Immunity and the Invertebrates

The fabulously complex immune systems of humans and other mammals evolved over hundreds of millions of years—in sometimes surprising ways

by Gregory Beck and Gail S. Habicht

In December 1882 a 37-year-old Russian zoologist named Élie Metchnikoff took a fateful stroll along the beach in Messina, a town on Sicily's northeastern coast. Returning to his cottage with the tiny, transparent larva of a common starfish, he pierced the creature with a rose thorn. When he examined it the next morning, he saw minute cells covering the thorn and attempting to engulf it.

He immediately recognized the significance of this observation—the cells were attempting to defend the larva by ingesting the invader, a process known as phagocytosis. Phagocytosis was already known to occur when certain specialized human cells encountered bacteria or yeast, but Metchnikoff's great realization was that phagocytosis actually plays a much broader role. It is in fact a fundamental mechanism by which creatures throughout the animal kingdom defend themselves against infection. With this keen insight and a subsequent lifetime of research, Metchnikoff created the discipline of cellular immunology. For this pioneering work, he shared the 1908 Nobel Prize in medicine with Paul Ehrlich, an early proponent of the importance of the other fundamental component of immunity, known as humoral immunity.

Impressive as Metchnikoff's achievement was, it was not the extent of his accomplishments. Significantly, his landmark experiment's subject, the starfish, was an animal that had remained virtually unchanged since its appearance at least 600 million years ago. After he punctured the starfish, Metchnikoff viewed a spectacle that was not much different on that December day in Sicily than it would have been in the earth's primordial sea tens of millions of years *before* the first living things with backbones—vertebrates—appeared.

Metchnikoff was well aware of this fact, and his studies would eventually show that the host defense systems of all modern animals have their roots in countless creatures that have populated this planet since life began. Thus was born yet another scientific discipline: comparative immunology. By studying various organisms—some very ancient—comparative immunologists gain unique insights that enable us to see from a different perspective one of the most complex and wondrous of all evolutionary creations: the immune systems of humans and other higher mammals. The discipline also often leads to insights into the nature of evolution itself: that invertebrates make up more than 90 percent of all the earth's species attests to the efficacy of their ostensibly "primitive" host defense mechanisms. Moreover, comparative immunology has enabled researchers to uncover several immune-related substances that seem to show promise for use in humans.

Recent advances in our knowledge and in the tools of immunology have engendered a fertile period in comparative immunology, a second golden age, as it were. Using molecular and cellular biological tools developed in recent years, researchers have built up an impressive body of knowledge on the host defense systems of such disparate animals as starfish, insects, sharks and frogs.

How Immunity Works

To appreciate fully the evolutionary twists and turns taken by immune systems over hundreds of millions of years, it is necessary to understand how they work. The most basic requirement of any immune system is distinguishing the cells, tissues and organs that are a legitimate part of the host body from foreign things, called "nonself," that

PHAGOCYTES attempt to engulf a rose thorn inserted into the transparent larva of a starfish. In 1882 the Russian zoologist Élie Metchnikoff (*photograph at right*) first noted this example of an innate host defense response. His subsequent studies established the field of cellular immunology.

might be present. The second job is to eliminate those nonself invaders, which are often dangerous bacteria or viruses. In addition, the immune system can recognize, and usually eliminate, "altered self"—cells or tissues that have been changed by injury or disease such as cancer. Most immunologists would agree that the immune systems of mammals, such as humans, have the most sophisticated mechanisms both for recognizing and for eliminating invaders.

Consider what happens when a weekend gardener pricks her finger on a rose thorn. Within minutes or immediately after the blood stops flowing, the immune system begins its work to eliminate undesirable microbes introduced with the wound. Already on the scene (or quick to arrive) are phagocytic white blood cells known as macrophages. These cells not only engulf and destroy any invading microbes but also release proteins that activate other parts of the immune system and alert other phagocytes that they may be needed.

This fast cellular response is sometimes called natural or innate immunity because the cells that execute it are already active in the body before an invader appears. All animals possess a defensive mechanism of this kind, which is believed to be the most ancient form of immunity. It was innate cellular immunity, for example, that Metchnikoff observed in that starfish larva.

Another component of innate immunity is known as complement. It is composed of more than 30 proteins in the blood. These proteins work in succession, in a kind of cascade, to identify and destroy invaders. Innate immunity usually suffices to destroy invading microbes. If it does not, vertebrates rely on another response: acquired immunity.

The soldiers of acquired immunity are the specialized white blood cells called lymphocytes that function together as an army. Moving through the blood and lymph glands, lymphocytes are normally at rest, but they become active and multiply if they encounter specific molecules called antigens that are associated with foreign organisms. Lymphocytes are of two classes—*B* and *T*. *B* lymphocytes secrete antibodies—defensive proteins that bind to antigens and help to eliminate them. The human body usually contains more than 100 billion *B* lymphocytes, each of which secretes an antibody that is different from most of the others. *T* lymphocytes serve a variety of purposes; they recognize and kill cells bearing nonself molecules on their surface, for example. They also help *B* lymphocytes produce antibodies.

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Acquired immunity is highly effective, but it takes days to mobilize because the response is so very complex. An invading microbe must come into contact with the right *T* or *B* lymphocytes; macrophages must be activated for assistance; the activated lymphocytes must divide; all the involved white blood cells must synthesize and release proteins that amplify the response; *B* cells must manufacture and release antibodies.

But acquired immunity also has a hallmark trait—immunologic memory—that can reduce the delay. Immunologic memory arises from the DNA-based mechanisms that allow the body's lymphocytes to recognize such a fabulous diversity of antigens even though each lymphocyte recognizes only one type of antigen. Essentially, each encounter with an invading microorganism stamps a genetic "blueprint" onto certain *B* and *T* cells. The next time these cells encounter that same invader, they use the blueprint in such a way that the response occurs faster and more powerfully than it did the first time. This phenomenon is what makes possible the familiar booster shots, or immunizations, given to children. The gardener of our example may with time forget her trivial cut, but her immune system never will.

In the Beginning

As we have described, the immune systems of such higher vertebrates as mammals can be broken down into two major types of response: innate and acquired. The latter includes immunologic memory as a significant, distinguishing characteristic. The responses are mediated by many different agents: macrophages and other phagocytic cells, *B* and *T* lymphocytes, antibodies and a multitude of other participating proteins. One of the central questions of comparative immunology is: How many of these features—or similar ones—appear in other, older groups of organisms?

