Immune Response: Evolution

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Phagocytosis in unicellular animals represents the most ancient and ubiquitous form of defence against foreign material. Multicellular invertebrates and vertebrates possess phagocytic cells and have evolved more complex functions attributed to immuno-defence cells that specialized into cellular and humoral immune responses.

Brief History of Immunology

Animals live successfully because they possess the full capacity to function in a variety of ways. Living beings are capable of eating, eliminating wastes, respiring, reproducing and responding to diverse environmental stimuli. The executors of these varied functions are the cells, tissues and organs of the digestive, endocrine, urinary, respiratory, reproductive and nervous systems. The immune system is likewise an indispensable organ system. In multicellular animals, immune systems contain different kinds of cells, tissues and organs and their molecular products that encompass and protect the whole organism against potentially harmful pathogens (such as bacteria, viruses, parasites) that inhabit the external environment. In fact, the immune system probably arose in evolution to defend organisms not only against external but also against internal pathological threats (such as cancer).

Self Versus Nonself

The immune system recognizes the differences between 'self' and 'not self' in its attempt to maintain a balanced internal milieu, but the distinction is not rigid as the opposite may occur – i.e. reactions to self may cause autoimmune disease to develop so that an organism self-destructs. Cancer is considered to be one of the dreaded internal threats that can lead to the death of an organism. Now that we have the rudiments of an immune system, what developed next? In vertebrates, an antigen is simply the ligand of an antigen receptor generated by recombination activator genes (RAG) and expressed only on vertebrate lymphocytes. According to another, less restricted definition, perhaps more applicable to invertebrates, antigens are any multitude of various chemical substances capable of stimulating an animal's immune system to respond by one of or a combination of several responses: phagocytosis; the more advanced cell-mediated immune response; or the even more complicated synthesis

> Our current biological concept of immunity has changed considerably from the original meaning of the word. Quite literally, its earlier usage referred to exemption from military service or paying taxes. Now, an 'immune system' protects us from certain diseases. The earliest history traces immunology as a science to the microscope, which made it possible to identify at least one group of microorganisms – the bacteria – that cause disease. Just before the nineteenth century, Edward Jenner, an English country physician, reported the first successful attempt to prevent a disease by vaccination. His approach grew out of an observation of nature. Dairy maids and farmers often became infected accidentally with cowpox and later seemed to be automatically protected against smallpox. Jenner deliberately inoculated a small boy with pus from a cowpox sore and found that the boy was immune to smallpox six weeks later. Later various modifications helped to produce vaccines so that smallpox and other infectious diseases are now virtually eradicated owing to the development and use of vaccinations. What does this health orientation have to do with evolution of immunity? The answer lies partially in the history of work on invertebrate immunity and how all animals react to foreign material by having an immune system.

focus. Second, the reality of this role was readily revealed, but not so quickly accepted, by the simple results of his prescient experiments using marine invertebrates, thus destroying the myth that immunodefence was restricted to vertebrates, especially mammals and of course humans.

Near the beach in Messina, Italy, Metchnikoff discovered that cells of starfish larvae rapidly gathered around and attempted to engulf a thorn that had been inserted experimentally into the body cavity. This represented the first observation of phagocytosis, an event that gave rise to the concept of cellular immunity. Phagocytosis in multicellular organisms is the process of cellular ingestion of foreign material and it is a component of the innate or natural immune system. Responses in this system usually do not require the specificity that accompanies adaptive responses that involve the elaborate interaction of several cell types followed by the synthesis and secretion of immunoglobulin or antibody. From the viewpoint of evolution, phagocytosis is probably the oldest of immune responses, traceable even to the protozoans; phagocytic cells are ubiquitous (**Figure 1**). To verify their presence, one need only expose an animal to carbon particles; shortly thereafter, phagocytes are blackened, heavily engorged with the ingested particles. Ingestion of bacteria by phagocytosis can contribute significantly to an animal's resistance to infectious organisms. Metchnikoff's discovery of phagocytosis among invertebrates was extended to humans and paved the way for reconciling the controversy between the importance of humoral immunity versus cellular immunity.

