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**MOLECULAR
BIOLOGY
INTELLIGENCE
UNIT**

THE PRIMORDIAL VRM SYSTEM

AND THE EVOLUTION OF VERTEBRATE IMMUNITY

John Stewart

R.G. LANDES COMPANY

AUSTIN

PREFACE

Every book owes more than can be told to many people – as scientific authors in particular we all stand on the shoulders of giants – and this one, however slender, is no exception. Its underlying theme was initially conceived in the course of a conversation with Antonio Coutinho about a substantial manuscript by a noted comparative immunologist who shall nevertheless remain nameless. My argument was that in spite of its defects, the article in question should be published because comparative immunology is a vital but gravely neglected resource for managing to keep sight of the wood in spite of the entangled trees represented by the explosive detail of experimental observations in contemporary immunology. Antonio's argument was, that the defects that I myself had pointed out were redhibitory but that as a counter proposal I should write an article myself. The terms of our compromise agreement were that we would write an article together, with me providing the conceptual framework of evolutionary biology and Antonio contributing the bulk of the empirical facts from his vast knowledge of the state of the art of contemporary immunology. The article was written, and intended as a chapter in a book on the evolution of the immune system. Two years later, this book whose editor shall also remain nameless was abandoned. Pending its appearance, I had published a succinct version of the principal points that Antonio and I had intended to make under the purposely provocative title "Immunoglobulins did not arise in evolution to fight infection". Shortly afterwards, I was pleasantly surprised to receive an invitation from R. G. Landes to write a book on the "Primordial VRM System" – a neologism that Antonio and I had coined to make our point that immunoglobulins are best defined structurally without using language that implicitly presupposes that their sole biological function is defense. Fondly imagining that the book was already 70% written in the form of the unpublished chapter, I rather imprudently accepted that appealing offer. The present book has virtually tripled in volume since that initial version, but whereas its idiosyncratic defects are entirely my own responsibility, any qualities that it may possess are more than shared with Antonio.

Going a little further back, this book would not have existed if I had not met Nelson Vaz and Francisco Varela at a week-long Symposium at Cerisy-la-Salle in 1987. Francisco and Nelson not only awakened me to their exciting and radically novel way of viewing the immune system, but also gave me renewed faith that scientific colleagues can also be personal friends and that this all too rare combination gives a special meaning to scientific work. In the preface to his book *"The Man Who Mistook His Wife for a Hat"*, Oliver Sacks recalls that the great Soviet neurologist Luria liked to speak of "romantic science". It seems to me that Nelson and Francisco are the inventors of "romantic immunology", in the best sense of the term: theirs is a way of doing science that is imbued with human meaning. At that time, Nelson invited me with warm insistence to visit him at the Department of Immunology at Belo Horizonte in Brasil, and this came to pass during the summer of 1988 when I notably had the privilege of meeting Humberto Maturana. My initial project was to write a History of Immunology which would articulate an "internalist" account of the intellectual structures of the competing paradigms with an "externalist" account of their social and political connotations. The military metaphors abounding in early Immunology are indeed in striking correspondence with the national chauvinism that was rampant, particularly in France and in Germany, in the same period between 1870 and the First World War. By contrast, the alternative approach to Immunology initiated by Vaz and Varela is rooted in the philosophy of Maturana, which holds that cognitive perception is intimately related to living being and expresses the values of autonomy and self-knowledge.

As a methodological tool for this socio-historical research, I was concerned to attain an "observer-participant" status and the opportunity came with Francisco Varela's invitation to attend the regular meetings of the "immune network" group that he and Antonio Coutinho organized at the Immunobiology Unit of the Pasteur Institute in Paris. Very rapidly I came to feel that rather than maintaining a distance as a sociological historian, it would be far more interesting to become a direct actor of the history itself. The value-associations I just mentioned remain present, but rather than being

the explicit object of a socio-historical account, they have become the underlying if largely implicit source of my scientific endeavors. I therefore started to work on computer simulations of the mathematical model of the immune network that Coutinho, Varela and Vaz had built in 1988. The Immunobiology Unit at Pasteur became my home, and as I have related it was in this context that I came to be interested in the evolution of the immune system.

I would like to comment here on what has been for me a particularly revealing aspect of this work. Before undertaking this study, I was of course well versed in the Darwinian *theory* of evolution but I knew virtually nothing about the actual course of phylogeny. It was fascinating news to me that after the origin of life some 4000 Million Years (MYr) ago, the first eukaryotic cells arose 1000 MYr ago, followed very closely by the first multicellular organisms (900 MYr). There was then an astonishing period of creativity (900-700 MYr) during which all the metazoan body plans were invented, no new plans having arisen since; and the first vertebrates arose shortly after, some 600 million years ago. Retrospectively, I was dismayed to discover that I could have passed for a professional biologist for over 30 years while being completely ignorant of such matters. Above and beyond my individual case, this seems to me a disquieting commentary on the trends in contemporary biology.

It is over seven years now that I have been a member of the Immunobiology Unit at Pasteur, and I would like to express my appreciation of the stimulating and friendly atmosphere of this group under the constant impulsion of Antonio Coutinho. The members of this group are too numerous to mention individually, but I particularly wish to thank my research student, Jorge Carneiro, whose knowledge of immunology far surpasses my own and who has been a constant and lively source of discussion on the issues raised in this book. I also wish to acknowledge Francisco Varela's specific contribution, notably concerning the themes of unicellular vs. multicellular organization and self-assertion. Tommaso Meo of the Department of Immunology at Pasteur helpfully informed me on the evolution of the complement system, and Hans Boman generously provided me with reprints of his work on the antibacterial peptides. I would also like to thank Rodney Langman, who has done so much to promote an evolutionary perspective on the immune system; he is sharply critical of my views on the question, probably with reason, but in spite of this he has been supportive of my endeavors to build a model of immune evolution and this seems to me a fine example of generosity in scientific debate which I deeply appreciate. Macmillan Publishing Company have granted me permission to reproduce a text of Emile Durkheim, and this has enabled me to introduce a book on biology by citing the theoretical conceptions of one of the founding fathers of modern sociology. Last but not least in this inevitably incomplete list of thanks, I would like to mention my editor, R G. Landes; without his spontaneous invitation to write this book, and his efficient support during its preparation, it would never have seen the day.

Finally, no words can adequately express what I owe to my wife, Véronique Havelange, whom I first met at that memorable Symposium at Cerisy-la-Salle which also marked my encounter with "romantic immunology".

John Stewart
Paris
March 3rd 1994

PROLOGUE

In this book we propose to study the most primitive and simple immune system which is actually known, to make an analysis of it, and to attempt an explanation of it. An immune system may be said to be the most primitive which we can observe when it fulfills the two following conditions: in the first place, when it is found in an organism whose organization is surpassed by no others in simplicity; and secondly, when it is possible to explain it without making use of any element borrowed from a previous immune system.

We shall set ourselves to describe the organization of this system with all the exactness and fidelity that a comparative physiologist or a student of evolution could give it. But our task will not be limited to that: biology raises other problems than evolution or comparative physiology. It does not seek to know the past forms of biological organisms with the sole end of knowing them and reconstructing them. But rather, like every positive science, it has as its object the explanation of some actual reality which is near to us... Then we are not going to study a very archaic immune system simply for the pleasure of telling its peculiarities and its singularities. If we have taken it as the subject of our research, it is because it has seemed to us better adapted than any other to lead to an understanding of the nature of the immune system of contemporary mammals...

It is undoubtedly possible to arrange immune systems in a hierarchy. Some can be called superior to others, in the sense that they call into play higher integrative functions and that they are richer in types of cells... But howsoever real this greater complexity and this higher discriminative capacity may be, they are not sufficient to place the corresponding immune systems in different classes. All are immune systems equally, just as all living beings are equally alive, from the most humble plastids up to man. So when we turn to primitive immune systems it is not with the idea of depreciating immune systems in general, for these immune systems are no less respectable than the others. They respond to the same needs, they play the same role, they depend on the same causes; they can also well serve to show the nature of immune function, and consequently to resolve the problem which we wish to study.

But why give them a sort of prerogative? Why choose them in preference to all others as the subject of our study? - It is merely for reasons of method.

In the first place, we cannot arrive at an understanding of the most recent immune systems except by following the manner in which they have been progressively composed in evolution. In fact, evolutionary analysis is the only means of explanation which it is possible to apply to them. It alone enables us to resolve a system into its constituent elements, for it shows them to us as they are born in time, one after another. On the other hand, by placing every one of them in the condition where it was born, it puts into our hands the only means we have of determining the causes which gave rise to it. Every time that we undertake to explain something biological, taken at a given moment in evolution, it is necessary to commence by going back to its most primitive and simple form, to try to account for the characteristics by which it was marked at that time, and then to show how it developed and became complicated little by little, and how it became that which it is at the moment in question. One readily understands the importance which the determination of the point of departure has for this series of progressive explanations, for all the others are attached to it. It was one of Descartes' principles that the first ring has a predominating place in the chain of scientific truths. But there is no question of placing at the foundation of the science of immune systems an idea elaborated after the Cartesian manner, that is to say, a logical concept, a pure possibility, constructed simply by force of thought. What we must find is a concrete reality, and evolutionary and comparative observation alone can reveal that to us. But even if this cardinal conception is obtained by a different process than that of Descartes, it remains true that it is destined to have a considerable influence on the whole series of propositions which the science establishes. Biological evolution in general has been conceived quite differently ever since it has been known that monocellular beings do exist. In the same way, the arrangement of facts concerning immune systems is explained quite differently, according to the form we put at the beginning of the

evolution. Even the most specialized scientists, if they are unwilling to confine themselves to a task of pure erudition, and if they desire to interpret the facts which they analyze, are obliged to choose a hypothesis, and make it their starting-point... Since it is impossible to avoid taking sides on this initial problem, and since the solution given is destined to affect the whole science, it must be attacked at the outset: that is what we propose to do.

Besides this, outside of these indirect reactions, the study of primitive immune systems has of itself an immediate interest which is of primary importance.

If it is useful to know what a certain particular immune system consists in, it is still more important to know what immune systems in general are... Since all immune systems can be compared to each other, and since all are species of the same class, there are necessarily many elements which are common to all. We do not mean to speak simply of the outward and visible characteristics which they all have equally, and which make it possible to give them a provisional definition from the very outset of our researches; the discovery of these apparent signs is relatively easy, for the observation which it demands does not go beneath the surface of things. But these external resemblances suppose others which are more profound. At the foundation of all immune systems there ought necessarily to be a certain number of fundamental features which, in spite of the diversity of forms which they have taken, have the same objective significance and fulfill the same functions everywhere. These are the elements which constitute that which is permanent and biological in immune systems; they form all the objective contents of the idea which is expressed when one speaks of immunity in general. How is it possible to pick them out?

