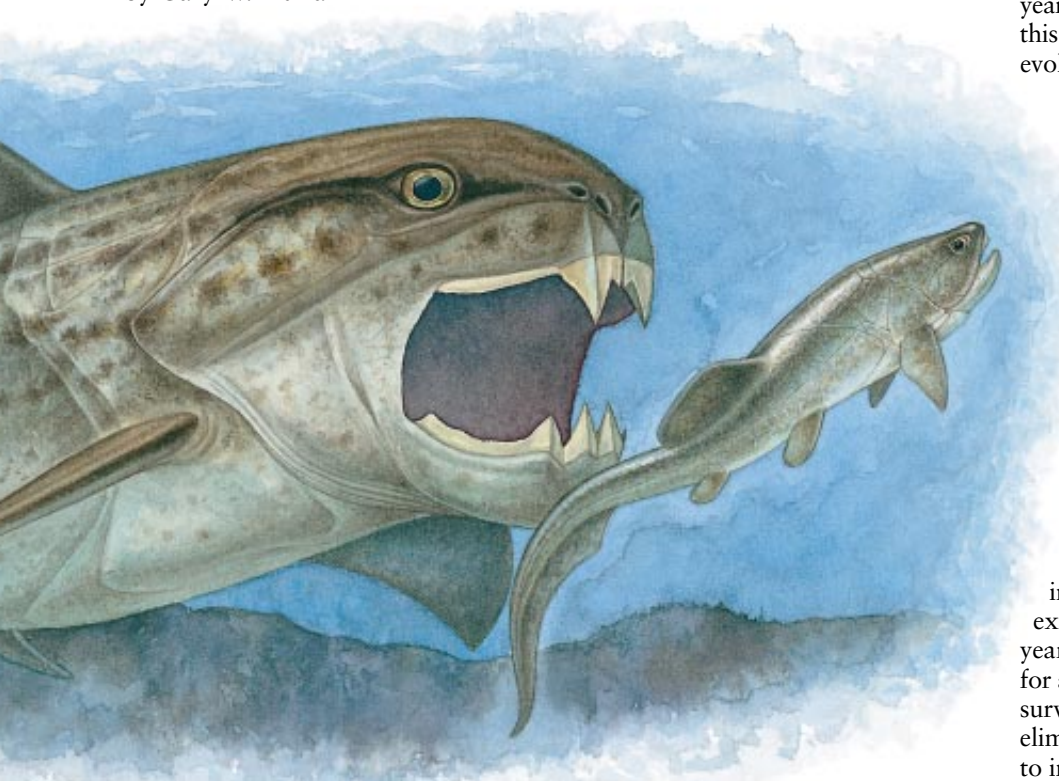
# Sharks and the Origins of Vertebrate Immunity

*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-



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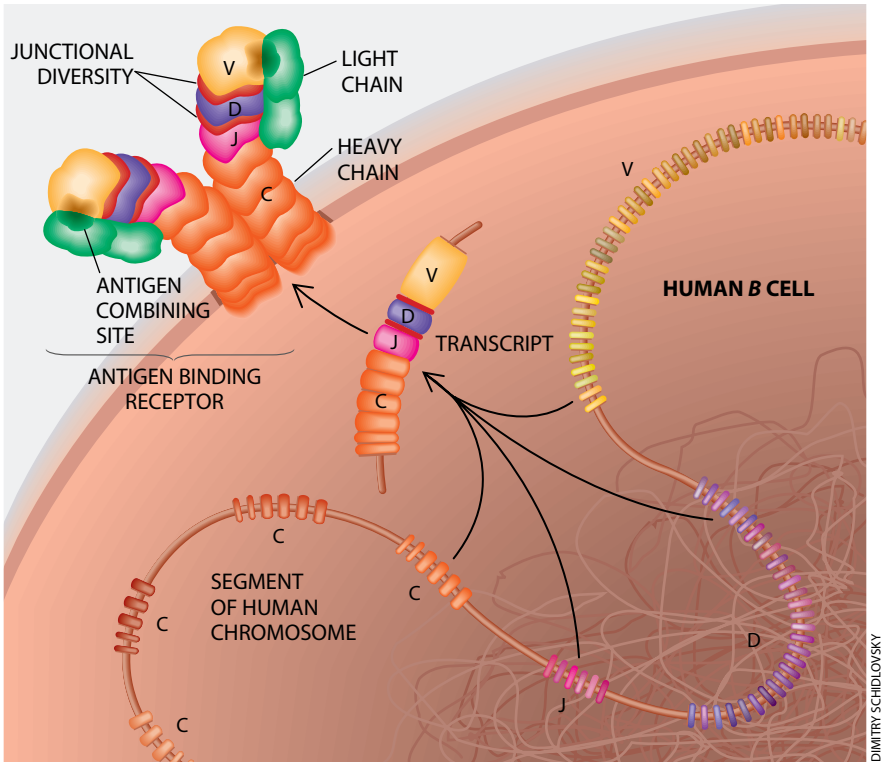
es, or antigens, on potentially harmful bacteria and viruses in the bloodstream. This binding enables other bodily entities to destroy the bacteria and viruses by various means. Antibodies are also known as immunoglobulins; humans have five major types of them.