Quite a few of them do; in fact, certain elements of immunity are detectable in almost all living things (phagocytosis is an example). Through the aeons, some of these elements carried over basically unchanged from one creature to the next. Other features are unique to higher vertebrates but bear intriguing similarities to aspects of invertebrate host defense systems. These similarities are important because they suggest that the invertebrate mechanisms are precursors of the corresponding later, vertebrate ones. Collectively, these links may be the most compelling evidence that the immune systems of humans and other mammals evolved from more ancient creatures over hundreds of millions of years.

Not surprisingly, host defense systems began when life did: with the protozoans, the simplest of all living organisms. Protozoans, which go back about 2.5 billion years, are single-cell life-forms; in other words, they accomplish every physiological function in just one cell.

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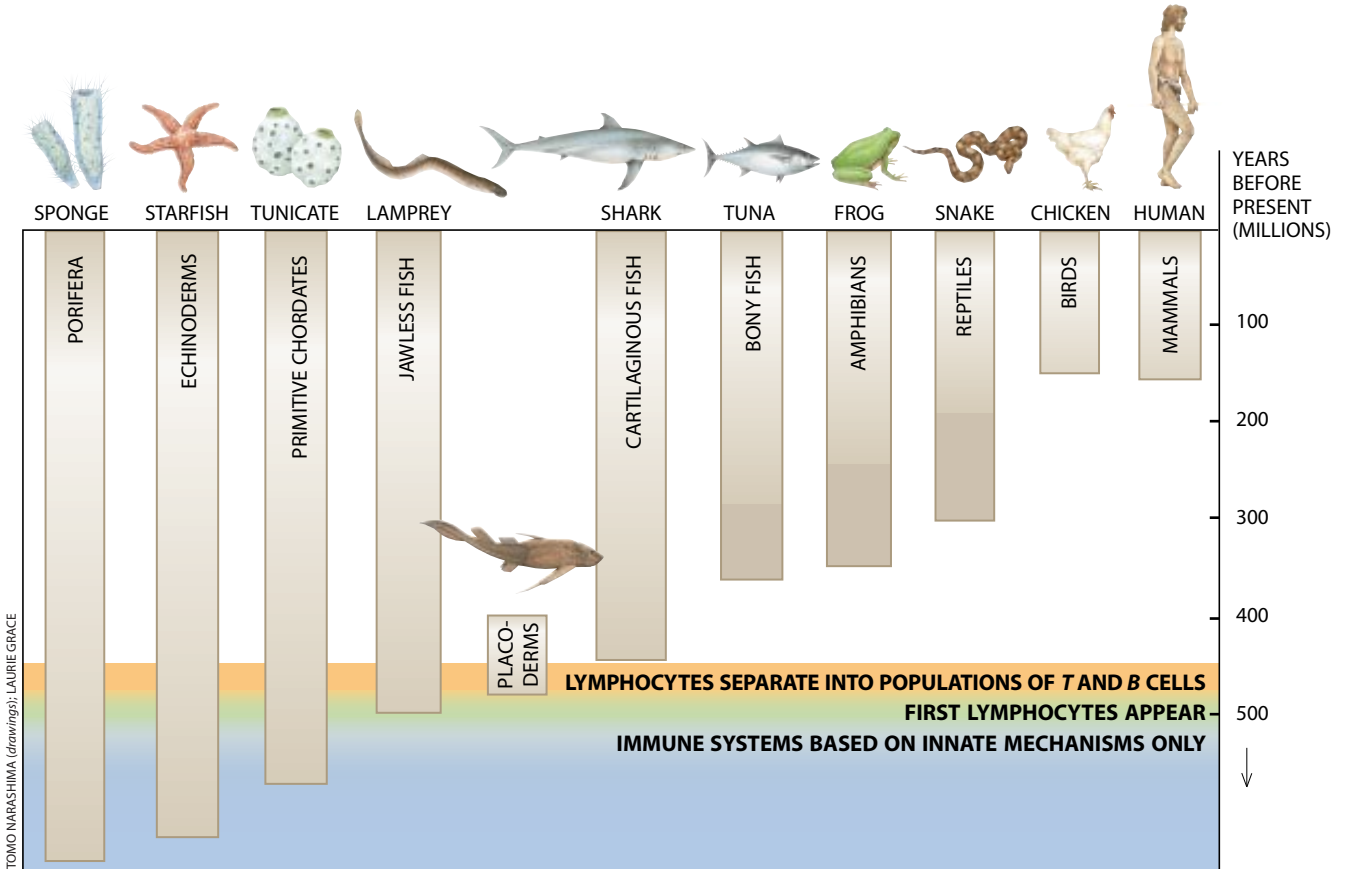


IMMUNOLOGIC MILESTONES occurred around the time that the first creatures with backbones (vertebrates) appeared. One of the most important of these milestones was the emergence of the first immune systems based on lymphocytes, possibly in jawless fish roughly 500 million years ago. These lymphocytes then separated into the two distinct populations discernible in all higher vertebrates. Invertebrates have a family of cells that resemble vertebrate immune cells in some respects.

In protozoans, respiration, digestion, defense and other functions are performed, at least in part, by phagocytosis. In its defensive function, protozoan phagocytosis is not very different from that accomplished by the phagocytic cells found in humans.

In animals ranging from starfish to humans, phagocytic cells travel through a circulatory system or (in the case of starfish) through a fluid-filled body cavity, or coelom. In multicellular animals that lack a body cavity and a circulatory system (such as sea sponges), the wandering phagocytic cells patrol the tissues and surrounding spaces.

Another fundamental aspect of immunity—the ability to distinguish self from nonself—also dates back to early in life's history. Some protozoans live in colonies of thousands of creatures and must be able to recognize one another. It is difficult to conceive of how either life in a colony or sexual reproduction could occur without the ability to distinguish self from nonself; thus, it is very likely that protozoans have this ability. Even the sponge, which in the view of some scientists is the oldest and simplest metazoan (multicellular animal), can distinguish self from nonself: its cells attack grafts from other sponges.

This rejection response is not identical to that found in vertebrates, however. In vertebrates, because of immunologic memory, if one graft from a donor is rejected, a second graft from the same donor will be rejected more quickly. In sponges and jellyfish, however, the second rejection is no faster than the first. These results suggest that the memory component of the immune response, a cornerstone of the vertebrate system, is missing. This conclusion is supported by experiments with starfish and other higher invertebrates, which also lack immunologic memory.