Metchnikoff's contribution was also instrumental in forging the discipline of comparative immunology, a branch of immunology that investigates the evolution of immune mechanisms by experimentation using different animal species. Invertebrate immunology is considered a subdiscipline of this since it deals with several related features (see **Table 1**). First, there is the need to understand the basis of innate, natural, nonspecific, nonanticipatory and nonclonal responses, largely restricted to invertebrates according to current evidence. Second, a thorough understanding of diverse mechanisms is essential if we are to define the various steps in the evolutionary development of

Table 1 Immunity in invertebrates is functionally similar to that in vertebrates

• **Protection against environmental threats, both internal and external**
• **Innate, natural, nonspecific, nonanticipatory, nonclonal** Antigen recognition, phagocytosis = invertebrate and vertebrate leucocytes
• **Adaptive, induced, specific, anticipatory, clonal** Antigen-specific = vertebrate T and B cells

pathogens, invertebrates can also respond in experimental situations in immunological laboratories that often utilize nonpathogenic materials (e.g. erythrocytes, yeasts). Equipped with an immune system, invertebrates are therefore capable of living out their normally programmed lifespans, which like those of vertebrates end in a period of senescence. To do this, they are endowed with the necessary network of leucocytes or white cells and the humoral products that these cells synthesize and secrete. Together, both the cells and humoral products (e.g. agglutinins, lysins) must be successfully coordinated in order to produce effector mechanisms that sequester potentially pathogenic material. As an organismic component, the immune system does not act alone. There is much information that reveals the necessity of coordination with the two other regulatory systems – the nervous and the endocrine. By this coordination, there is established an efficient internal network that maintains a harmonious living being.

Progenitors of Immunocytes

The leucocytes of invertebrates in many instances have a remarkable morphological resemblance to those of vertebrates. These same cells effect analogous functions and there are some clues beginning to emerge that suggest structural and functional homologies. Leucocytes of invertebrates appear like the ubiquitous macrophages that are capable of spreading on glass and extending pseudopodia in anticipation of phagocytosing a foreign particle (**Figure 2**). There are others, however, that in every respect appear like the typical small lymphocyte with its distinguishing characteristics: low nucleocytoplasmic ratio, and therefore sparse cytoplasm; well developed Golgi apparatus; and hardly any pseudopodia (**Figure 3**). It is relatively easy to envision how these cells could serve both as immunologically competent cells (on the basis of morphology alone) and as precursors of the vertebrate immunocytes by first developing on their surface membranes the molecules essential for recognition – the receptors. Thus, two developments may be required for conversion to an adaptive immune system. The first is an increased flexibility

