones started with increasing frequency, one anticipates discovery of novel defense-related proteins throughout phylogeny. Anti-trematode molecules will likely fall in this category. Efforts to isolate from snail plasma and to characterize molecules responsible for killing trematodes (Sapp and Loker, 2000) remain to be brought to fruition.

This cornucopia of defense-related products and cells in a species representing others that have been around for 520 to 530 million years, and that are members of the Lophotrochozoa, emphasizes the power and redundancy of innate immune systems that had evolved long before the origin of the vertebrates.

MERELY ATTITUDE ADJUSTMENT, OR A PARADIGM SHIFT?

Even towards the end of the 20th century, one might have expected to read in textbooks on immunology that immunocompetence requires a lymphoid immune system that includes the genetic raw material and the recombinase enzymes for making antibodies and T cell receptors, accessory (antigen-processing and presenting) cells, co-stimulatory ligands and receptors, cytokines and a major histocompatibility system. This is what we have all been taught. I suggest, however, that a 21st century consensus might read like this; immunocompetence requires cells and molecules that recognize and neutralize potentially dangerous infectious
agents, and such cells and molecules are essential components of all eukaryotic organisms.

Figure 5 conveys the notion that the adaptive (lymphoid) arm of the vertebrate immune system was, at the time of its origin, subervient to the dominant innate arm of immunity. A pre-existing, competent, diversified armory of defensive mechanisms was joined by an army of small cells (lymphocytes) endowed with the novel capacity to produce receptors capable of specifically recognizing a huge array of epitopes. Whether these cells retained the novel receptors on the cell surface or secreted them, the receptors did little more than enable the innate immune system to better aim its offensive armory against specific targets. By analogy, one might envisage a band of hooligans armed with grenades, mace and shot-guns recruiting novices with rifles—insruments to achieve better aim. The ammunition, however, had already been developed. Among others in the comprehensive armory, there were these: a complement system with at least one activation pathway and the ability to target a diversity of molecules (Sunyer et al., 1998); a similarly promiscuous α-2-macroglobulin (Mutsuro et al., 2000); and phagocytic ancestors of dendritic cells already armed with Toll-like receptors communicating with gene-activating mechanisms (Escoubas et al., 1999), cytotoxic molecules, and the abilities to make toxic radicals and cytokines. Importantly, a well developed acute phase response was likely present, as it is today in insects (Hoffmann et al., 1996) and teleosts (Bayne and Gerwick, 2001). Furthermore, immunoglobulin superfamily members were expressed on cell surfaces (Rader et al., 1996) and may even have had defensive roles (Greenberg et al., 1996; Mendoza and Faye, 1999; Zhang et al., 2001). Lacking at the time were jaws, lymphoid tissues, RAG 1 and 2, and bone marrow.

FURTHER SPECULATIONS

For most of the long history of the evolution of life on this planet, the essential components of self defense systems have existed. Both surveillance to detect dangerous chemical signatures and aggression to neutralize the bearers of those signatures arose early and were retained. The armory of aggressive “weapons” diversified and evolved to encompass practically all of the gene systems that now effect immune defenses in modern mammals. Late in evolution (ca. 430 million years ago), the horizontal transfer of a transposase from a prokaryote to a primitive vertebrate provided the means to create large numbers of distinct receptors that were recruited into the service of the defense system.

Whereas in vitro assays of lymphocyte responsiveness, widely used in the mid-twentieth century, created the impression of an autonomous adaptive immune system, it has more recently become appreciated that the armies of lymphocytes serve at the behest of phagocytic cells of the myeloid series. It remains to be determined if any aspects of lymphoid function operate autonomously in vivo.
Is the adaptive immune system an evolutionary off-shoot of the vertebrate nervous system?

As though to tickle the imagination, it appears that the acquisition of RAG genes may have coincided with the elaboration of neural crest and tissues derived from it, such as jaws, at a time of increasing complexity of the nervous system. The possible relevance of this becomes evident when we consider insights suggested first by Bill Hildemann and his associates (Hildemann et al., 1979), who drew attention to the fact that the vertebrate immune and nervous systems share the properties of specific recognition, memory and the abilities to mount and target aggressive responses. A growing number of “neuro”-transmitters are now known to be made by and are active in the immune system (Schauenstein et al., 2000), and a growing number of “cytokines” are made by and active in the nervous system (Dunn, 2000). Indeed, it has been convincingly established that neuro-immune cross-talk is essential to homeostasis throughout the vertebrates.

What is most intriguing here is that at least some of the signaling molecules mediating this communication are shared by neural and immune systems. Lately, the understanding of immune processes has benefited from the concept of the immunological synapse, a cell-cell interface structurally and functionally reminiscent of the neural synapse (Krummel and Davis, 2002). Given all these considerations, perhaps it is not so revolutionary to suggest that the adaptive immune system is an evolutionary off-shoot of the vertebrate nervous system! This notion is encouraged by the recent discovery that immunoglobulin superfamily members facilitate correct targeting of neuronal axons in the developing nervous systems in animals as primitive as C. elegans (Aurelio et al., 2002). The proper wiring of complex nervous systems, of course, requires very large numbers of specific ligands and receptors. The hypothesis suggested here predicts the existence, in the developing nervous systems of all gnathostomes, of a system for generating high levels of diversity in such an address system.

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