Two other features of the vertebrate immune system—complement and lymphocytes—are also missing from invertebrates, but for both there seem to be invertebrate analogues. In place of complement, several phyla of invertebrates, including various insects, crabs and worms, exhibit a similar response, called the prophenoloxidase (proPO) system. Like the complement system, proPO is activated by a series of enzymes. A cascade of reactions ends with the conversion of proPO to the fully active enzyme phenoloxidase, which plays a role in encapsulating foreign objects. Kenneth Söderhäll of the University of Uppsala in Sweden and Valerie J. Smith of Gatty Marine Laboratory in Scotland have shown that the system serves other purposes as well, including blood coagulation and the killing of microbes.

Invertebrates lack lymphocytes and an antibody-based humoral immune system. Nevertheless, they do have mechanisms that seem to be precursors of those aspects of vertebrate immunity. For example, lymphocytelike cells have been found in earthworms—which probably appeared 500 million years ago. Perhaps more significantly, all invertebrates have molecules that appear to function much like antibodies and may be their forerunners. These molecules, a group of proteins called lectins, can bind to sugar molecules on cells, thereby making the cells sticky and causing them to clump. Lectins must have evolved quite early because they are ubiquitous; they are found in plants, bacteria and vertebrates, in addition to invertebrates.

The role of lectins in immune responses is not known exactly; they appear to play a part in tagging invading organisms, which are probably covered with different sugar molecules. Lectins isolated from earthworms, snails, clams and virtually every other invertebrate animal participate in the coating of foreign particles, thus enhancing phagocytosis. Numerous lectins with different sugar specificities can be found in each animal phylum. Lectins isolated from the flesh fly, *Sarcophaga peregrina,* and from the sea urchin are related to a family of vertebrate proteins called collectins. In humans, collectins serve important roles in innate immunity by coating microbes so they can be more easily identified by phagocytes and by activating immune cells or complement.

And although antibodies are not found in invertebrates, molecules that are structurally and even functionally similar to them are. Antibodies (also known as immunoglobulins) belong to a very large, very old family of molecules—the immunoglobulin superfamily. Molecules in this group all have a characteristic structure called the Ig fold. They serve diverse functions but in general are involved with recognizing nonself as well as other types of molecules.

The Ig fold probably emerged during the evolution of metazoan animals, when it became necessary for specialized cells to recognize one another. The fold could have originally been a pattern-recognition molecule involved in identification of self; later, it evolved into something that could recognize antigens as well, setting the stage for the emergence of true immunoglobulins.

Hemolin, a protein isolated from the blood of moths, is a member of the immunoglobulin superfamily. It binds to microbial surfaces and participates in their removal. Studies have identified other superfamily molecules in several invertebrates (grasshoppers and flies), as well as in lower vertebrates. These observations suggest that antibody-based immune responses, though restricted to vertebrates, have their roots in invertebrate defense mechanisms.

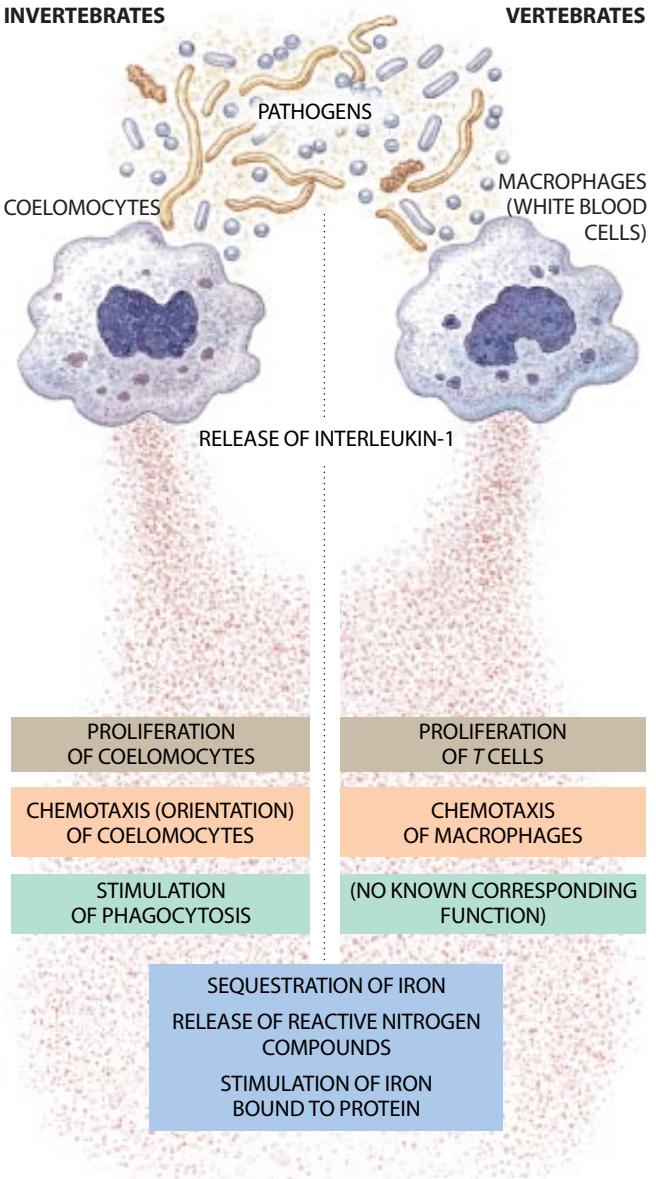
Precursor of Immune Regulation

Evolution seems to have conserved not only many aspects of host defense mechanisms found in invertebrates but also many of the control signals for these mechanisms. Our own work has recently focused on isolating molecules in invertebrates that resemble the cytokines of vertebrates. Cytokines are proteins released by various activated immune (and nonimmune) cells that can either stimulate or inhibit other cells of the immune system and have effects on other organs as well. Cytokines include the interferons, the interleukins (such as IL-1 and IL-6) and tumor necrosis factor (TNF). These molecules are critical regulators of every aspect of vertebrate immunity.

We suspected that invertebrates would have IL-1 or a similar ancestral cytokine for several reasons. First, these molecules regulate some of the most primitive mechanisms of vertebrate immunity. Second, the structure and defensive functions of IL-1 are similar in many different vertebrates, suggesting that the molecules evolved from a common precursor. Finally, macrophages, the type of white blood cells that produce IL-1, are ubiquitous throughout the animal kingdom.