Surely it is not by observing the complex immune systems which appear in the course of evolution. Every one of these is made up of such a variety of elements that it is very difficult to distinguish what is secondary from what is principal, the essential from the accessory. Suppose that the immune system considered is like that of a particular species of bird or mammal. In one or another of these systems it is quite possible to find the means of making a profitable study of some particular fact which is specially developed there; but how is it possible to find the common foundations of the immune activity underneath the luxuriant vegetation which covers it?

Things are quite different in the lower organisms. Differences and variations are reduced to a minimum. And while all is uniform, all is simple as well. That which is accessory or secondary, the development of luxury, has not yet come to hide the principal elements. All is reduced to that which is indispensable, to that without which there could be no immune system. But that which is indispensable is also that which is essential, that is to say, that which we must know before all else.

Primitive organisms offer privileged cases, then, because they are simple cases... It is for this same reason that the discovery of unicellular beings, of which we just spoke, has transformed the current idea of life. Since in these very simple beings, life is reduced to its essential traits, these are less easily misunderstood.

But simple immune systems do not merely aid us in disengaging the constituent elements of immunity; they also have the great advantage that they facilitate the explanation of it. Since the facts there are simpler, the relations between them are more apparent. In proportion as an immune system progresses in evolution, the causes which called it into existence, though remaining active, are no longer perceived. The psychological gap between the cause and the effect, between the apparent cause and the effective cause, has become more considerable and more difficult for the mind to leap. The remainder of this book will be an illustration and a verification of this remark on method. It will be seen how, in the primitive immune systems, the mark of their origins is still visibly carried: it would have been well-nigh impossible to infer these origins merely from the study of the more developed immune systems.

The study which we are undertaking is therefore a way of taking up the problem of the origin of immunity... What we want to do is to find a means of discerning the ever present causes upon which the most essential forms of immune activity depend. Now for the reasons which were just set forth, these causes are proportionately more easily observable as the organisms where they are observed are less complicated. That is why we try to get as near as possible to the origins. It is not that we ascribe particular virtues to the lower immune systems. On the contrary, they are rudimentary and gross; we

cannot make of them a sort of model which later immune systems only have to reproduce. But even their grossness makes them instructive, for they thus become convenient for experiments, as in them, the facts and their relations are easily seen. In order to discover the laws of the phenomena which he studies, the physicist tries to simplify these latter and rid them of their secondary characteristics. For that which concerns biological systems, nature spontaneously makes the same sort of simplifications at the beginning of evolution. We merely wish to put these to profit. Undoubtedly we can touch only very elementary facts by this method. When we shall have accounted for them as far as possible, the novelties of every sort which have been produced in the course of evolution will not yet be explained. But while we do not dream of denying the importance of the problems thus raised, we think that they will profit by being treated in their turn, and that it is important to take them up only after those of which we are going to undertake the study at present.

CHAPTER 1

INTRODUCTION

EVOLUTION AS A PERCEPTUAL TOOL

The immune system of present day mammals is characterized by its truly bewildering complexity. The major components of the system, the lymphocytes, are divided into B-cells and T-cells which are further subdivided into different types on the basis of cell-surface markers (CD1-40 and their corresponding ligands). The molecules that mediate the multiple interactions between these cell-types are also diverse: interleukins 1-10, interferon, cytokines and so on. Hardly a month passes without the identification of new molecules, and it sometimes seems that the list of components of the immune system is limited only by the ever-increasing sophistication of experimental techniques and the amount of work invested. This being so, if immunological science is to amount to anything more than meaningless fact-collection the overriding and ever more urgent problem is to find a way of keeping sight of the wood without getting lost in the trees. The need is to simplify our perception of the immune system in a way that will bring into focus the truly essential features of its organization.

The text presented in the Prologue to this book is not, as one might have thought, the work of a biologist. The author is Emile Durkheim¹, the father of modern sociology, in the Introduction to his classical work "The Elementary Forms of the Religious Life". In this text, Durkheim gives an exemplary exposition of the methodological rationale for "choosing in preference to all others as the subject of study...the most primitive and simple religion which is actually known". It is quite uncanny how well this text reads when one substitutes, as I have taken the liberty of doing, "immune system" for "religion", "evolution" for "history", and "organism" for "society". The basic theme of this book is thus that the reconstruction of phylogenetic origins can provide a privileged tool for appropriately simplifying our perception of a complex biological system. This transposition of Durkheim's method from sociology to biology is actually a fitting way of returning a compliment, for Durkheim himself refers to the discovery of primitive unicellular organisms in biology as a source of his own inspiration.

THE RECONSTRUCTION OF EVOLUTION

The main idea of referring to evolutionary origins as a means of grasping the essential organizational features of a biological system is, in its overall outline, quite clear. However, the actual application of this method is not as straightforward as it might seem, for reasons which require comment. The principal difficulty is that evolution is a historical process which occurred in the past and is therefore not amenable to direct observation. There is of course the fossil record; but this record is both partial (only certain aspects of anatomy, notably skeletal remains, are available; neither physiology nor behavior leave traces) and fragmentary (due to the chance vicissitudes of fossilization and discovery). The immune system in particular leaves no fossil traces. In the endeavor to build a picture of the primordial immune system as it first arose in evolution the fossil record is of course important; but it is only one of several lines of evidence which must be integrated in the framework of a comprehensive theory of the evolutionary process if we are to succeed in reconstructing phylogeny.

The major line of empirical evidence which can be used in reconstructing evolution is the diversity of species which are extant at the present time. The first idea which comes to mind is that some of these species may be more "primitive" than others and thus directly representative of the "primordial" forms we seek to identify. Durkheim himself makes an assumption of this sort when he rather uncritically considers that the societies of contemporary Australian aborigines are "primitive" compared to those of the great historical civilizations. In its simplistic form, this assumption is incorrect; all extant forms of life, including human societies, have exactly the same elapsed

chronological age since the origin of life some 4000 million years ago, and so one cannot simply assume that some are more "evolved" than others. The correct formulation is that the varying degrees of similarity between extant forms can be interpreted in terms of more or less remote common ancestors, and this can be used to construct hypotheses concerning these common ancestors. However, it turns out that this correction is to some extent over-cautious. The point is that the rate of evolutionary change in organic forms is not the same in all branches of the evolutionary tree. Classical Darwinian and neo-Darwinian theory lead to an expectation that evolutionary change is due to the gradual accumulation of small differences, and hence that the rate of change is approximately constant. While this is basically correct for the accumulation of "neutral" mutations at the levels of nucleotide sequence in DNA and amino-acid sequence in proteins (hence the possibility of calibrating "molecular clocks"), it is not correct concerning the anatomical and functional aspects of living forms. The reason is that living organisms are characterized by a circular organization², in which each of their multiple parts only make sense by virtue of their relationships to other parts. Normally each part is thus constrained by these relationships and cannot change in isolation without compromising the viability of the whole. At the same time, if change does occur it is likely to involve a sizable network of interacting parts. There is thus an underlying systemic rationale for the phenomenon of "punctuated equilibria" first formulated by Eldredge and Gould³. Most of the time, living forms are remarkably stable, remaining virtually unchanged for millions or even hundreds of millions of years; however when changes do occur, they tend to be rapid and large (as for example the flowering of a whole host of new forms after the appearance of multicellular organisms, or the adaptive radiation of mammals after the disappearance of the dinosaurs). This being so, the simplistic view that some living forms are more "primitive" than others is not as wrong as might have been thought. Thus, contemporary unicellular organisms are surprisingly representative of the organisms extant 1000 million years ago when all organisms were unicellular. To take another example, particularly relevant for the theme of this book, the taxonomic series: jawless fishes (lampreys, hagfish)-cartilaginous fishes (sharks, rays)-bony fishes-amphibia-reptiles-mammals, is a surprisingly good approximation to what we imagine must have been the ancestral sequence in vertebrate evolution. Of course, care must be taken in the interpretation to distinguish truly primitive features from those that are indeed subsequent developments: for example, contemporary lampreys and hagfish are all parasitic, but it would not be correct to conclude that the ancestral vertebrates were also parasitic. However, with this proviso, the diversity of extant forms does provide a substantial basis for the reconstruction of evolution. Comparative immunology is thus an essential resource for our study, on the important condition that it be considered not as an end in itself (in which case it degenerates and actually aggravates the problem of "fact-collecting" by multiplying the number of species), but as a theoretically disciplined effort to reconstruct the evolutionary process which has given rise to contemporary mammals.

Another line of empirical evidence that can be used in the reconstruction of phylogeny stems from the relationship between phylogeny and ontogeny. Here again, there is a simplistic idea that is wrong but not quite as wrong as it might seem. In this case, the simple idea, due to the 19th century German biologist Haeckel, is that ontogeny "recapitulates" phylogeny or, in other words, that the developmental process leading from the fertilized egg-cell to the adult is a sort of accelerated summary of the evolutionary process leading from unicellular organisms to the present forms. The correct formulation is that since evolution occurs by modifications not of the adult form as such, but by unit modifications of the *processes* of development, there is always a relationship between phylogeny and ontogeny. To the extent that evolution occurs by the simple addition of novel prolongations of ontogeny, the early part of ontogeny remaining unchanged, ontogeny will indeed recapitulate phylogeny. This is sometimes the case, and accounts for the germ of truth in Haeckel's idea, an example being the fact that mammalian embryos pass through a fish-like stage with gills reminiscent of their evolutionary ancestors. However, this is not always the case; some of the most striking innovations in evolution arise by *neoteny*, i.e., a process whereby the early stages of the ancestral form are amplified and modified, the later stages being simply discarded. A major case in point is the origin of vertebrates themselves: the latter probably derive from the free-swimming *larval* stages of tunicate-like protochordates, the metamorphosis of these ancestors to a sessile form being postponed and

ultimately (after the neo-larval form acquired the capacity for sexual reproduction) being dispensed with altogether.

Independently of the correctness or otherwise of the idea of recapitulation, the fact that evolution occurs by modifications not of the adult form as such but by unit modifications of the processes of development is important for our understanding of the evolutionary process and hence for any reconstruction of phylogeny. Evolution occurs by processes of variation and natural selection. In classical neo-Darwinian theory, variation is considered to be random so that the only systematic organizing force is that of selection. However, although variation at the genetic level of mutational modifications in chromosomal DNA does seem to be essentially random, it does not follow that the resulting phenotypic variation will also be random. As Waddington⁴ has emphasized, the developmental processes are themselves organized in such a way that they are easy to shift in certain directions and less easy in others, so that the changes produced by mutation will often be exhibited as one of the patterns which the developmental system has a tendency to assume. A new gene mutation can cause an alteration only to a character which the organism already possessed in previous generations. For example, it could not produce a lobster's claw on a cat; it could only alter the cat in some way, still leaving it essentially a cat. An understanding of the epigenetic organization of development is thus important for understanding evolution; we shall see that this point has quite definite implications for our attempt to reconstruct the evolution of the vertebrate immune system.