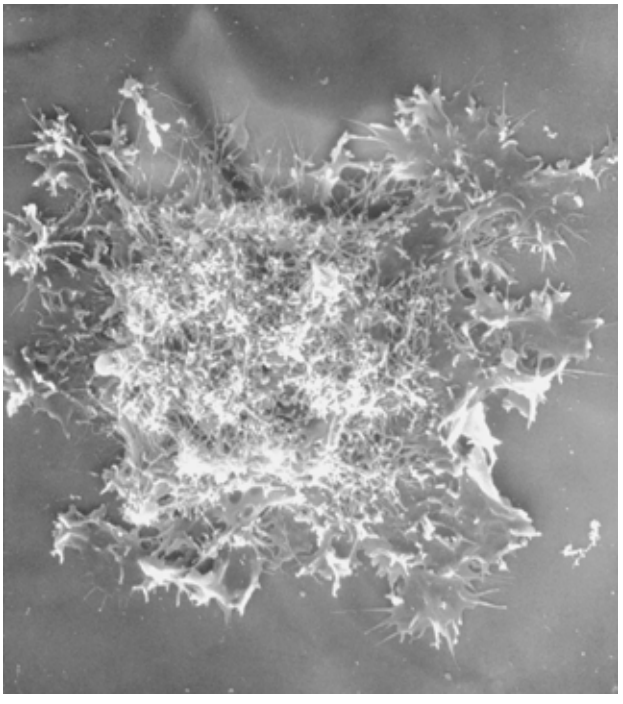


Figure 2 Neutrophilic coelomocyte of the earthworm (scanning electron micrograph). During the healing and subsequent rejection of transplants, it is this cell that migrates across the squamous epithelial barrier that separates the body wall from the coelomic cavity. This cell is highly phagocytic and, *in vitro*, it phagocytoses debris after the cell in **Figure 3** kills the tumour K562 and forms granulomas around the remains.

on the part of the somatic genome (coupled) with coding for the pattern of these recognition units. This conceivably arose during periods of changing patterns or mechanisms of differentiation. Its function was to provide a means of greater diversification of pattern and to associate this diversification with an increasingly absolute phenotypic restriction. Thus, there would be an increasing number of foreign patterns that could be recognized and a concentration on a few cells of high ability to deal with any one specific pattern of foreignness. The second requirement is that contact of the foreign pattern antigenic determinant with the recognition unit's combining site should, under appropriate conditions, allow proliferation of those cells concerned with retention of flexibility throughout the descendent clone. This, at the cellular level, is the essential feature that makes an immune system adaptive.

We now have leucocytes (coelomocytes, haemocytes in invertebrates; lymphocytes, plasma cells in vertebrates) that produce an unknown substance x, retaining it on the cell surface or releasing it into the coelomic cavity or blood. In residence there, cells stand ready to recognize and sequester an antigen. According to Sir MacFarlane Burnet writing in 1968:There is at least a limited capacity to recognize foreignness in the haemocytes of invertebrates. There are also proteins in invertebrate body fluids which

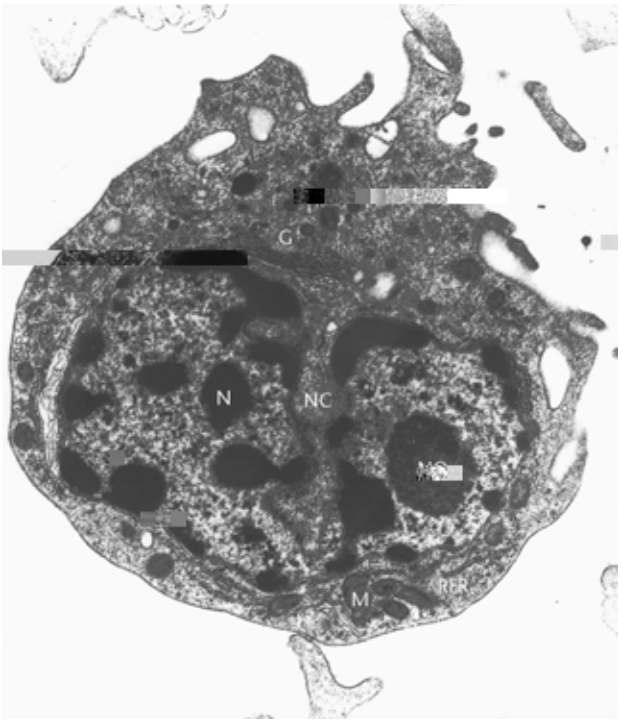


Figure 3 Transmission electron micrograph of the lymphocytic coelomocyte of the earthworm, involved in rejection of transplants and the killing of the tumour K562 *in vitro*. Note that the size of the cell is roughly that of a small lymphocyte in vertebrates. The cytoplasm is occupied almost entirely by the nucleus and the cytoplasm contains well-developed organelles: mitochondria, Golgi apparatus and endoplasmic reticulum. ($\times$ 34 000). G, Golgi; N, nucleus, No, nucleolus; NC, nuclear cleft; M, mitochondrion; RER, rough endoplasmic reticulum.

have pseudo-immunological capacities, and can, for example, agglutinate mammalian red cells…What recognition of foreignness there is in invertebrates is a function of wandering phagocytic cells that are in some sense ancestral to the immunocyte, polymorphonuclear and macrophage of the vertebrates.