From the coelomic fluid of the common Atlantic starfish *Asterias forbesi,* we isolated a protein that behaved like IL-1 in many respects: its physical, chemical and biological properties were the same; it stimulated vertebrate cells responsive to IL-1; and antibodies that recognized human IL-1 recognized this protein, too. Subsequently, we have found that many invertebrates possess molecules related to vertebrate cytokines. Worms and tunicates (sea squirts) carry substances similar to IL-1 and TNF. One of us (Beck) has found molecules resembling IL-1 and IL-6 in the tobacco hornworm. Thus, invertebrates possess correlates of the three major vertebrate cytokines.

The invertebrate cytokines seem to perform functions similar to those in vertebrates [*see illustration at left*]. We found that in starfish, cells called coelomocytes (the equivalent of macrophages) produce IL-1. In experiments conducted with Edwin L. Cooper of the University of California at Los Angeles and David A. Raftos, now at the University of Sydney, we showed that IL-1 stimulated these macrophage equivalents to engulf and destroy invaders. Invertebrate cyto-



CYTOKINE RELEASE can stimulate many functions in invertebrates and vertebrates alike, including dozens aimed at defending the host. Recently the authors found that the cytokine interleukin-1 serves defensive functions in the starfish that are either analogous or identical to those it serves in vertebrates.

kines therefore appear to orchestrate much of their host's defensive response, just as vertebrate cytokines do in innate immunity.

Medicine from a Frog

Comparative immunology does not consist solely of looking for the analogues of vertebrate defenses in invertebrates. On the contrary, studies of invertebrates have sometimes uncovered novel types of defenses that were only later identified in vertebrates as well.

For instance, key defensive molecules in invertebrates are the antibacterial peptides and proteins. These molecules—some of which have potentially significant applications as medications for humans—are usually released from an organism's blood cells early on in the innate response. The most widespread antibacterial protein isolated from invertebrates is lysozyme, which was also the first to be isolated. Insects produce lysozyme when infection sets in or when exposed to proteins that make up bacterial cell walls. Interestingly, lysozyme is also part of the innate defense in humans. For example, in saliva it acts to defend the oral cavity against bacteria.

In 1979 a group at the University of Stockholm led by Hans G. Boman discovered peptides with bacteria-eliminating properties in the silk moth, *Hyalophora cecropia*. This class of peptides, which they named cecropins, can kill bacteria at concentrations low enough to be harmless to animal cells. They act by perforating the bacteria, causing the cells to burst. Recently five different molecules related to cecropins were isolated from the upper part of the pig intestine, where they help to regulate the bacterial contents of that animal's digestive tract. They are currently being developed as antibacterial agents for use in humans.

Jules A. Hoffmann and his colleagues at the CNRS Research Unit in Strasbourg have been studying another group of antibacterial peptides, called defensins, in insects. Defensins have been isolated from several insect orders and appear to be the most common group of inducible antibacterial peptides. Like cecropins, defensins are relatively small protein molecules. Unlike cecropins, the way in which they kill bacteria is not well understood. Mammalian defensins are also small but have little else in common with insect defensins. These facts suggest that small antibacterial peptides are a fundamental part of the animal front line of rapidly deployed defenses.

Lower vertebrate species are yielding unique host defense molecules as well. In 1987 Michael Zasloff, then at the National Institutes of Health, noticed that African clawed frogs required no antibiotics or other treatments to completely recover from nonsterile surgery—in spite of the fact that they recuperated in bacteria-laden water. Searching for the source of this extraordinary protection, he eventually isolated two peptides—which he termed magainins 1 and 2—from frog skin. ("Magainin" is derived from the Hebrew word for shield.) The compounds exhibit a broad range of activities against bacteria, fungi and protozoa. Antibodies that bind to magainin also bind to cells of human epithelial tissues, such as the skin and intestinal lining, which suggests that humans synthesize similar molecules as a first-line defense against pathogens.

Pervasive Legacy

The fact that peptide antibiotics (along with other unique host defense strategies) were originally discovered in invertebrates may help stimulate the study of esoteric defense systems that have languished in the "tidal pools" of immunology research. Who knows how many potentially lifesaving compounds remain to be discovered?

It is surprising that so little attention has been focused on the host defense systems of invertebrates, because such studies pay off in so many ways. New and diverse defense functions characteristic of all living things are being discovered, and by looking at ancient, ancestral organisms, we are learning about their evolutionary descendants as well.

In the end, the intricacies of the vertebrate immune response can only be fully understood by analyzing less complex systems, such as those found in invertebrates. Surely this work has extensive implications for understanding not only basic evolution but also more immediate problems of human health and disease. In these and other endeavors, Metchnikoff's legacy is pervasive. SA

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GREGORY BECK and GAIL S. HABICHT began working together in 1989, when Beck was a graduate student and Habicht his thesis adviser at the State University of New York at Stony Brook. Beck is now assistant professor of biology at the University of Massachusetts at Boston. He earned a B.S. from S.U.N.Y. at Albany in 1982 and his Ph.D. from S.U.N.Y. at Stony Brook in 1994. Habicht, vice president for research and professor of pathology at Stony Brook, earned her Ph.D. from Stanford University in 1965. She held postdoctoral positions, first at the Rockefeller University and then at the Scripps Clinic and Research Foundation, between 1965 and 1971, when she moved to Stony Brook. This is their second article for *Scientific American*.