Finally, we may mention another reason why the study of evolution is an indispensable tool for the understanding of any biological system. The point is this: if a human engineer, starting from scratch, were to design a system to perform a particular function, he would almost certainly never adopt the organization of the actual biological system. The reason for this discrepancy between a rationally designed system and the actual biological organization is that the latter never starts from scratch: every biological system has been constructed by evolutionary "tinkering" through a linear series of single steps with the constraint that the system must be viable at each step^{5,6}. This constraint is, indeed, the source of a central, recurrent enigma in evolutionary theory. Biological systems are typically composite entities with circular relations between their parts; for example, a hormone only makes sense if there is a receptor, but the receptor only makes sense if there is a hormone. As with the chicken and the egg, the enigma is: which came first? The task of answering such questions is rarely an easy one; but the answers, if they can be found, provide keys to understanding the bizarre design features which are so characteristic of biological systems in general. As noted by Cairns-Smith⁷, quoting Sherlock Holmes, the bizarre enigmas are not actually the most difficult to unravel; whereas the banal cases have many plausible explanations, if any solution at all can be found to a bizarre problem it is likely to be the correct one.

STRUCTURE AND FUNCTION

In this endeavor to reconstruct an account of evolution, there is a methodological principle which should be underlined as it is often ignored with unfortunate consequences: in evolution, what is conserved is *structure*, not *function*. Structures, be they molecules, cells or organs, do of course change through evolution; but they change more or less continuously in such a way that homologies can be reliably recognized. It is, indeed, on the basis of structural homologies that the phylogenetic relationships between contemporary species can be elucidated – these phylogenetic interpretations being of course consolidated by studying the structures in the fossil record. Functions, on the other hand, are typically *not* conserved; they evolve *discontinuously*. In the opportunistic tinkering which is so characteristic of the evolutionary process, related structures are frequently *redeployed* for qualitatively different (and on occasion completely novel) functions. For example, mammalian forelimbs with clearly recognizable anatomical homologies can be flippers (seals), wings (bats), hooved legs (horses), or arms and hands (rodents, monkeys). Conversely, apparently similar functions can be performed by evolutionarily unrelated structures. For example heads – defined as a regrouping at the anterior end of an animal of the mouth, the major sensory organs (functionally responsible for sight, hearing and smell) and the brain (a neuronal network predominantly composed of interneurons) – evolved quite independently in insects and the higher vertebrates, since their most recent common

ancestors were headless worms. To avoid misunderstanding, I should make it clear that I am definitely *not* espousing Francis Crick's famous doctrine "If you don't understand a function, study a structure". As Langman⁸ has pointed out, structures are certainly moulded by natural selection in relation to their function, and this is precisely what gives rise to striking phenomena of convergent evolution. One example is the case just cited of heads in insects and vertebrates; another is provided by the astonishing similarity of a whole range of animals from wolves to flying squirrels between the marsupial forms in Australia and their mammalian counterparts in Europe and elsewhere⁹. But the point is that functional similarities of this sort are precisely unreliable and indeed actually misleading if we are seeking to elucidate phylogenetic relationships. Summing up then, *from an evolutionary point of view* related structures can serve unrelated functions, and unrelated structures can serve related functions.

This being so, it is a serious mistake to try to understand the evolution of a biological system in terms of a specified function. Unfortunately, the besetting sin of much of what passes for comparative immunology is to commit precisely this mistake. Specifically, the immune system is habitually *defined*, constitutively, as the system which performs the twin functions of defense and self versus non-self discrimination. The problem is that whereas these functions are virtually universal among all living organisms, the structures and indeed the strategies deployed in order to fulfill these functions are highly diverse and phylogenetically very largely unrelated. The defense strategy of insects, for example, is structured around the existence of an impermeable, chitinous exoskeleton; that of fungi around the biochemical production of natural antibiotics. It is not particularly illuminating, to say the least, to compare these structures with the "immune system" of mammals. The situation with respect to histocompatibility and allorecognition is if anything even worse. For example, sponges express on their surface polymorphic determinants that prevent sponges of the same species from forming a single colony on the sea floor. Hermaphroditic plants have polymorphic determinants that prevent self-pollination. But this does not mean that the mechanisms and molecules involved have much to do with each other, or with the MHC system in mammals. Comparative immunologists have made repeated attempts to identify allorecognition molecules in invertebrates as members of the immunoglobulin superfamily, using antibodies, DNA probes and peptide sequences; these attempts, which have all failed¹⁰, do at least serve to illustrate the futility of trying to base comparative immunology on the postulate of conserved function.

The conclusion that studies of the evolution of the immune system should be based on the conservation of structure rather than that of function does not mean, however, that a consideration of function is unimportant. On the contrary, an elucidation of structural homologies, with a concomitant interpretation in terms of a series of more or less gradual step-by-step phylogenetic modifications, is itself only really interesting if it serves in turn as the basis for *understanding* the qualitative functional redeployments that have occurred. It is, after all, this sort of understanding which is necessary to illuminate the essential organizational features of complex contemporary systems.

DEFINING WHAT THE "IMMUNE SYSTEM" IS

The methodological prescription that the object of evolutionary studies should *not* be identified in terms of a specific function does, of course, pose one awkward question. At the present time, the consensual definition of what the immune system is refers constitutively to the function of defense – an assumption so deeply ingrained that it is inscribed in the very name we give to the system and to the discipline, immunology, which studies it. If we wish to postulate that the principal function of the "immune system" (the inverted commas are now necessary) has not necessarily always been that of defense, how *are* we to identify the object we are talking about?

In this book, we shall define the focus of our attention as *the system of "variable-region molecules" and the cells that produce them* (i.e., lymphocytes). The structural diversity of the "V regions" of immunoglobulins (Igs) and T-lymphocyte receptors (TCRs) is at least as great, and probably greater, than that of all the other molecules in the vertebrate body put together. The whole vertebrate genome encodes some 5×10^4 different proteins. The precise extent of the actual V-region diversity in a given individual is a complex question. Theoretical considerations (based on the number of V region gene segments and the processes leading to their recombination) show that those processes

are unlikely to generate precise repeats in nucleotide sequence, and lead to estimates of the potential diversity of the order of 10^{10} or more. However, it is virtually certain that such a large potential diversity is never fully expressed, if only because V regions are expressed on a clonal basis so that the upper limit to their number is the total number of lymphocytes that the individual contains at any point in its life-time; this is always smaller than the size of the potential diversity. Certain authors^{11,12} have suggested that the expressed diversity may actually be as small as 10^4 . Empirically, the repeated isolation of the same V-region from genetically identical animals (mouse strains) has proven to be a very rare event, in spite of many thousands that have thus far been analyzed. Overall, it seems fair to conclude that the actual diversity is less than the potential diversity, but nevertheless very great indeed.

The question of the extent of the actual and potential structural diversity should be distinguished from the question of the size of the "antigenic" universe that a single immune system can specifically recognize¹³. Classical experimentation has led to the generalized conviction that the domain of V regions is "complete" or "open-ended"; that is, "immune systems" seem to be capable of identifying any molecular shape whatsoever. Moreover, animals of quite different size (from an elephant to a tadpole with only 10^4 lymphocytes) all seem to be endowed with "completeness" of recognition. Estimates of the number of structurally distinct "antigenic sites", based on the classic work of Landsteiner¹⁴, are of the order of 10^{17} ; this is of course far higher than the actual diversity of V regions. This was long considered to be an overwhelming argument in favor of an "instructionist" theory of antibody formation¹⁵. A possible solution to this apparent paradox has been suggested by Inman¹⁶: it is likely that individual V-region molecules are "degenerate" (recognizing from 0.01% to 1% of all antigenic epitopes), but that a *serum* containing many different immunoglobulins (which have in common their cross-reactivity to a particular antigen) is far more "specific" than each immunoglobulin taken individually.

Whatever its precise extent, however, the main point that is relevant for our present purposes is that this striking diversity is generated by quasi-random processes during somatic lymphocyte development, breaking the central dogma of DNA conservation. As we shall see in detail in Chapter 2, the molecular mechanisms involved are now well known, and it appears that the biochemical machinery necessary to construct and express V-region genes in somatic cells is strictly limited to lymphocytes. By focusing our attention on the mechanism which generates V region structural diversity, we satisfy the requirement of having a clear structural definition of the object of our study.

It is of course customary at present to use the term "immune system" to refer indiscriminately both to immunoglobulins and lymphocytes (i.e., structures), *and* to the system responsible for defense (i.e., a function). In order to avoid unnecessary confusion, and to underscore our deliberately agnostic position with respect to function, we shall refer to such diversified molecules by coining the term "variable region molecules" (or VRM for short) and call the system which produces these molecules the "VRM system". This will permit us to follow normal usage by reserving the term "immune system" to designate the system which is principally responsible for the defense function at any one time in evolutionary history. On this point, I emphatically agree with Warr and Cohen¹⁷ who express the view that immunity is best defined functionally as resistance to infection or other harmful effects of foreign agents. They note that this definition may not be considered appropriate by those who regard the mammalian immune system as the paradigm; and we may remark that it is also likely to be challenged by comparative immunologists who seem to take it almost as a personal slight to consider that invertebrates cannot show "anticipatory" immune responses or engage in "self-nonsel" discrimination. Warr and Cohen note, however, that few if any of those working in the field of the evolution of immunity still argue that homologs of T and B cells exist in the protostomates or that immunoglobulin affords invertebrates protective humoral immunity. They express the belief that continuing to focus attention on the issue of "anticipatory" responses only avoids dealing with some of the most interesting questions concerning evolutionary relatedness between vertebrate and invertebrate immunity.

In order to underscore the consequences of our proposed conceptual distinction between structural and functional definitions, I shall now briefly evoke the evolutionary histories of three components of the immune systems of multicellular animals which illustrate Warr and Cohen's contention that a fruitful study of "some of the most interesting questions concerning evolutionary

relatedness between vertebrate and invertebrate immunity" does indeed require abandoning the view that the mammalian immune system is the paradigm for all immunity. These three components are cellular immunity, and two components of humoral immunity (the complement system, and antibacterial peptides). It will hopefully become abundantly clear that none of these components has any intrinsic relation to the VRM system.