Invertebrate immunocyte receptors may be related to the common agglutinins in the coelomic fluid wherein coelomocytes are suspended. In fact, after injecting an invertebrate such as an earthworm with an antigen, there is repeated evidence of agglutinin synthesis, which is rapid within 24 hours (**Figure 4**). The coelomic fluid, therefore, with its coelomic cells is like vertebrate blood, carrying certain immune cells. Although there are immunoglobulins in invertebrates, it is not clear how specificity and (perhaps ultimately) memory are mediated. We may speculate that the complex structure of immunoglobulins evolved and that certain invertebrates should possess molecular configurations with (obvious) resemblances to immunoglobulins or the T-cell receptor. Although there are immunoglobulin superfamily members present in invertebrates, none according to current information shows diversity generated by recombinase. In lieu of immunoglobulin, agglutinins that can act as lectins, unique to invertebrates, may function as invertebrate immunocyte receptors. In this context, we must remember that vertebrate phagocytes and natural killer (NK) cells use lectins (as well as immunoglobulin, which evolved later).

Can We Compare Vertebrate and Invertebrate Immune Responses? From Eating to Immunity

Cells are essential for either a cellular or humoral immune response. Antigen (presumably) receptor molecules of vertebrate immune cells are T-cell receptors or immunoglobulins that remain bound to the cell's surface. There may be invertebrate cells that bear receptor molecules, but their intricate nature in all invertebrates remains largely unexplored. Notwithstanding this cautionary note, there is a remarkable convergence of events when we consider the protozoans *vis-à-vis* vertebrates. We have already seen how unicellular invertebrates combine food-getting with defence in the single act of phagocytosis. Yet defence preceded by recognition does not reveal the entire complexity and capability of unicellular animals as has been observed in two protozoans. We can conclude that the roots of fundamental immunodefence functions, even at the molecular level, are already expressed – a situation that underscores the importance of a universal approach to biological problems.

The enteric protozoon *Entamoeba histolytica* is the cause of worldwide human amoebiasis. The amoebae produce a protein capable of forming ion channels or pores in lipid membranes and of depolarizing target cells. Despite the repeated occurrence of the membranolytic function within the animal kingdom, the unanticipated sequence resemblance of amoebapores to NK-lysin, an effector molecule of mammalian killer cells, is remarkable. Amoebapores are sufficient to induce lysis of bacteria and eukaryotic cells, and may be viewed as structural fossils in comparison with the mammalian NK-lysin. In another protozoan, a ciliate, *Euplotes raikovi*, there are known mating types governed by gene-controlled pheromones that are obviously important in cell communication. Sequence information reveals significant homology between the pheromone and IL-2, an interleukin essential for cell communication during certain immune responses.

If we leave the protozoans and look at a group of successful and long-surviving metazoans, the insects offer considerable functional evidence at the molecular level that reveals homology. In the cecropia moth and in *Drosophila*, invertebrate immunologists have discovered that many of the genes that control antibacterial proteins share a common upstream motif, similar to the binding site for NF-κB (nuclear factor-κb). NF-κB is a mammalian