Further Reading

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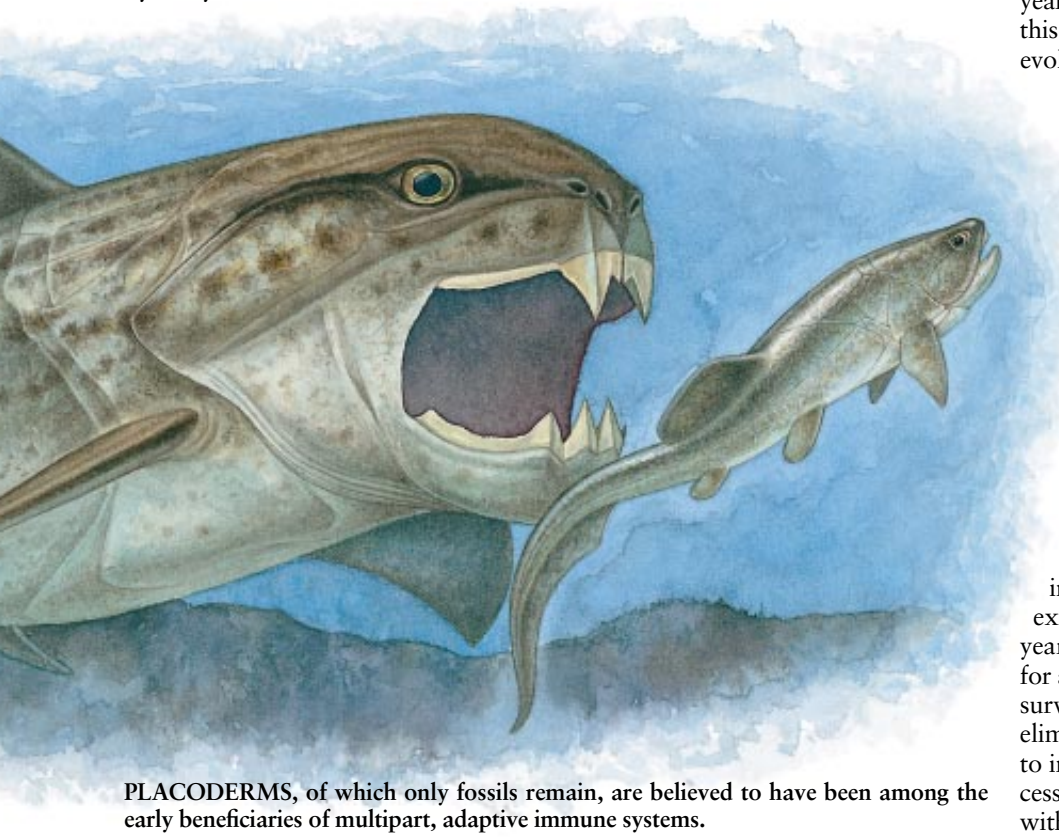
Sharks and the Origins of Vertebrate Immunity

Sharks, which have existed for as many as 450 million years, offer glimpses of a distant period in the evolution of the immune system

by Gary W. Litman



PLACODERMS, of which only fossils remain, are believed to have been among the early beneficiaries of multipart, adaptive immune systems.

Some 500 million years ago the ancestor of all jawed vertebrates emerged in the warm waters of the earth's vast primordial sea. Although its identity is shrouded in mystery, some paleontologists believe that this ancestor resembled certain members of a later group of fish known as placoderms, which are known, at least, from the fossils they left behind. These ungainly creatures, some of which apparently grew to lengths of about seven meters, had a head and pectoral region encased in protective bony plates.

A living placoderm, or one of the other possible ancient vertebrate forerunners, would of course add immeasurably to our understanding of evolution. Perhaps most significantly, we would be able to see the workings of one of the most complex of bodily constituents—the immune system—that existed shortly after some vertebrates made the critical transition from jawless to jawed form. The transition is a key one in evolution because it is a link in the course leading to more advanced animals, including those that eventually crawled onto land and evolved into humans. It is likely that multicomponent, adaptive immune systems began with the first vertebrates. The immune systems of surviving invertebrates, which are probably similar to those of ancient ones, do not have the remarkable adaptive capabilities of vertebrate immunity.

Although the placoderms and their ancestors are long gone, we do have the next best thing: several of their phylogenetic relations, including sharks, skates, rays and ratfishes. These creatures—with immune systems that have also probably changed little if at all since their earliest appearance hundreds of millions of years ago—may provide a window onto this distant and extraordinary period in evolution.

During the past several years, my colleagues and I have studied the immune systems of some of these creatures. As might be expected, immunity in these living fossils is different from that in such later animals as frogs, monkeys and humans. Yet intriguingly, when it comes to protecting their hosts against disease, infection and other ills, these ancient immune systems appear to be every bit as effective—if not more so—than their more modern counterparts.

Perhaps this is not surprising; the subclass of elasmobranchs, which includes sharks, skates and rays, has existed for as many as 450 million years (*Homo sapiens* has been around for approximately half a million years), surviving several mass extinctions that eliminated countless species. It is hard to imagine how such evolutionary success could have occurred in creatures with immune systems that were anything less than unusually effective. Our efforts to identify the features that have made elasmobranch immunity so successful have had a valuable side benefit: insights into human immunity.

The Two Parts of Immunity

The adaptive immune system has two basic parts, called humoral and cellular. The agents of humoral immunity are known as *B* lymphocytes, or *B* cells. *B* cells produce protein molecules, or antibodies, that bind to foreign substanc-

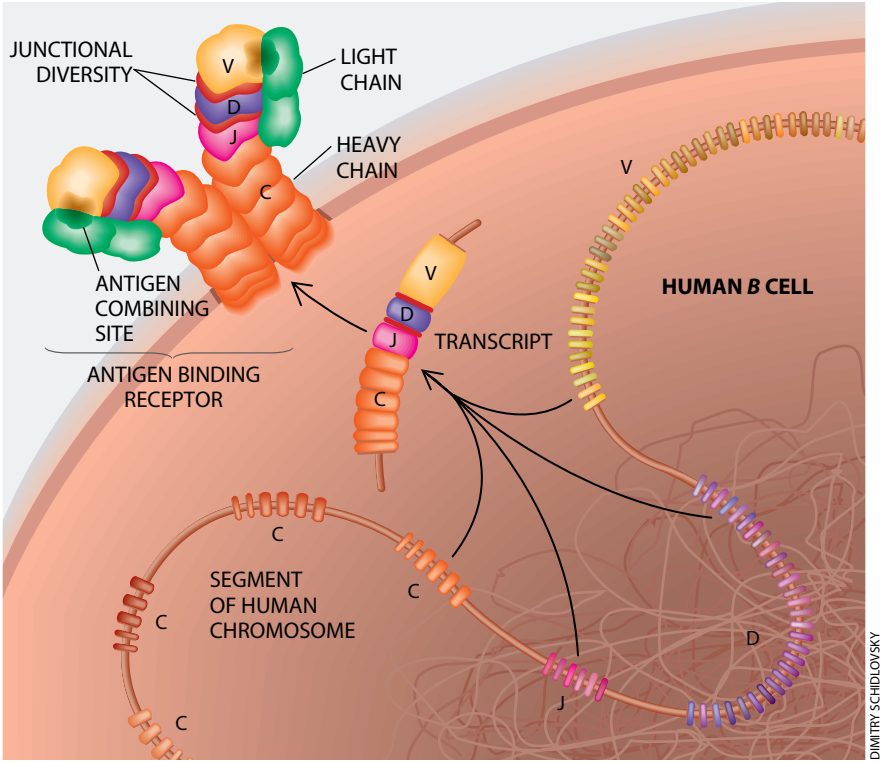
es, or antigens, on potentially harmful bacteria and viruses in the bloodstream. This binding enables other bodily entities to destroy the bacteria and viruses by various means. Antibodies are also known as immunoglobulins; humans have five major types of them.