METCHNIKOFF AND THE EVOLUTIONARY ORIGIN OF CELLULAR IMMUNITY

The fact that much comparative immunology to date is based on the mistaken postulate that functions are conserved in evolution has generated considerable conceptual confusion. This is all the more disappointing in that the early history of immunology was marked by an outstanding contribution which is a fine model of the methodology capable of rendering an evolutionary approach fruitful. The Russian Metchnikoff was exceptional, not only among the immunologists of his day but (unfortunately) among those of succeeding generations as well, in that his background was neither that of a chemist nor of a medical clinician, but truly that of a theoretically informed biologist. Metchnikoff's life work, apparently characterized by an impressively eclectic diversity, was in truth the coherent working-out of a single great theoretical idea. Strangely, this fact has been poorly appreciated, both at the time and since, in spite of being clearly explained and movingly documented in a little book by Besredka¹⁸ who was one of Metchnikoff's own disciples. Interestingly, exactly the same point has been made recently, apparently independently, by two contemporary historians of science^{19,20}. Without going into details, for which the reader is referred to the above-mentioned references (and to Metchnikoff's original work), the gist of the matter is this. Metchnikoff, greatly impressed by Darwin's theory of evolution, looked to comparative embryology and a consideration of the relations between ontogeny and phylogeny in order to find confirmation of this theory and its corollary, an underlying unity behind the apparent diversity of living organisms. Concerning the passage from single-cell to multi-cellular organisms, Metchnikoff concluded that the primitive multi-cellular organism must have retained a capacity for intracellular digestion or phagocytosis. In what can serve as a paradigm case of what I have called the "redeployment" of a structure, the subsequent development of a gastric cavity and extracellular digestion meant that phagocytosis was released for new functions – among which Metchnikoff situated the digestion of pathogenic parasites and hence cellular immunity. As Chernyak and Tauber¹⁹ insist strongly, Metchnikoff's conceptual category of "phagocytosis" was thus not a lucky guess, nor an isolated empirical observation, but the deeply-reflected result of a coherent chain of thought. One conclusion we can retain, and to which we shall return, is that in terms of its evolutionary history immunity has been intimately associated with the processes of digestion and nutrition.

Chernyak and Tauber²⁰ have also pointed out that an important theme in Metchnikoff's thought is the potential conflict between different cell-lines in the context of a multicellular organism. As we shall see, this theme has been recently taken up by Buss²¹; it will play a basic role in the hypothetical account of the evolution of the immune system that I present in Chapter 3 of this book.

THE COMPLEMENT SYSTEM

The complement cascade constitutes a molecular mechanism for punching holes in cellular membranes and is an important component of antibacterial defense systems. In mammals, there are two distinct pathways, the so-called "classical" and "alternative" pathways²². The proteins involved in these two pathways are structurally very similar, and so they almost certainly arose by gene duplications from a single ancestral pathway. In particular, the C2-B gene pair marks the duplication that preceded the development of the two main pathways for the assembly of the C3/C5 convertases²³. The classical pathway includes a component, C4, that does not exist in the alternative pathway; moreover, the alternative pathway, which has the same end result, does not depend on immunoglobulins for its initiation. These findings converge to suggest that the "alternative" pathway, although discovered later, actually corresponds more closely to the ancestral form. This conclusion is further supported by the fact that C3 in the lamprey, a primitive vertebrate which does not possess immunoglobulins, retains close similarity to the common ancestor of mammalian C3 and C4²⁴.

Tracing the evolutionary ancestry of the complement system even further back, Tosi et al²³

conclude that the C2 component descends from an atypical member of the serine protease family. C3, C5 (and C4), on the other hand, descend from what they term the α_2 -microglobulin in recognition of the broad biological significance of this protein and the evolutionary conservation of its function, which can be detected even in invertebrates²⁵. The major function of α_2 -microglobulin is to tag through a covalent bond active endopeptidases of any class destined for removal from the circulation. In this suicidal action the macroglobulin undergoes a conformational change that exposes a site recognized by receptors on macrophages which endocytose the complexes. It is not hard to imagine that the basic purging functions of complement, cell opsonization and lysis of biological membranes, were acquired through the recruitment and the structural refinement of these descendants of α_2 -microglobulin. Finally, all three proteins have become instrumental collaborators of C2, a special descendant of the serine proteases that their primitive relative, α_2 -microglobulin, scavenged indiscriminately.

The conclusion to be drawn from this brief history of the complement system is that this defense mechanism preexisted in invertebrates, before the evolutionary appearance of VRMs in vertebrate evolution. Of course in contemporary mammals, the VRM system has been recruited to serve as a trigger for setting in motion the complement cascade. However, the pre-existence of the complement system raises the possibility, which will be one of the main themes of this book, that the VRM system itself may have arisen in evolution for reasons initially unrelated to the defense function, and only later been redeployed to play a role in triggering preexisting effector functions.

ANTIBACTERIAL PEPTIDES

The complement system is not the only humoral defense mechanism which arguably predated the appearance of VRMs in vertebrate evolution. All higher organisms can in some way defend themselves against their natural flora of bacteria. Even in humans, when the immune system is impaired by AIDS or immunosuppressive agents, it is the natural flora and opportunistic pathogens that take over. In this section, I shall briefly discuss three kinds of antibacterial peptides that are widely distributed in the animal kingdom: defensins, magainins and cecropins²⁶.

Phagocytosis was discovered more than 100 years ago, but the mechanisms by which engulfed bacteria are killed were only elucidated 80 years later. Among the molecules responsible are the defensins, originally isolated from rabbit granulocytes and neutrophils; these small peptides all contain 29-34 amino acid residues, have three intramolecular disulfide bonds, and are quite basic. They act on a wide variety of bacteria, but usually better on gram-positive than on gram-negative bacteria. They also work on fungi and are somewhat toxic for normal eukaryotic cells. However, this does not seem to cause any problems, because defensins are not normally released but are sequestered in granules of neutrophils.

Many insects respond to an injection of live bacteria with the production of a potent cell-free antibacterial activity²⁷. The principal molecules in question, the cecropins, were originally identified in immune hemolymph from the cecropia moth. They are all molecules with 31-39 residues, devoid of cysteine, with a strongly basic N-terminal region and a long hydrophobic stretch in the C terminal half. In two dipterans, *Sarcophaga* and *Drosophila*, the amino acid sequences of the cecropins are identical, although there is only 73% homology at the nucleotide level. Thus, there appears to be strong selective pressure to conserve the cecropin protein sequence in different insects.

Frog skins contain glands that produce a large number of biologically active peptides, whose secretion can be induced by epinephrine or norepinephrine. Some are general toxins: crude toxin from the frog *Bombina* was early reported to act on bacteria. Twenty years later *Xenopus* had become a widely used laboratory animal from which oocytes were removed surgically. These frogs could thrive in waters quite dense with bacteria, yet the wounds from their operation never became infected. This observation led to the finding of the magainins, two closely related antibacterial peptides produced in the skin of *Xenopus*. Another frog skin peptide, PGLa, has also been shown to be antibacterial. Magainins have only 23 residues, are devoid of cysteine, and form an amphipathic α helix.

When they were first discovered, the defensins, the cecropins and the magainins were each thought to be specific to that group of animals in which they had been identified. However, that perspective changed when defensin-like peptides were discovered in insects and when a cecropin was

isolated from pig intestine²⁸. Subsequently, other peptides were found in the horse-shoe crab, in bovine tracheal mucosa, in other insects, and even in barley. Antibacterial peptides with new properties have also been obtained by making hybrid molecules with one domain from a cecropin and another from the bee venom toxin melittin. The detailed molecular mechanisms of action of the defensins, the cecropins and the magainins are different, but all three groups of peptides have been shown to form channels in artificial membranes. In vivo, their primary targets are the inner and outer bacterial membranes. Taken together, these data strongly indicate that antibacterial peptides are widespread in nature.

Several lines of argument converge to suggest that antibacterial peptides arose early in evolution. Defense systems as such must have evolved in concert with the eukaryotic cell, because even the earliest eukaryotes had to compete with bacteria. In a primitive organism it will be important for the defense system to have three characteristics: polyspecificity, self-nonsel discrimination, and rapid response kinetics.

Janeway²⁹ has pointed out that it is reasonable to assume that in the initial stages of evolution, defense reactions were triggered by a single "pattern recognition" event. Cecropins and other antibacterial proteins in insects are indeed induced by a single signal, such as a degradation product of murein or free lipopolysaccharide. These common microbial constituents cannot be modified by the pathogen without changing its core structure, which would require many separate mutations that might destroy its infectious capacity altogether. Moreover, in most species studied, the antibacterial peptides come in several closely related copies with genes that can be very similar; in no case is there a bacterium on which only a single factor acts. This makes it virtually impossible for a susceptible bacterium to produce mutants which are resistant to the humoral immunity of the host. We are thus indeed dealing with a system of broad-spectrum antibacterial molecules.

Secondly, antibacterial peptides lyse both gram-positive and gram-negative bacteria, but cause little or no harm to eukaryotic cells. Cecropins will also lyse artificial liposomes composed of phospholipids having zwitterionic or negatively charged head groups. The rate and extent of lysis are dependent on the cecropin concentration, indicating that the cecropins act stoichiometrically and not catalytically; there is every reason to believe that this applies equally to defensins and magainins. Thus, self destruction is largely avoided by membrane specificity of the effector mechanism, presumably without positive recognition of any bacterial proteins at this level because peptides with only D amino acids are just as active as the natural ones. Of course, self-nonsel discrimination will be maintained in such a system by the simple expedient that organisms generating self-destructive responses will be lost. Finally, rapid response kinetics is achieved by having all the cells that bind a given microbial pattern rapidly becoming effectors. Since antibacterial peptides are inducible and probably do not require specialized cells for their synthesis, there is no need for clonal expansion and differentiation to effector function. Overall, then, there is ample justification for supposing that antibacterial peptides represent a defense system that predated the separation of vertebrates and invertebrates.

THE AIM OF THIS BOOK

To summarize, the question I seek to address in this book is the evolutionary origin and subsequent history of the VRM system. In accordance with my own methodological prescriptions, I shall of course start by considering genes, molecules, cells and organs, for these are the structural components of the system; as such they are relatively well conserved, so that the continuity of evolution is most readily perceptible at that level. This will enable us, in Chapter 2, to reconstruct a reasonably reliable description of the course that the evolution of the VRM system has taken. Following the methodological injunction that the object of evolutionary study should be defined, in the first instance, in purely structural terms brings a reward. By defining the object of study as the VRM system, a striking fact springs sharply and neatly into focus. Variable-region molecules exist in nearly all vertebrates, from sharks to humans; they do not however exist in the most primitive extant vertebrates (hagfish and lampreys) and they do not exist in any invertebrate animals.