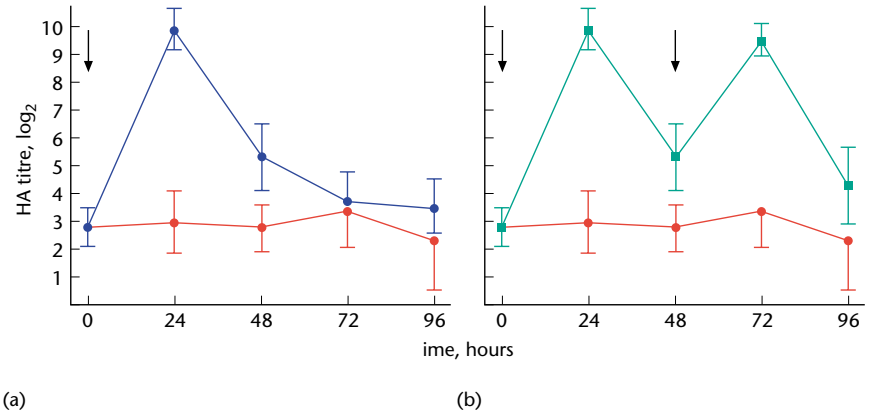


Figure 4 Agglutinin titre curve that represents the typical humoral response of the earthworm to an injection of antigen (rabbit red blood cell, RRBC). (a) Single injection of RRBC (blue circles) or saline (red circles). (b) Two injections of RRBC (green squares) 48 hours apart.

member of the Rel family of transcription factors. In mammals, NF-κB and other Rel proteins play a central role in the transcriptional activation of immune-related factors such as immunoglobulins, interleukins and the proteins of the acute-phase response. These very same transcription factors are also exploited by the human immunodeficiency virus when it infiltrates the human immune system. This kind of evidence strongly supports the need for a revision in approaches to the evolution of immune mechanisms.

Many immunologists believe that invertebrate coelomocytes are the evolutionary precursor of all known vertebrate immunocytes. According to one view, all vertebrate immunocytes evolved from an invertebrate precursor cell that could recognize and react to antigen. However, unlike vertebrate lymphoid cells, surface receptors of invertebrate immunologically competent cells may not be numerous, so that the invertebrate is incapable of responding to a wide variety of antigens in a specific manner. Therefore, this limited number of receptors may result in limited, specific recognitions and subsequent memory responses for invertebrates. In contrast, invertebrates may be endowed with pattern-recognition receptors, an hypothesis that may be viewed in the following manner. The mammalian macrophage mannose receptor is in many ways the prototypic pattern-recognition receptor. The receptor is expressed mainly on tissue macrophages and dendritic cells. It is a lectin-like molecule that directly recognizes a wide array of microorganisms. Ligation of the receptor results either in endocytosis or phagocytosis of the ligand–receptor complex. Moreover, the mannose receptor plays an important role in the recognition of microbe-derived glycolipids, which are processed via a newly described CD1b pathway. (As we will see later, CD refers to cellular differentiation antigens that are present on leucocytes and are essential in the immune system.) Thus, the mannose receptor provides a clear link between adaptive immunity and the innate immune response. This is especially meaningful because it is associated with the complement-related serine proteases in tunicates and vertebrates.

In the transition from invertebrate to vertebrate immune systems, certain points are essential to consider. With regard to phagocytosis and immunity, both defence and food-getting among the less differentiated invertebrates (e.g. protozoans and sponges) are probably reactions governed by cell surface receptors. In the phylogenetic progression, food processing cells become differentiated from defence or antigen-processing cells with this specialization. Whereas a single-cell amoeba combines both defence and nutrition in one act, the metazoan's phagocytes are involved with defence and not with nutrition, which is relegated to other cells. Other cells cope with nutrition and still others effect defensive responses. In other words, there has been a division of labour. To execute these functions, the phagocytes of simple metazoans may be coated with molecules having structures that are complementary to both food and components of microbes. More advanced organisms possess a greater number of different receptor molecules having various steric configurations. This enables them to distinguish between a wider range of microorganisms and between what would be a defence and/or a nutritive response.

Invertebrate animals possess diverse cell types similar in morphology to vertebrate blood cells. In fact, their coeloms or body cavities are comparable to vertebrate haematopoietic sites such as bone marrow in that they possess leucocytic types. With increasing taxonomic and anatomical complexity, which seems to parallel immune response complexity, isolated invertebrate cells can, like those of vertebrates, exhibit characteristic immune reactions. For example, humoral agglutinins acting as lectins in sea urchins will agglutinate many types of vertebrate erythrocytes and also promote adhesion of autologous leucocytes. This activity is Ca^{2+} dependent but not Mg^{2+} dependent and is heat stable, which suggests a C-type