All the antibodies on a single *B* cell are of the same type and bind to a specific antigen. If this antibody encounters and binds to its corresponding antigen, the *B* cell is stimulated to reproduce and to secrete its antibody. Most of the human body's billions of *B* cells make antibodies that are different from one another, because during the formation of each *B* cell a genetic process that has both random and inherited components programs the cell to produce a largely unique "receptor"—the part of the antibody that actually binds to the antigen. It is this incredible diversity among antigen receptors that gives such vast range to humoral immunity.

Cellular immunity is carried out by a different group of immune cells, termed *T* lymphocytes, or *T* cells. In contrast to *B* cells, *T* cells do not produce antibodies; rather they recognize antigens bound to a type of molecule on the surface of a different kind of cell. For this purpose, they are equipped with a specialized class of molecule, called a receptor. Typical manifestations of *T* cells at work include such diverse phenomena as the rejection of a foreign skin graft and the killing of tumor cells.

Antibodies, or immunoglobulins, and *T* cell receptors are the primary means by which the body can recognize specific antigens. Although humoral and cellular immunity have basically different functions and purposes, they interact during an immune response. *T* cells, for example, help to regulate the function of *B* cells.

In some ways, shark and skate immunity is similar to that of humans. These fish have a spleen, which, as in humans, is a rich source of *B* cells. When a shark is immunized—that is to say, injected with an antigen—*B* cells respond by producing antibodies. The similarities extend to cellular immunity. Like humans, sharks and skates have a thymus, in which *T* cells mature and from which they are released. Sharks also have *T* cell receptors. Recent work by me and Jonathan P. Rast, now at the California Institute of Technology, showed that, as in humans, diversity in these receptors arises from the same kind of genetic mechanisms that give rise to antibody diversity. Finally, skin grafted from one shark to another ultimately results in rejection.



HUMAN AND SHARK ANTIBODY GENE SYSTEMS have striking differences in the arrangement of the gene segments that recombine to specify an antigen binding receptor. Shown here is a simplified version of the process that specifies the "heavy-chain" molecule that makes up part of the antigen binding receptor. The receptor is part of a large antibody molecule known as IgM, which actually has five such recep-

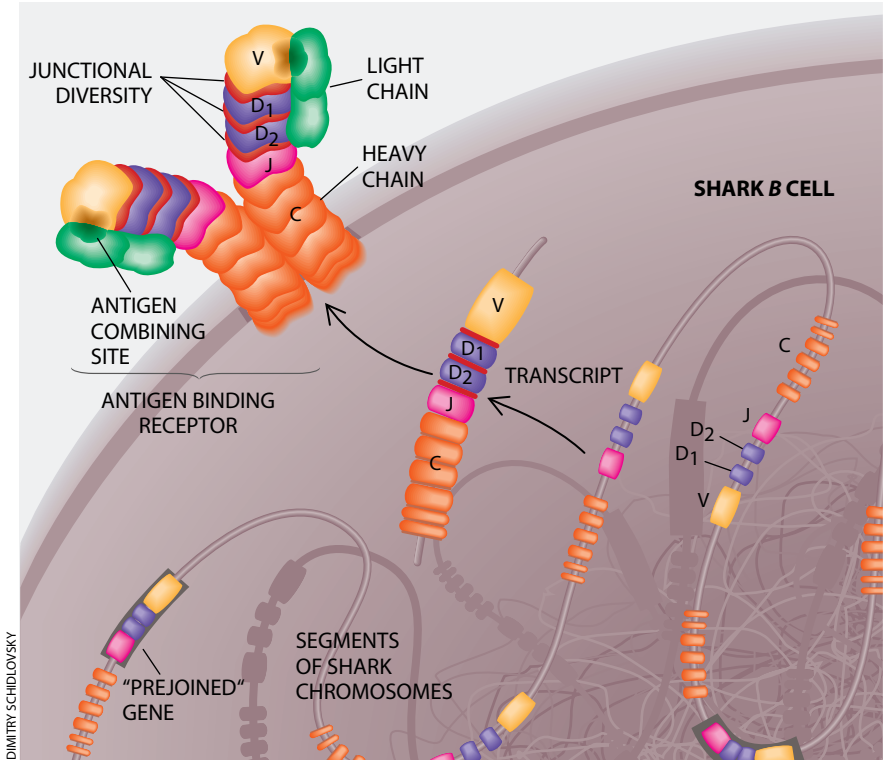
These similarities notwithstanding, there are some significant and fascinating differences between the immune systems of such cartilaginous fish as sharks and of humans. For example, cartilaginous fish have four different classes of immunoglobulin, only one of which is also among the five major types in humans. Furthermore, these shark antibodies lack the exquisite specificity that permits the recognition of, among other things, the subtle differences between two similar types of bacteria.

In addition, these antibodies lack the capacity of human antibodies to bind more and more strongly to an antigen during the course of a prolonged immune response—a decided advantage in fighting infection. A difference in cellular immunity is implied by the fact that sharks do not reject skin grafts vigorously and quickly, as humans do, but rather over a period of weeks.

Do these facts mean that the immune systems of sharks and skates are less suited to the needs of the host in comparison with those of humans and other mammals? Not at all. Indeed, the idiosyncratic nature of this ancient immune system illustrates well the twists and turns that occurred during the evolution of immunity. This sinuous course, moreover, suggests that evolution, at least where the immune system is concerned, may not have always proceeded in the inexorable, successive way in which it is often portrayed.