Having clarified this fundamental point, however, I wish to make it clear from the outset that this structural account is only a preliminary. The ultimate object of this book is to examine the VRM *system* as a functional whole. Thus, on the basis of the structural account given in Chapter 2, I shall go on in

Chapter 3 to examine the (shifting) organizational *relations* between these components that give rise to systems with novel emergent properties and corresponding (shifting) functionalities. More precisely, I shall seek to identify the successive functional redeployments of the structural precursors of contemporary mammalian VRM systems. And the nexus of this inquiry will be the first appearance of the VRM system, which by some criteria seems to have sprung fully-fledged and quasi-miraculously out of nowhere early in vertebrate phylogeny. Where *did* the VRM system come from? What was the unique opportunity, or the unique novel necessity, or the unique conjunction between an opportunity and a novel functional necessity, that can explain this sudden appearance of the VRM system as such? In other words, what was the function of the VRM system at this crucial point in evolutionary history?

In our present state of relative ignorance, it is of course likely that any answer to this question will be little more than a speculative hypothesis. Nevertheless, the attempt seems worthwhile, if only to provide heuristic guidelines for future research. What then are the elements at our disposal for the construction of such a hypothesis? A systematic review of all the possible elements would of course be a vast undertaking, and it is not my intention to attempt an exhaustive encyclopedic review which would be beyond my competence. My aim is rather to illustrate the way in which a synthetic theoretical viewpoint can serve to focus attention on truly *significant* details.

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CHAPTER 2

ELEMENTS FOR THE CONSTRUCTION OF A HYPOTHESIS

Faithful to the principles outlined in the introduction, I shall prepare this account of the various functions of the evolving VRM system by a review of the evolution of the structures involved: genes, molecules, cells and organs. In other words, the aim of this chapter is to summarize the major lines of evidence which I shall seek to incorporate into this account.

GENERAL BACKGROUND

I shall start by reviewing a certain amount of background information necessary to situate my description of the evolution of the structural elements of the VRM system.

TAXONOMY AND CHRONOLOGY

Whereas an "immune system", based on other defense strategies well capable of eliminating bacteria, viruses and other foreign bodies, is already clearly present in invertebrates, the *VRM system* as I have defined it first appears in vertebrates. The general framework for my account, which makes it possible to relate the different lines of evidence to each other, is thus provided by the chronology of the major taxonomic events leading up to and including the evolution of vertebrates. The data shown in Table 2.1 are based primarily on comparative anatomy and the fossil record, supplemented by calculations of "molecular clocks" based on the accumulation of random mutations in functionally non-critical regions of DNA and proteins.

Date (MYr ago)	Taxonomical Event	Biological/Ecological Event
900 ¹	First unicellular eukaryotes	
900-700 ¹		Cellular differentiation
700 ¹	Metazoan phyla established Invertebrates	Increased atmospheric O ₂ Macroscopic animals
600 ⁶	Protochordates	
500 ⁶	First vertebrates	Spinal cord, central nervous system
410 ² , 485 ³	(Agnatha-Gnathostomes)	First endocrines
400 ⁴	Bony fish	Initial development of the head
450 ⁶	(Chondrichthyes-teleost)	
350 ⁴	Amphibians	Move from water to land
385 ² , 387 ³	(Teleost-tetrapod)	Double heart and separate lung-tissue circulation; Long bones
300 ⁴	Reptiles	Amniote egg; XY and ZW sex chromosomes
345 ² , 260 ³	(Amphibian-amniote)	
300 ² , 180 ³ , 300 ⁵	(Ancestral bird-mammal divergence)	
150 ⁴	Birds (from reptiles)	Warm-blooded animals
130 ⁴	Mammals (from reptiles)	
75 ² , 90 ³ , 70 ⁴	Extinction of dinosaurs; rapid radiation of eutherian mammals from bats to whales	

Table 2.1. The evolutionary context of the emergence and development of the VRM system.

References: (1) Buss¹. (2) Ladd Prosser²: paleontology. (3) Ladd Prosser²: protein clocks.

(4) Nei³. (5) Ayala⁴. (6) Du Pasquier⁵.

MULTICELLULAR VERSUS UNICELLULAR ORGANISMS

One of the most fundamental stages in evolution, after the origin of life itself, was the passage from single-cell to multicellular organisms. The first eukaryotic cells appeared some 900 million years (MYr) ago. There followed a relatively short period of 200 MYr which saw the rapid evolution of a variety of different metazoans, multicellular organisms made up of a number of terminally differentiated cell-types. The body plans of contemporary metazoans fall into a number of discrete categories. These fundamental "bauplans" (together with a number of other bauplans, bizarrely unlike any contemporary forms, which rather rapidly became extinct) all appeared during this short period; by 700 MYr there are metazoans clearly recognizable as members of all the phyla that are extant today. Since then, no new body plan has arisen; for over 600 million years, metazoan development has continued to follow the same basic formulae. In an important and highly original book, Buss¹ has remarked on this striking pattern, emphasizing that it calls for explanation. He propounds the view that a cardinal consideration in this respect is the potential conflict between natural selection at the level of cell lines, and selection at the level of the multicellular individual: the latter requires that the "interests" of cell-lines be subordinated to the requirements of the individual organism as a whole. He has argued convincingly that the evolutionary patterns of metazoan development can be largely understood in terms of a necessary resolution of this potential conflict. I shall return to this thematic opposition between cell-lines and the individual multicellular organism in Chapter 3, since it will provide the starting point for the construction of my hypothesis concerning the functional biology of the VRM system at the time of its evolutionary origin.

BIOLOGY OF THE FIRST VERTEBRATES

The VRM system made its evolutionary appearance very early in vertebrate phylogeny. In order to examine the possible function of the primitive VRM system, it is therefore necessary to characterize the general biology of these first vertebrates. What sort of animals were they? What was their "life strategy", their "ecological niche"? More specifically, what was the significance of the innovation of a vertebrate spinal cord? What were the particular life-problems for which this innovation was relevant? And what were the new problems which this innovation caused in turn?

Although the origin of vertebrates is still somewhat clouded in mystery, the initial answers to these questions are rather disappointing. The first vertebrates were aquatic animals with a simple, elongated body displaying bilateral symmetry. They were jawless fishes without any appreciable discontinuity between head and body. Their living representatives (the hagfish and lampreys of the class *Agnatha*) are parasitic on other fish, but this "life strategy" is clearly a subsequent, degenerative development. Altogether, they seem to have been rather unassuming, unremarkable creatures; there is certainly nothing so excitingly dramatic as the move from aquatic to terrestrial life.

The answers only begin to be more interesting when we turn to their internal organization. Any account of this must start with a description of their embryology. *The* great innovation of the chordates in this respect was the development of a spinal cord (initially cartilage rather than true bone), with the gut situated ventrally and – a particularly important novelty – a *dorsally* situated neural tube running the whole length of the animal⁶. Already a small piece of the jigsaw puzzle seems to drop into place: a re-organization of embryological processes of this order must surely have involved a diversification and redeployment of the cell adhesion molecules, and in particular, those which regulate the morphogenesis of nervous tissue. It is suggestive to note here that the genes coding for NCAMs and numerous other adhesion molecules belong to the Ig-gene superfamily⁷⁻⁹.

Moving on from embryology to anatomy and physiology, the dorsal neural tube was the foundation stone for the development of a true *central* nervous system. From this time on, the sensory inputs (principally from eyes, ears and nose) were progressively centralized and coordinated at the anterior end of the neural tube: the cephalization of this anterior region was to give rise to the development of the brain. To be sure, this innovation did not immediately reach the heights it was to achieve later in birds and mammals; a Bdellostome, living representative of these primitive vertebrates,

is quite capable of swimming vigorously away five hours after being decapitated¹⁰. Nevertheless, the trend was set.

Another physiological innovation that dates from this time, and which was also destined for a glorious future, was the first *endocrine* organ: the thyroid, up until then an exocrine organ, now for the first time secreted its product internally into the blood stream⁶. An endocrine function implies of course, as a pre-requisite, an effective *circulatory* system: and indeed the first vertebrates did possess a real if rudimentary cardiovascular system. They had blood, with plasma and red and white blood cells, and there was clear arteriovenous differentiation, with a central heart (although this organ was as yet without innervation, and there were still accessory venous "hearts"). These animals also possessed a lymphatic system draining the tissues; this system too was at the very beginning of its differentiation being still a hemo-lymphatic system containing red blood cells. We may mention also the existence of a rudimentary kidney; and the fact that the respiratory function was localized in an organ derived from the pharyngeal region of the digestive tract, opening towards the exterior, the gills ejected the water ingurgitated through the mouth.

Finally, we may note that the first vertebrates made their appearance a surprisingly short time after the move from unicellular to multicellular organisms. This suggests that the thematic opposition between cell lines and the individual organism as a whole, noted by Buss¹, was in all likelihood still an active issue. In this respect Ohno¹¹ has expressed the view that the multitude of vertebrate oncogenes which encode autocrine (intracellular) growth factors are evolutionary relics of the past. Unicellular eukaryotes have no choice but to depend on autocrine growth factors, and growth and differentiation of many multicellular eukaryotes such as insects appear to be cell autonomous, suggesting continued dependence on autocrine factors. With the development of the cardiovascular system, vertebrates have forfeited the cell autonomous mode of growth and differentiation. The functions of autocrine growth factors were replaced by extracellular endocrine factors and their specific membrane receptors, in order to deprive individual somatic cells of their last vestige of autonomy. The propensity of oncogenes to go astray and cause cancers due to autonomous cell growth might reflect their status as evolutionary relics which have remained in a functional state not because of positive natural selection, but because of their internal repetitiousness which makes them relatively impervious to deletions and insertions.

In summary, we see that in these first vertebrates, the main features of the physiological organization that will be further developed and refined in the course of subsequent evolution are already laid out. On the one hand, there is a localization of discrete functions in specialized organs – heart, gills, kidney, eyes, ears, nose. On the other hand, and simultaneously, we see the emergence of "central systems" which coordinate these differentiated functions: the central nervous system, a developed circulatory system, the endocrine system, and (so I shall argue) the VRM system.