lectin. These results coupled with more detailed characterization point out the importance of these molecules in defence against invading bacteria or altered cells and a fundamental role as cell adhesion molecules involved in cell–cell and cell–matrix interaction. Other interesting molecules play a role in the humoral immune response of echinoderms. Isolated from starfish leucocytes, these IL-1-like proteins exhibit a wide range of biological activities, such as inflammatory reactions identical to delayed-type hypersensitivity, inhibition of macrophage migration, activation of macrophages for cytostasis or suppression of T-dependent but not T-independent immune responses. Later results have revealed that sea urchin coelomocytes activated with lipopolysaccharide (LPS) respond to immune challenge from LPS with significant elevation of profilin, a small actin-binding protein involved in signal transduction. Comparison of deduced amino acid sequences with known protein sequences revealed significant matches with diverse proteins such as an invertebrate homologue of a vertebrate complement component, clotting factors, complement receptor or regulatory proteins, C-type lectin, protease inibitors, serine protease with similarities to thrombin, elastase and plasmin, proteins involved in signalling, lysosomal proteins and cytoskeletal proteins.

Despite their simpler organization and body plan, invertebrates exhibit reactions that are functionally equivalent to vertebrate cellular responses such as rejection of foreign transplants and the killing of various tumour cell targets cocultured with them *in vitro*. In earthworms, certain cellular differentiation (CD) antigens have been identified and associated with particular functions that seem to be mediated primarily by leucocytes, although the intervention of and cooperation with humoral components cannot be excluded. As mentioned earlier, the value of CD antigens is enormous. Specific identification has allowed immunologists to functionally classify the cells participating in various immune responses, to isolate them and to individually analyse their specificities, response patterns and effector mechanisms. The monoclonal antibodies used to delineate particular cells have also been used to define specific alterations in particular lymphocyte subsets that might occur in various diseases. Further investigations of the effects of monoclonal antibodies on lymphocyte function have shown that these surface proteins are not merely phenotypic markers but are themselves involved in several lymphocyte responses. For example, in mammals most helper T lymphocytes are CD3 + CD4 + CD8 − and most cytotoxic lymphocytes are CD3 + CD4 − CD8 + . The two most frequent functions attributed to various CD antigens are: (1) to promote cell–cell interactions and adhesion, and (2) to transduce signals that lead to lymphocyte activation.

Using this approach, certain CD markers have been revealed in earthworms, leeches sipunculid worms and snails. It is important to note that certain other markers are absent. In earthworms (*Eisenia fetida*), it was demonstrated that their coelomocytes (leucocytes) can kill the human tumour cell line K562 when cocultured with it. To clarify which cells are responsible, and using cytofluorimetric analysis, microscopy and mouse anti-human monoclonal antibodies, two types of coelomocytes have been identified: (a) small (8–11 μm) electron-dense cells (SC) that were stained by the monoclonal antibodies for epitopes present on human cell adhesion molecules (CD11a, CD45RA, CD45RO, CDw49b, CD54) and those for β_2-microglobulin and Thy-1; and (b) large (12–15 μm) electron-lucent cells (LC) that were negative for these same markers. β_2-microglobulin, Thy-1 and Lyt-2 (CD8a) have been previously demonstrated by serological methods. Both cell types were negative for numerous other CD antigens in all of the animal models and MHC class I and class II markers in earthworms. In vertebrates these markers play important roles during the intricate mechanisms of cytotoxic reactions and immunoglobulin synthesis.

The mechanisms in earthworms have several characteristics. Rather precise characteristics appear to be associated with the killing response. First, there appeared to be a division of labour reflecting the ancient response as well as a perhaps more recent response. SC were active during recognition, rapidly binding to K562 targets and probably effecting the killing. In contrast, LC were phagocytic, vestiges of the ancient reaction. Second, release of ^{51}Cr, the assay system for cytotoxicity, revealed rapid, significant and equal levels of killing at 4, 20 and 37°C. Third, after cytotoxicity is complete *in vitro*, there are reactions associated with nonspecific inflammatory responses. Thus, the debris that cannot be sequestered by phagocytosis is encapsulated, with the formation of granulomas. Finally, perforin-positive granules are present in the cytoplasm of coelomocytes. Since perforin is involved in cell lysis, sequence information on the putative perforin molecule is needed to confirm that it may be partially responsible for lysis of tumour cell targets.