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



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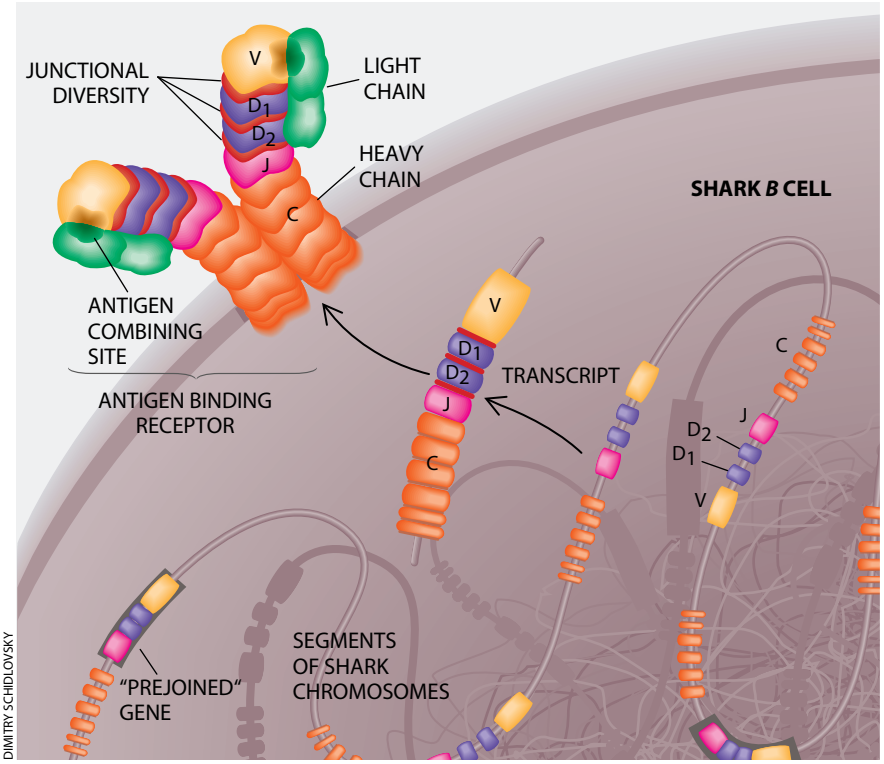


**tors; it is the only antibody that humans and sharks have in common. In humans the gene segments that come together to specify the receptor are scattered along a relatively long length of one chromosome. In sharks the gene segments are already next to one another as a kind of package that can be on 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


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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.

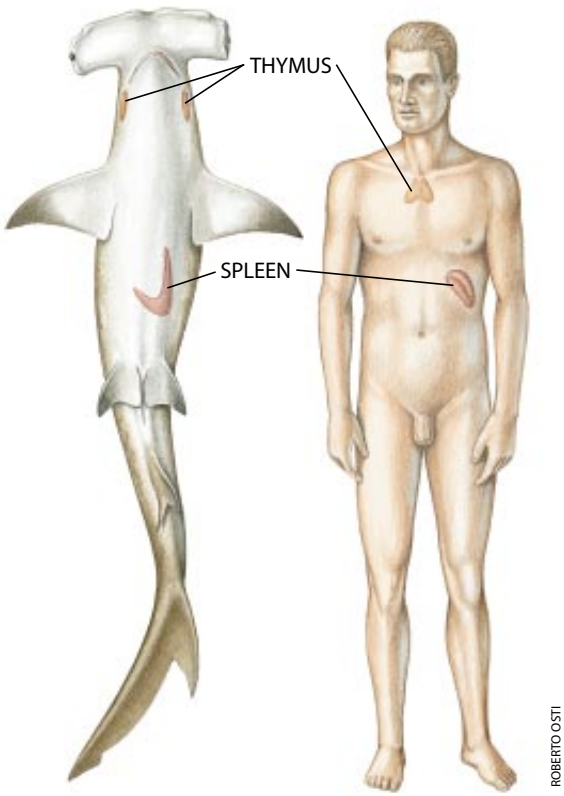
Sharks, on the other hand, rely heavily on a form of inherited diversity. This form, the most distinctive feature of the shark immune system, allows the animals to avoid depending on a chance occurrence—for example, a fortuitous combination of DNA base pairs attained through junctional diversity—to generate 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.




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

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.

### 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-



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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.**

## *The Author*

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