VERTEBRATE GENOMES

On the basis of observations of chromosome number and DNA content per cell, Ohno¹² has provided an account of the evolution of vertebrate genomes. *Amphioxus*, an organism considered to be closely related to the immediate ancestors of the first vertebrates, has a DNA content which is 17% that of contemporary mammals. This value is close to that of 20% which is the minimum found in present-day fishes: arguably, it represents the "primordial" vertebrate genome. Ohno distinguishes two types of duplication: tandem duplication (by unequal crossing-over), which produces copies of short segments adjacent on the same chromosome; and polyploid duplication (notably auto-tetraploidy followed by diploidization, an event that has apparently occurred recently in salmonoid fish), which produces a multiplication of the entire genome, the copies being on different chromosomes. Of the two mechanisms, the latter is theoretically the more significant, because after tandem duplication, the mechanisms of gene regulation are such that it is difficult to achieve functional diversification. Ohno suggests that during the period when the vertebrate genome was in flux, tetraploid duplication occurred in the fish/amphibian ancestors of reptiles, birds and mammals. More precisely, he suggests that two distinct instances of tetraploid duplication occurred in vertebrate evolution. The first took place in the ancestors of the Diapsidian reptiles which gave rise to dinosaurs, snakes and lizards, and birds: this genome subsequently stabilized with a ZW system of sex chromosomes and a DNA value 50-60% that

of mammals. The second tetraploidy occurred somewhat later in the common ancestors of the Anapsidian reptiles (subsequently turtles, tortoises, alligators and crocodiles) and the Synapsidian reptiles which gave rise to mammals. This genome subsequently stabilized with an XY system of sex chromosomes and a DNA value 80-100% that of mammals. In summary, Ohno has made the major proposal that the genomes of all contemporary vertebrates other than fish and amphibia are characterized by a tetraploid duplication of the "primordial" vertebrate genome.

If true, this major event would provide a central reference point in early vertebrate evolution. Unfortunately, it is by no means clear that such an event has really occurred. Theoretically, if there was indeed a major polyploid event, there should be numerous examples in higher vertebrates of duplicate genes on different chromosomes, and dating by molecular clocks should give a convergent estimate for the date of this event. In fact, examples which have been explicitly interpreted in this way are virtually non-existent in the literature. The genes for the vertebrate hemoglobin alpha and beta chains, which are often on different chromosomes, seem at first sight to fit the required pattern: the difference between the alpha and beta chains within a single species is greater than the difference in alpha (or beta) chains from one species to another. Moreover, the hemoglobin of protochordates and lampreys is a single-chain molecule¹³. However, in newts the genes for alpha and beta chains are close on the same chromosome, which suggests that in fact these genes arose by tandem duplication with subsequent dispersal to different chromosomes¹⁴.

The same pattern holds for the immunoglobulin light and heavy chains, and Ohno¹² explicitly cites this as a major example of genes arising from the hypothetical tetraploid duplication. However, all species of vertebrates, including primitive fishes such as sharks, have circulating immunoglobulins with distinct light and heavy chains; this excludes the hypothesis that the genes in question arose from a tetraploid duplication in lower vertebrates at the later stage of bony fishes or amphibians. In addition, we may note that the genes for the kappa and lambda light chains in mammals are also on different chromosomes, as are those for the various T-cell receptor chains (with the sole exception of the δ and α genes, which as we shall see are in tandem on the same chromosome). Since there has been at most one tetraploid duplication, this means that the pattern of related genes on different chromosomes must have arisen in most if not all cases by tandem duplication followed by dispersal to different chromosomes.

In conclusion, there is unfortunately no convergent evidence in favor of an identifiable tetraploid duplication in the ancestry of all higher vertebrates. Thus, other putative examples, such as the two genes for phospholipase A which has been adapted as a component of venom in snakes¹⁵, are also likely to have arisen by tandem duplication with subsequent dispersal.

The period of major flux in vertebrate genomes came to an end with the invention of the amniote egg and the elimination of a free-living larval stage at the time of the first reptiles. The reason is that whereas in fish and amphibians the sex chromosomes are undifferentiated, in reptiles (and subsequently in birds and mammals) the XY or ZW mechanism means that polyploid organisms are sterile and indeed often non-viable. This had the effect of stabilizing the main organizational features of the genome, which have remained unchanged since that time in reptiles, birds and mammals.

REPTILES, BIRDS AND MAMMALS

The evolutionary relationships between reptiles, birds and mammals are complicated but important for our subject¹⁶. Contemporary reptiles consist of three main orders of animals which are quite widely separated from each other in evolution: the turtles and tortoises, the snakes and lizards, and the crocodiles and alligators. The reptiles, which are ectotherms, are related to the endotherms in a complicated bush. Mammals diverged from the common ancestor only shortly before the various reptile branches diverged from each other (or perhaps even after the turtles diverged from the rest of the amniotes). Birds derived from archosaurs (which include the famous dinosaurs) which are most closely related to the crocodylians, and are thus more closely related to one branch of the reptiles than the various branches of the reptiles are to each other. Hence, any similarities between the avian and mammalian immune systems will be either found in all reptiles as well (if they have not been lost by secondary evolution) or will be due to convergent evolution (perhaps in relation to endothermy). As we shall see, these considerations are important for the question of MHC evolution¹⁷.

GENES

The genes necessary for the synthesis of variable-region molecules have been studied in great detail. Rather than attempting an exhaustive review of the primary experimental evidence, I shall present here a summary of five topics of particular interest for our reconstruction of the evolution of the structures of the VRM system: the immunoglobulin superfamily; the molecular mechanisms of somatic DNA rearrangement; the question concerning the existence of VRMs in primitive jawless vertebrates; genome organization; and the relationship between $\alpha\beta$ and $\gamma\delta$ TCRs. In this section I shall concentrate on the evidence from the linear sequences of nucleotides in DNA and of amino acids in proteins.

THE IG SUPERFAMILY

When immunoglobulin (Ig) chains were first sequenced, segments within the constant regions of H and L chains showed sequence similarities, and this led to the idea that the Ig chains had all evolved from a primordial gene coding for about 100 amino acids⁸. The domains within the Ig chains all contained a characteristic intrachain disulfide bond, and the hypothesis that the domain represents an independent structural unit was confirmed when the structures of the V and C domains were shown to have a common fold that is stabilized by the conserved disulfide bond.

Subsequently, many new molecules have been identified and sequenced, and the Ig-related family now includes not only the T-cell receptors (TCR), lymphocyte markers (CD1-8), MHC class I and class II, but also molecules which are not specifically components of the vertebrate immune system such as the differentiation antigen Thy-1, the cell adhesion molecules (CAMs) which play a key role in embryogenesis⁷ and the carcinoembryonic antigen (CEA). The molecules of the Ig superfamily thus have a diversity of functions, but the common denominator seems to be a general role in cell surface recognition¹⁸.

SOMATIC DNA REARRANGEMENT

The V-region in each chain of a VRM is translated from a single mRNA which also encodes the respective C-region. V-region genes, however, do not exist in germ-lines, and are formed somatically by an extraordinary process of DNA arrangement that brings together two or three gene segments in the genome of the differentiating lymphocyte. The chemistry of this recombination process, which as far as we know is unique to the VRM system, has revealed a very important feature for our discussion. In all rearranging groups of gene segments known to date, and in all species analyzed (including sharks, frogs, alligators, chicken and mammals), the same "signals" (nucleotide sequences at the joining sites; length of the "spaces" between them) are conserved. This important sequence is known as the "recombination signal sequence" or RSS¹⁹. Furthermore, mutants have recently been isolated in the mouse, the so-called rag knockouts, that fail to accomplish such rearrangements in all gene complexes, and thus produce no VRMs at all; this shows that the same enzymes and chemical processes are uniformly used for all VRM genes. It follows that in all likelihood, this extraordinary process of somatic DNA rearrangement was invented only once in evolution, and was thereafter recurrently used for all V-regions.

VARIABLE-REGION MOLECULES IN AGNATHA

The most primitive known vertebrates belong to the order Agnatha, the jawless fishes whose living representatives are the hagfish and lampreys. These animals obviously possess an immune system capable of defending them adequately against bacterial and viral parasites, and their sera contain an inducible protein which functions as an "antibody". It is a striking testimony to the confusion generated by automatically equating VRMs and the defense function that until very recently these "antibodies" were generally assumed to be immunoglobulins^{5,19}. However, the physicochemical properties of the serum protein fraction associated with these "antibodies" are dissimilar to the properties associated with immunoglobulins and they lack the typical multimeric disulfide bond. In fact, the hagfish humoral defense protein is not clonally derived, and exhibits structural and functional homology with mammalian complement components²⁰. The clinching evidence has recently come from

DNA sequences of the genes concerned in hagfish²¹ and lamprey²² which show that this molecule represents a complement polypeptide which retains close similarity to the common ancestor of mammalian C3 and C4. Exhaustive efforts to detect immunoglobulin gene homologues in both lampreys and hagfish have been uniformly unsuccessful with a variety of probes that complement highly conserved regions of vertebrate immunoglobulins²³. The basal protochordate species presumably possess an ancestral member of the immunoglobulin superfamily, but this molecule does not seem to be a VRM and so far it has not been identified.

GENOME ORGANIZATION

The most primitive vertebrates in which VRMs have been identified are the cartilaginous fishes (sharks and rays). These molecules are immunoglobulins which already possess the basic structure consisting of two 4-domain heavy chains and two 2-domain light chains typical of all higher vertebrates. Moreover, the V-region of the heavy chain is split into V, D, J segments and that of the light chain into V and J segments, and these segments are remarkably similar in their DNA sequences²³. However, the genomic organization of these genes is markedly different from that of higher vertebrates. In teleost fish, amphibians, reptiles and mammals there are multiple V segments adjacent to each other, followed by multiple D segments (for the heavy-chain genes) and multiple J segments. One element of each class is combined by the deletion of the intervening DNA regions to create a variable-domain exon which after transcription is spliced to the exons for the unique domains of the constant C-region for the immunoglobulin M heavy chain. There is thus a combinatorial source of variation based on the choice between several hundred Vs, some 20 different Ds and 4 different Js. In sharks, by contrast, the genes are structured as a single V region linked to a single D region linked in turn to a single J region, all of these linked to a single set of C-region exons. This entire pattern is then reiterated hundreds of times. For one of these genes to function, the intervening DNA is deleted between a neighboring V and D, and that D and its J, to create the V-D-J exon which is then connected by transcription and splicing to its constant domains. Diversity in the shark has thus two sources: inherited diversity from the many copies of the (V + D + J) + C sets, each one of which has drifted individually; and the junctional diversity on their assembly.

This alternative gene structure shows that at the moment of divergence of the two lines, some 450 million years ago, the common ancestor would have had a single copy of a (V + D + J) + C gene. Down one evolutionary road this entire unit was duplicated and re-duplicated many times, developing new variants after that. Down the road that leads to mammals, the parts of this unit – the V segments, the D segments and the J segments – were separately duplicated, creating greater possibilities of variation through the combination of the different regions²⁴.

$\alpha\beta$ AND $\gamma\delta$ TCRS

In mammals, T-cell receptors are differentiated into two classes: $\alpha\beta$ and $\gamma\delta$ receptors. In view of their similarity, it is likely that one arose by duplication of the other; the question is, which came first? At present, there is no evidence either for or against the presence of TCRs in cartilaginous fishes²³. Since in mammals $\gamma\delta$ T cells are remarkably unobtrusive and were discovered later with some difficulty, this absence of evidence is in itself a slight argument for the priority of the $\gamma\delta$ TCRs. This argument is however obviously insufficient; in order to obtain a more conclusive answer to the question of priority it is necessary to turn to indirect evidence.