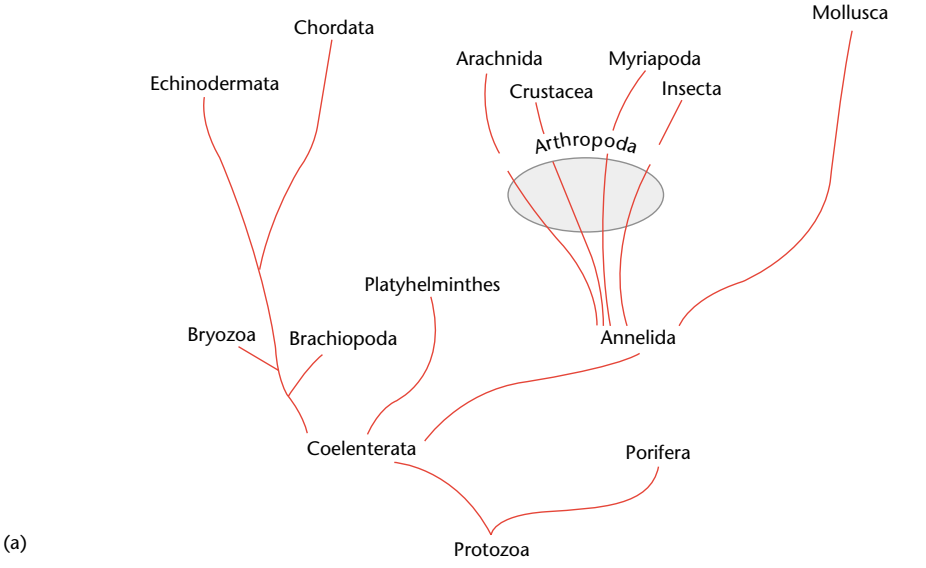
Vertebrate Immune Systems

Immunology is now a broad discipline, ranging in subject matter from the oft-quoted phagocytosis in unicellular invertebrates to antibody synthesis even in the most primitive species of the present-day vertebrates, the cyclostomes. In the context of comparative immunology, it is essential now to encompass major themes, perhaps inherited during evolution, in order to piece together this enormous mosaic of responses. Advanced invertebrate lines diverged at the level of the coelenterates. The chordate line, to which vertebrates belong, consists of animals characterized by the deuterostomate pattern of embryogenesis. In such a pattern, the blastula possesses two separate openings that become the mouth and anus.

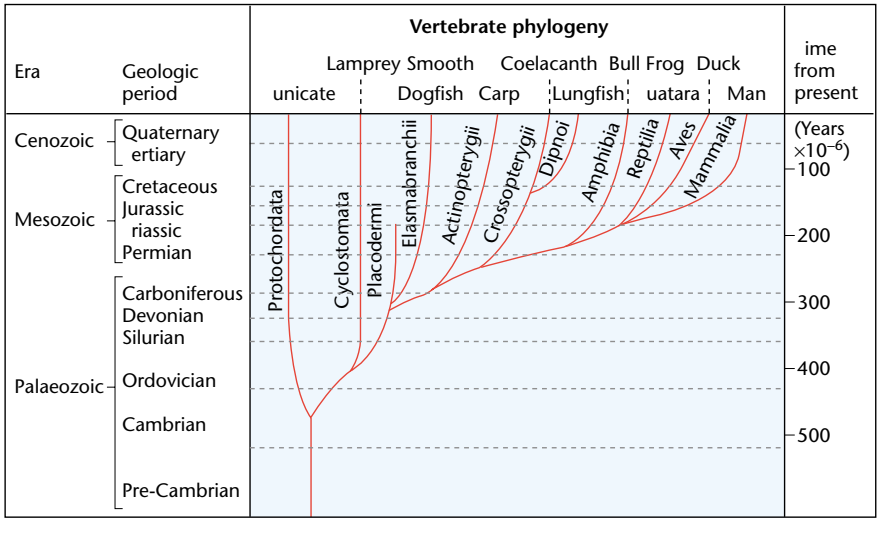
Echinoderms such as the sea stars share this trait with vertebrates. Thus, study of the evolution of immunity through this line may have direct bearing on how the vertebrate immune system evolved. By contrast, most other invertebrates belong to a group referred to as the protostomates. During blastogenesis, it is the blastopore alone that splits to form the mouth and the anus of either an adult or larval form depending on the animal species.