A Receptor for Every Antigen

Much of our work so far has been devoted to elucidating the humoral immune system of the horned shark, a spotted creature that usually grows to about a half meter in length. In this animal, as in all vertebrates, the diversity in antigen receptors has a genetic basis. Specifically, each antibody's antigen receptor is formed through the interactions between two amino acid chains, which are protein molecules, characterized as heavy and light. With few exceptions, the basic antibody molecule has two pairs of such chains and therefore two antigen receptor sites. Exactly which antigen a receptor will bind to depends on the type and arrangement



tors; it is the only antibody that humans and sharks have in common. In humans the gene segments that come together to specify the receptor are scattered along a relatively long length of one chromosome. In sharks the gene segments are already next to one another as a kind of package that can be on any one of several chromosomes. For simplicity, the details of the multistage transcripting process have been omitted.

of the amino acids in the chains that make up the receptor.

Regardless of where they are produced in the body, amino acid chains are created in cells and specified by genes—which act as a kind of blueprint—in the cell's nucleus. In the case of an antigen receptor, the amino acid chain is specified by gene segments, also known as antibody genes, in the *B* cell's nucleus. There are three types of gene segments for this purpose; they are designated V ("variable"), D ("diverse") and J ("joining"). The amino acids in the heavy chain are specified by all three types of gene segments; the light chain is encoded by the V and J only. A fourth type of gene segment, designated C ("constant"), determines the class of antibody.

In humans the functional V, D, J and C segments are found on a single chromosome. As in most higher vertebrates, the segments are located in clusters, with, for example, some 50 functional V, 30 D, six J and eight C elements in a single location, occupying roughly a million components, or "rungs," of the DNA molecular "ladder." (These rungs are the base pairs.) When a *B* cell's gene-reading mechanisms produce an antibody, various cellular entities first recombine single V, D and J segments adjacent to a C segment in a multistep process. This genetic material is then "read out" to the cell's protein-making systems. The recombination of these gene segments determines the antigen-binding characteristics of the antibody. In such higher vertebrates as humans, this joining of different V, D and J elements, which is called combinatorial diversity, is an important factor in antigen receptor diversity.

In sharks, too, antibody gene segments are organized in clusters. A shark heavy-chain cluster, however, contains only one V segment, two Ds, a single J and a single C. There are more than 100 such clusters, distributed on several different shark chromosomes. When the protein-making machinery in one of the shark's *B* cells produces an antibody, only the four gene segments (V, D1, D2 and J) from a single cluster are recombined (the C segment is already linked to the J). As in the mammalian case, their genetic message is read out and translated into a protein that makes up an antigen receptor.

Does the recombination of only the V, D1, D2 and J elements found in one cluster limit the shark immune system's ability to produce a great diversity of antigen receptors? It probably would, except (as mentioned earlier) there are hundreds of different antibody gene clusters spread over several different shark chromosomes. Furthermore, neither the shark nor mammalian immune systems depend solely on combinatorial diversity to generate many different antibodies. In fact, in sharks and other cartilaginous fish, two other phenomena are much more significant in fostering this diversity; they are termed junctional diversity and inherited diversity.

Where Diversity Comes From

To understand junctional diversity, we must return to the joining of V, D and J gene segments that specifies an antigen receptor chain. Junctional diversity occurs when, say, V and D or D and J segments come together. At the joining boundary where the two segments unite, before their actual fusing, several DNA base pairs are removed, and new bases are added in a nearly random manner. This localized alteration in genetic content ultimately changes the amino acid sequence and therefore the characteristics of the antigen receptors that are created.

Therein lies the real advantage of the extra D gene segment in the shark antibody-producing system. With four different gene segments, there are three places where this diversity can occur: between V and D1, between D1 and D2, and between D2 and J. Thanks to junctional diversity, millions of different variants of an antibody molecule, each possessing slightly different receptor structures, can be created from each cluster. In mammals, on the other hand, junctional diversity can occur typically in only two locations: between V and D segments and between D and J. Therefore, junctional diversity leads to somewhat less variation in mammals.

This ability to generate many different antibodies is conceptually attractive for protection against a vast array of foreign invaders. But a large—and potentially fatal—gap exists between the ability to generate antibody diversity and the efficient use of this diversity. In light of this fact, junctional diversity is a double-edged sword: in theory, it can generate enough antibody specificity to handle almost any situation. Yet broadly speaking, it could in practice take too

much time to generate enough antibodies, select the best ones, expand their numbers and then deal with the invading pathogen; in other words, the host could lose a race with the infectious agent.

To try to keep the host from losing that race, the body relies on mechanisms that rapidly select the "blueprint" of the immediately needed antibody gene. This blueprint is first expressed by one *B* cell among the body's billions. In mammals, specialized cellular compartments and complex intercellular communications mobilize and expand the immune system for this purpose.

Sharks, on the other hand, rely heavily on a form of inherited diversity. This form, the most distinctive feature of the shark immune system, allows the animals to avoid depending on a chance occurrence—for example, a fortuitous combination of DNA base pairs attained through junctional diversity—to generate the right antigen receptor at the right time. In a shark, a large percentage of the gene clusters in every cell are inherited with their V, D1, D2 and J gene segments already entirely or partially "prejoined."

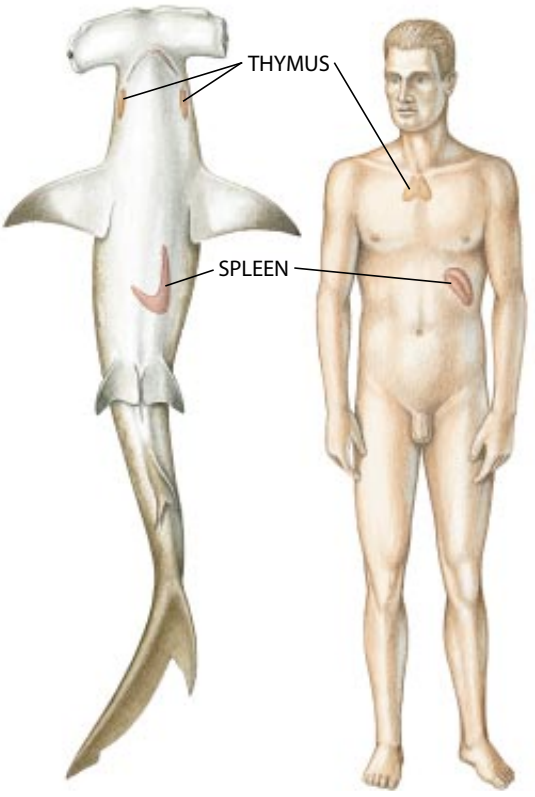
In such clusters, there is limited capacity, or none at all, for junctional diversification. Analyses of hundreds of these prejoined or partially prejoined clusters have shown their gene segments to be remarkably similar to those of ordinary clusters, suggesting that one type derived from the other at some point in evolution.