The mechanisms accounting for the VRM diversity are essentially the same in these two classes, and hence provide no arguments concerning their evolutionary relationship. However, the whole of the δ locus (V, D, J and C) is imbedded in the α locus, so that the rearrangement necessary for the expression of a TCR α -chain eliminates all the δ genes from that chromosome²⁵. This detail of gene organization strongly suggests that the $\gamma\delta$ receptor is the more primitive form.

The diversity of the $\gamma\delta$ receptors is much less than that of the $\alpha\beta$ receptors. This difference arises in part because $\gamma\delta$ TCRs are assembled from a significantly lower number of germ-line gene segments than those available to $\alpha\beta$ TCRs (or Igs), a fact which tends to confirm that the $\gamma\delta$ receptor is probably the more primitive form. In contemporary $\gamma\delta$ T cells, two independent processes accentuate this

difference in diversity. Firstly, certain subsets of $\gamma\delta$ T cells, which show very restricted diversity, are generated from precursors at a stage in development when V-gene rearrangement is very selective for 3' genes in the locus, and the enzymes which introduce functional diversity are not fully expressed. Secondly, there is a mechanism of cellular selection for uniformity in V regions that is actually so precise that it requires novel theoretical considerations not available this far.

I will discuss the relationship between $\alpha\beta$ and $\gamma\delta$ T cells more fully in the section on cells below.

MOLECULES

THE IMMUNOGLOBULIN SUPERFAMILY

The Ig superfamily is notable because no extracellular sequence includes an enzymatic activity or segments from more than one protein superfamily. Even more important, these molecules are characterized by their ability to associate either with themselves (homophilic) or with other (heterophilic) members from within the Ig superfamily²⁶. Examples are the homophilic binding of NCAM and the binding of TCRs to MHC. These molecules also commonly form dimers, and binding between domains can be homophilic or heterophilic. Stable dimers are often disulfide-linked, but metastable interactions can also occur; for example, immunoglobulins, TCRs and MHC are all heterodimers in which the domains of the two constituent polypeptide chains show strong tendencies to associate even in the absence of covalent bonds. When such homophilic or heterophilic binding occurs between molecules on opposed membrane surfaces, it gives rise to the typical adhesion functions or binding functions which trigger a subsequent event at the cell surface. Thus, in all functions in which Ig-related domains are known to be involved, the domain can be considered as providing a stable platform for the display of specific determinants for recognition reactions.

In terms of functional evolution the phenomenon of interactions within the superfamily is strongly suggestive that the primordial function involved a single domain interacting with itself probably between opposed membranes. Such a function has been suggested for the P₀ myelin protein²⁷. If correct, this may be an interesting model for the function of the primordial domain. Heterophilic receptor pairs presumably evolved from a homophilic interaction system. It seems likely that the first functions were related to adhesion or triggering at cell surfaces to control the behavior of cells within a multicellular organism. A possible functional antecedent for the role of vertebrate immunoglobulins is the phenomenon of programmed cell death. In the invertebrate *Caenorhabditis elegans*, 25% of developing neural cells die in a predictable way, and in some cases one cell appears to kill another.

The Ig-related molecules that function in neural tissues may also be mediating functions of the primitive type. A particularly interesting case is that of Thy-1, the smallest member of the Ig superfamily and thus arguably a "primitive" molecule. In mammals, Thy-1 is a major cell-surface component expressed by several tissues; the protein, carbohydrate and gene structures of this molecule are known, yet its function is not. It is highly expressed in nervous tissue, where it appears on virtually all neurons after the cessation of axonal growth. Tiveron et al²⁸ have shown that in tissue culture, expression of Thy-1 by a neural cell line inhibits neurite outgrowth on mature astrocytes; this inhibition could be reversed by low concentrations (nanomolar) of soluble Thy-1. If a similar interaction occurs in vivo, this would suggest that Thy-1 directly initiates a growth-inhibitory signal across the membrane in the case of cell-cell contact. As we shall see in Chapter 3, this is an attractive model for the function of the immediate ancestor of the first variable-region molecules.

Structural and functional evolution would be greatly illuminated if invertebrate Ig-related molecules could be identified. Thus far only one invertebrate sequence that might be Ig-related is known, and this is a glycoprotein of 84 amino acids that was identified in a search for Thy-1-like molecules from squid neural tissue. The squid glycoprotein has some interesting sequence similarities to Thy-1 and Ig V domains, but since it does not have a standard domain pattern it cannot at this stage be added with confidence to the Ig superfamily⁸.

IMMUNOGLOBULINS

All living vertebrates from sharks onwards have circulating immunoglobulins; these are

molecules composed of four-chain units, assembled by homodimerisation of two identical halves each consisting of one heavy and one light chain. Immunoglobulin molecules can consist of only one (IgG) or up to five (IgM) such units assembled in various ways. Thus they are molecules with at least two and as many as ten identical V regions or combining sites.

Immunoglobulin diversity is much less in cold-blooded vertebrates than in mammals^{5,26}. In Agnatha, hagfish and lampreys can make "antibodies" against sheep red blood cells, KLH, bacteriophage, Brucella, and human erythrocytes, but as we have seen the molecules in question are not immunoglobulins. In Chondrichthyes both biological and structural data indicate that the repertoire of these species is low. In Teleosti the anti-DNP response of the carp consists of IgM antibodies; the number of isoelectric focusing (IEF) antigen binding bands per individual is small and there is little variation from one outbred individual to another. It is impossible to say how many antibody types the IEF bands correspond to, but certainly fewer than 23; moreover, some anti-DNP cross-reactive idiotypes are present at a high frequency in an outbred carp population. Antibodies from three Cyprinid fish – carp, goldfish and tench – raised against penicillin show a rather low heterogeneity and striking IEF similarities even between unrelated fish of the same genus. In amphibians the primitive urodeles express a very restricted repertoire of antibodies; in *Xenopus*, as in the shark, the amino acid sequence indicates low antibody heterogeneity. It is important to note that there are high levels of natural antibodies: if there are few categories of antibodies, all of them are represented at a relatively high concentration in the serum. Early work on tadpoles proved that anuran larvae could respond specifically (with less than 10^6 lymphocytes) to many antigens; however, the heterogeneity of the response is much more restricted than in mammals. The antibody diversity of anuran amphibians has been estimated at 10^4 different antibody molecules.

If all species use more or less the same mechanism to generate immunoglobulin genes (see below), where does the difference in expressed repertoire between cold-blooded vertebrates and mammals come from? As already mentioned, it seems to lie in part in the gene organization. The shark Ig gene complex consists of hundreds of clusters of V, D, J and C elements, but the elements rearrange exclusively within one cluster. Such rearrangement without combination will clearly reduce the expressed diversity compared to what the same number of elements, organized in the mammalian way, would have done. However, this cannot be the whole explanation, because bony fish already have a gene organization which seems similar to that of mammals, and a multiplicity of germ-line V_H segments which actually approaches the complexity seen in mouse and man. Furthermore, the chicken germ-line V_H and V_L diversity is quite reduced, but their expressed repertoires (generated by gene conversion in the bursa of Fabricius) are large.

One reason for the differences in antibody diversity recorded in cold-blooded versus homeothermic animals may be that all studies have been carried out after immunization, and thus concern the antibodies in hyperimmune sera. In mammals it is well established that most of the antibodies produced under such conditions derive by somatic mutation from rearranged genes that were expressed in unmutated form before immunization. To date, there is no evidence of somatic hypermutation of VRM genes in cold-blooded animals. It is thus possible that the pre-immune repertoires are relatively comparable, the greater recorded diversity being due in part to somatic mutation.

Another possibility, which would account for differences in diversity even at the level of the "natural antibodies" which circulate in non-immunized animals, is that the introduction of non-template insertions was a relatively late evolutionary event. Overall, however, it must be admitted that although structural diversity certainly increases through evolution from cyclostomes to mammals, the evidence is insufficient at present to decide conclusively on the precise evolutionary sequence in which the various mechanisms for generating structural diversity in mammals were introduced.

Finally, we may note here that V-region chains are actually themselves mosaics of conserved and variable residues. Thus, the variability is restricted to a limited number of hypervariable or "complementarity-determining regions" (CDR1, CDR2, CDR3); these regions are separated by a number of "framework regions" (FR1, FR2, FR3) which have been highly conserved in evolution and are actually much less variable from one species to another than the "constant" C-regions. When the

linear primary amino-acid chain folds to form a 3-dimensional molecule, the three CDR regions are juxtaposed and together form the specific "combining site" of the molecule. Ohno et al^{29,30} have pointed out the probable significance of this finding: unrestricted variation in the whole V-region would produce many molecules with no specific binding capacity at all. The "mosaic" arrangement ensures that free-wheeling variation in the hypervariable regions will nevertheless produce molecules which conserve the overall domain structure and thus bind specifically to some antigen (or idio type) or other.

MHC

The major histocompatibility complex has been identified in every mammalian species that has been studied, so it is probably safe to assume that all mammals possess an MHC. In birds, the MHC has been demonstrated so far only in the domestic fowl, but there is every reason to believe that all other species of this vertebrate class possess this complex. Information about the presence of MHC in vertebrates other than mammals and birds is sketchy; the evidence, such as it is, is negative since gene probes have failed to detect MHC in *Xenopus*, trout, sharks or tunicates¹⁶. MHC evolution must have included events such as the formation of the primordial MHC molecule from a member of the Ig superfamily, the divergence of class I and class II molecules from their presumed common ancestor, the emergence of polymorphism, the association of peptides with MHC and the recognition of MHC by ancestral TCR, CD4 and CD8 molecules. In the absence of evidence, however, hypotheses concerning the timing, relative order, and structural and functional aspects of these events are pure speculation.

Turning to indirect arguments, there is now evidence that MHC Class I molecules, at least, are actually "heterotrimers" of the polymorphic Class I heavy chain, invariant β_2 -microglobulin, and one of the peptides (8-9 amino acids long) that can bind to that particular heavy chain. In other words, "empty" Class I molecules do not normally exist; even if they can be assembled and transported in the absence of a peptide, they are extremely labile and short-lived. It would seem to follow that throughout the evolutionary history of MHC, "peptide presentation" or "complex formation" with peptides internal to the cell has been an important theme.

We have already seen that the evolutionary relationships between reptiles, birds and mammals are such that any similarities between birds and mammals will be either found in all reptiles as well (if they have not been lost by secondary evolution) or will be due to convergent evolution (perhaps related to endothermy). Thus, although there is no good structural evidence for an MHC in any reptilian species, the fact that MHC exists in both birds and mammals suggests that it might well have existed in the ancestral reptiles. There is indeed evidence that some reptilian molecules exist which are very similar to MHC molecules of mammals and birds¹⁶. The class II molecules immunoprecipitated from the Egyptian sand snake, the red striped terrapin and the Caiman by cross-reactive xenoantisera are all glycoprotein heterodimers with physico-chemical similarities.