The phylum Chordata consists of two important subphyla for our purposes: the protochordates (tunicates) and the Vertebrata (vertebrates, including humans). Protochordates share certain developmental characteristics with vertebrates. They possess a dorsal hollow nerve cord, a notochord and, at some stage during their life histories, pharyngeal gill slits. (This pharyngeal gill region is of great interest when we consider homology, since lymphocytes are found there in protochordates and it is the origin of the thymus in vertebrates.)

The earliest vertebrates were the ostracoderms, fish-like forms that lacked jaws, paired fins and a single dorsal nostril and occurred during the Ordovician period some 400 million years ago. Although these are now extinct, the nearest living relatives of the ostracoderms are the present-day hagfish and lamprey, jawless cyclostome fishes that apparently descended from two different ostracoderm types. True jaws first appeared among the placoderms,



(a)



(b)

Figure 5 The phylogenetic scheme of the animal kingdom. (a) Phylogeny of invertebrates. (b) Phylogeny of vertebrates. (From Cooper, 1976).

extinct fishes that arose during the Devonian period. The cartilaginous fishes (chondricthyes) and the bony fishes (osteichthyes) arose from different placoderm lines during the Devonian period (**Figure 5**). The crossopterygians were ancestral to the amphibians; the nearest living relative is the lungfish, order Dipnoi. The cotylosaurs were the stem-reptiles that arose from the labyrinthodont amphibians or the microsaur amphibians during the Carboniferous period. From the stem-reptiles there emerged two forms, the thecodonts and the therapsids, which gave rise to birds and mammals, respectively. We surmise that immune response patterns developed phylogenetically, based on the data gained from living or extant animals that are relatives of extinct species. Certainly the fossil record can tell us nothing about a functioning immune system. Thus, it is essential to examine present-day living forms in order to attempt a synthesis and to make informed deductions concerning origins.

We may now ask what the dominant themes were that coincided with the advent of vertebrates. Without listing the numerous cells, molecules, tissues and organs of the immune system, there were probably two major events: movement from water to land and development of the vertebrate body plan. First, there was the movement from an aquatic environment inhabited by fishes to the land when the amphibians emerged. All of the equipment for effecting immune responses was present in the sea, but the development of the so-called terrestrial body plan caused a tremendous change in the location and distribution of relevant cells, tissues and organs. Probably the greatest redistribution involved the sites for haematopoiesis. Bone marrow as we know it in terrestrial vertebrates did not exist in the sea. Instead, blood cell formation occurred in other sites that were retained but distributed differently. In this regard, amphibians, especially the anurans, were crucial in having developed long bones that housed bone marrow, which was then responsible for seeding the body with precursors of immunocompetent cells and their descendants. This body plan of haematopoietic sites housed in long bones was retained in reptiles, birds and mammals as a successful model. From then on there was probably no other major event such as the movement from water to land that could have affected the evolution of the immune system. The focus of the thymus became more pronounced, perhaps a relic from our protochordate ancestors in whom, in the pharyngeal region, there is strong evidence of lymphoid precursors. After all, the thymus in this region is essential for generating the master regulators of the immune response. This includes the helper, cytotoxic and suppressor cells so crucial in all of the immune responses asTlx