But why? As in so many areas, our knowledge of genetic mechanisms has far surpassed an understanding of their relation to function. Still, it would be entirely reasonable to theorize that the humoral immune systems of cartilaginous fish have evolved to combine the best of two possibilities: a large number of genes that can recombine and thus provide immunologic flexibility, as well as some genes with fixed specificities that can be mobilized quickly to make antibodies against pathogens that these species encounter all the time.

Combinatorial, junctional and inherited forms of diversity are not the extent of diversity-producing mechanisms. In addition, the two types of gene clusters undergo additional change through mutation, which occurs at a very high frequency in the antibody genes of higher vertebrates. These mutations are directed at altering the characteristics of the antigen receptor sites of antibodies.

One interesting conclusion from a comparison of human and shark humoral immunity is that some 450 million years of evolution did relatively little to change the molecules of antibody immunity; the protein structures of shark and human antibodies are very similar. Moreover, the V, D and J sequences of gene segments that specify the creation of antibodies are similar. What evolution did radically alter is the way these gene segments that specify antibodies are organized; it placed greater emphasis on junctional and especially inherited diversity in sharks, for example. Though relatively simple, the mechanisms of genetic diversification in the shark's immune system seem in many ways more efficient than those in such higher vertebrates as humans.

This finding confirms, not surprisingly, that evolution has a way of uniquely adapting systems to their hosts' immediate needs. In the case of immunity, evolution also has to provide for unexpected challenges as well. The surprise is that in order to make that efficiency possible, enigmatic evolutionary leaps of uncharacteristic magnitude apparently sometimes occur, at least in antibody immunity, over relatively short periods.



ROBERTO OSTI

SHARKS AND HUMANS share a number of immunologic features, including a thymus and a spleen.

Cellular Immunity

Many of the basic principles put forth in the discussion so far—the rearrangement of widely spaced gene segments scattered along a stretch of chromosome and the reading out and alteration of their genetic information to specify the creation of antigen receptors made up of amino acid chains—apply to cellular as well as humoral immunity. After all, *T* cells, just like the antibodies secreted by *B* cells, must also recognize and bind to an almost limitless assortment of antigens.

T cells and antibodies both have receptors that are specified by similar gene segments. The basic mechanisms of gene segment reassembly that produce antibody molecules also create *T* cell receptors. But a *T* cell receptor is found only on the cell's surface and only recognizes foreign material bound to a specialized molecule on a different cell. *T* cells' affinities for foreign materials are low in comparison to some antibodies, and they do not undergo mutation in the same manner as antibodies.

In the past, many immunologists believed that cellular immunity predated humoral immunity. Yet the aforementioned chronic nature of skin graft rejection in sharks suggests that, if anything, cellular immunity in the shark is not robust and possibly lacks specificity. This notion, in turn, implied to some observers that sharks do not have *T* cells.

In order to test this hypothesis, my colleagues and I set about determining whether the horned shark has *T* cells. Unequivocal proof of the existence of *T* cells requires identification of their antigen receptors. For this purpose, the conventional approaches available until recently were inadequate. The breakthrough came with the development several years ago of a technique known as the polymerase chain reaction (PCR), which can produce millions of copies of a small section of DNA. We used a form of the PCR technique as part of a process that produced great numbers of *T* cell receptor genes in order to character-

ize them. Recently we found all four classes of mammalian *T* cell antigen receptors in the skate and have evidence suggesting their presence in the shark.

Extensive characterization of one of the classes of shark *T* cell receptors showed it to be about as complexly diversified as its human equivalent. This finding surprised us, indicating that in contrast to antibody gene organization, *T* cell receptor genes seem to have undergone no major changes since the time of the divergence of the sharks from the evolutionary line leading to the mammals some 450 million years ago. The antibody gene system and the *T* cell receptor gene system may well have diverged from a common ancestor that more closely resembled the latter, although the opposite can also be argued—that it was an antibody-gene-like ancestor that gave rise to both categories of immune gene systems.

As the genomes of sharks and their relatives continue to be characterized, we now recognize a variety of different gene clusters. For example, a group led by Martin F. Flajnik at the University of Miami recently found gene clusters that resemble those of both antibodies and *T* cell receptors. Intriguingly, the genes in these clusters undergo extraordinary rates of mutation.

Ongoing studies have also suggested that immune system genes from different clusters have "mixed and matched" with one another during evolution. With hundreds of clusters and plenty of genetic backup, exchange between clusters may have been a very efficient means of generating novel gene clusters. It is quite possible, too, that our continuing studies will identify even more receptors in the shark immune system.

With respect to this exchange among different clusters, the peculiar redundancy of different immune receptor gene clusters in the shark—the groupings of essentially identical V, D1, D2 and J segments repeated over and over on various chromosomes—can be seen in an entirely new light. In short, this recombination, along with other unique features of the shark's genetic mechanisms, affords a means for rapidly evolving new families of receptor molecules. In mammals the gene segments are isolated to single chromosomes, and little structural redundancy is evident; these facts mean that the opportunity for this type of recombination is remote.

Furthermore, duplication of gene segments—the existence of multiple Vs, Ds and Js, a hallmark of the mammalian immune system—appears to come at the price of introduction and retention of significant numbers of nonfunctional genetic elements. In sharks and skates, on the other hand, nonfunctional elements are uncommon and probably are lost quickly from the genome.

As surviving representatives of a very ancient line, sharks, skates and their relations may be our only remaining link to the distant origins of *T* and *B* cell immunity. These fish offer a unique glimpse of a pivotal moment in the course of evolution. Through this window we may someday begin to see the elements that drove the evolution of a system that in different ways is as protective, if not more so, as the armor plates of the ancient placoderm.

If we are correctly reading the evolutionary record, several questions come to mind. Was it the relentless nature of the challenge from pathogens that led to relatively sudden, radical changes in the way that antibody genes are organized? Do these lessons from the prehistoric vertebrates and the profound differences seen in contemporary mammals suggest that the immune system is poised for quick change? This scenario may well be the case, forcing us to rethink our notions of evolutionary selection and adaptation. SA

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HORNED SHARKS are among the most ancient creatures in which *T* cells, the agents of cellular immunity, have been conclusively identified.

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