In conclusion, it is indeed a sad commentary on current fashions in biological research that adequate information on the molecular structure of MHC (and its precursors) in the different non-mammalian vertebrate classes, from Agnatha to reptiles and birds, is not available at the present time. On the basis of indirect evidence it may be very tentatively suggested that MHC made its first significant appearance in the reptilian ancestors of birds and mammals.

CELLS

B CELLS AND T CELLS

It has been traditional in immunology, for historical reasons, to consider that the immune system consists of two major subdivisions: the cellular component and the humoral compartment. Although it is now recognized that the humoral component of the VRM system (circulating immunoglobulins) is produced by secretory *cells*, the old division subsists in the form of a distinction between the B-cell compartment and the T-cell compartment. In order to square this distinction with a linear stepwise process, there are only three possibilities: either (i) secretory B cells derive from a primitive line of T cells, or (ii) T cells derive from a primitive line of B cells, or (iii) both B cells and T cells derive from

the differentiation of a common precursor.

We have seen that on the basis of the genes coding for the VRM receptors, B cells and T cells have a common ancestral lineage at least during the period when gene rearrangement was invented, up to and including the point when the second episome insertion gave rise to the D-segment in the V-D-J-C complex on one of the two chromosomes carrying these genes. On the other hand, still from the receptor viewpoint, the simple two-domain structure of the T cell receptor, the lack of complicated class switch, and the lack of secretory forms or of somatic mutations make this molecule a candidate for a conserved primitive recognition molecule⁵. This suggests that just before the time of divergence, the common ancestor to B cells and T cells resembled present T cells more than present B cells.

B-1 CELLS

Recent research has led to the identification of a characteristic subclass of B lymphocytes known as B-1 cells, as distinct from the B-2 cells which correspond to the normal B-lymphocytes produced in the adult bone marrow³¹. B-1 cells characteristically express the CD5 marker (in humans) and the Ly-1 marker (in mice). Their discovery has given rise to the concept of a developmentally layered system in which the B-1 cells represent the primordial form. Human B-1 lymphocytes are indeed the dominant clonotypes in the early B-cell repertoire, and B-1 cells account for most B cells in fetal and newborn mice. The fetal omentum is a source only of B-1 cells. The system is "layered" because the B-1 compartment subsists throughout adult life, where it coexists with the later-developing B-2 compartment. In unimmunized adult mice, B-1 cells are not generated by differentiation from precursors in the bone marrow. In fact, B-1 cells are self-replenishing, arising from cells emerging and therefore rearranging their Ig genes during fetal or early postnatal life. Another "primordial" characteristic is that even in the adult, athymic nude mice have normal levels of B-1 cells in the peritoneum, so that functional T cells are not necessary for their development.

If we seek to characterize this "primordial" compartment, several facts are worthy of note. Only IgM has been seen to be produced by B-1 cells in the mouse peritoneum or in blood. The majority of available data state that in B-1 cells, IgV genes are expressed in germline form, without any somatic mutation. In the normal adult, B-1 cells are very rare in peripheral blood and the spleen and are virtually absent from lymph nodes, but are very frequent among free-living lymphocytes in the peritoneal cavity and also among B cells in the lamina propria of the gut. It has also been suggested that B-1 lymphocytes constitute the physiological equivalent of the neoplastic elements in various lymphoproliferative disorders.

Finally and most significant of all for the theme of this book, B-1 characteristically include a significantly high proportion of cells committed to the production of immunoglobulins that bind a variety of self-antigens, including hormones, nucleic acids and phospholipids in addition to isologous and heterologous serum proteins, erythrocytes and tissues. Immunoglobulins with these properties arise independently of known specific immunization and have been referred to as *natural antibodies*³². A considerable proportion of natural antibodies are polyreactive, i.e., they are known to bind to two or more different antigens. Because of their ability to bind self-antigens, including other immunoglobulins, natural antibodies would provide the structural correlate for the high degree of interclonal "connectivity", i.e., idiotypic cross-reactivity of the early B-cell repertoire³³⁻³⁵. We shall see that these characteristics are strikingly analogous to those of what I shall call the phylogenetically "primordial" VRM system.

$\alpha\beta$ AND $\gamma\delta$ T CELLS

As regards the evolutionary relationship between $\alpha\beta$ and $\gamma\delta$ T cells, there is no direct evidence because TCRs of either type have only so far been identified in mammals (where they have been found in all species examined so far: humans, mice, rats, sheep, cattle and pigs) and in birds (chickens)³⁶. The molecular genetic identification of TCRs in lower vertebrates is an obvious area for fruitful investigation.

However, turning to indirect evidence, five characteristics suggest an earlier origin of $\gamma\delta$ T cells or, rather, their higher relatedness to the primordial T lymphocyte from which both T cell lines

evolved. First, $\gamma\delta$ cells precede $\alpha\beta$ T cells in development and are thus the first TCR bearing cells to appear in ontogeny³⁷. At a molecular genetic level, rearrangements and surface expression of γ and δ genes precede those of α and β genes³⁶. Second, in contrast to $\alpha\beta$ T cells, some $\gamma\delta$ T cells differentiate in the absence of a thymus and could thus have preceded evolutionarily the rather late development of this organ. Third, most $\gamma\delta$ T cells do not express the CD4 or CD8 accessory cell-adhesion molecules which are essential in the process of thymic selection of $\alpha\beta$ T cells. Fourth, the VRM diversity (both potential and somatically generated) of $\gamma\delta$ T cells is considerably lower than that of $\alpha\beta$ T cells and seems predominantly directed at MHC class I genes that are expressed in a tissue-specific manner and show little polymorphism in the species. In addition, neither γ nor δ chains appear to undergo somatic hypermutations, an important mechanism for diversifying immunoglobulin V regions following antigenic stimulation³⁷. Fifth, as already mentioned, the V, J and C genes for the δ -chain are contiguous on the chromosome, being flanked on one side by the V genes for the α -chain and on the other side by the J and C genes for the α -chain of the TCR. This arrangement is natural if the α -chain genes arose by tandem duplication from the δ -chain genes, but would be completely bizarre if the evolutionary relationship were the other way round.

Finally, we may note that Raulet³⁷ explicitly agrees with our conclusion, suggesting that several paradoxes can be resolved by recourse to an evolutionary perspective in which the $\gamma\delta$ cell receptor preceded the $\alpha\beta$ cell receptor and functioned originally to survey tissues for a limited array of predictable antigens.

$\gamma\delta$ CELLS

In view of our conclusion that $\gamma\delta$ cells represent the primordial lymphocytes in which variable-regions molecules were first expressed, and from which B and T cells derived, they are of particular importance for our attempt to reconstruct the evolutionary origin of the VRM system. We shall therefore briefly review what is currently known about this class of lymphocytes.

In defining subclasses of lymphocytes, it is usual to start by detecting specific functional activities. In contrast, progress in understanding $\gamma\delta$ cells is proceeding in the reverse order, from genes to TCR and from TCR to a new class of lymphocytes³⁶. The long odyssey of immunologists searching for the T-cell receptor came to an end when genes rearranging in T cells but not in B cells were identified and cloned. However, this technique led to the discovery not only of the expected $\alpha\beta$ TCR, but also of a hitherto unsuspected receptor, the $\gamma\delta$ TCR; this was followed by the discovery of a novel class of lymphocytes bearing this receptor³⁷. Ever since their discovery in 1986, the biological function of these cells has been the object of intense study, but to date the results are remarkably inconclusive. A major reason for this is certainly the preconceptions derived from our knowledge of $\alpha\beta$ T cells: most investigators searching for $\gamma\delta$ cell function have designed their experiments as if $\gamma\delta$ cells were just other $\alpha\beta$ T cells³⁶. If, as I argue in this book, the $\gamma\delta$ cells are evolutionarily primitive, pre-existing even B lymphocytes, and $\alpha\beta$ T cells correspond to a later specialization, this unexamined assumption is likely to lead us seriously astray. I shall therefore concentrate on summarizing the evidence that $\gamma\delta$ cells are *not* like $\alpha\beta$ T cells.

Firstly, as already mentioned, some $\gamma\delta$ cells can develop independently of a thymus and their designation as "T cells" is thus questionable. Secondly, $\gamma\delta$ cells have not been noticed by cellular immunologists in innumerable studies of humoral and cell mediated immune responses, and several findings indicate that $\gamma\delta$ cells do not normally function as helper cells for B cells³⁶. It is true that, like all other lymphocytes, $\gamma\delta$ cells are under strict control of their cell-surface receptors. However, $\gamma\delta$ cell populations do not seem to contain many cells that recognize allogeneic MHC proteins or any other polymorphic proteins; the specificity of clones that do recognize MHC proteins is unusual in that it is broadly cross-reactive for the products of different alleles³⁶.

More positively, it has been suggested that $\gamma\delta$ cells have a surveillance function against cancer. The following observations can be cited to support this idea³⁶. First, IL-2 activated $\gamma\delta$ cells can kill many different tumor cells. The molecular basis of the distinction between normal cells and tumor cells by the lymphokine-activated $\gamma\delta$ cells is not known. Second, a subset of human $\gamma\delta$ cells appears to recognize a superantigen on the surface of some Burkitt lymphoma cells. Another subset recognizes

Epstein Barr virus transformed B cells. Third, some $\gamma\delta$ cells that were isolated from patients with Burkitt lymphomas in complete remission were shown specifically to recognize autologous tumor cells. Fourth, $\gamma\delta$ cell lines could be established from populations of tumor infiltrating lymphocytes. These observations are of course far from conclusive, but they are nevertheless suggestive.

Finally, we may note that the TCR repertoire of $\gamma\delta$ cells generated outside the thymus is characterized by restricted V-gene usage and high junctional diversity. This finding accords well with the notion that $\gamma\delta$ cells are representative of an evolutionarily primitive form of VRM producing cells.

ORGANS

GENERAL

Invertebrates do not possess any organized lymphoid tissue, only individual lymphoid cells scattered through the body. This condition probably also pertained in ancestral vertebrates, so that the lymphoid cells moved freely through the tissues, but at later stages some of them began to assemble into lymphoid foci which are particularly abundant in certain regions of the gut – the mouth and pharynx, the throat region, esophagus, and intestinal wall. Masses of lymphoid tissue are still found at these sites in virtually all vertebrates³⁸. On the classic view of the immune system as a defense system, this early predominance of gut-associated lymphoid tissue (GALT) would be related to the fact that the gut is a major route of entry for pathogenic microorganisms. In our view, the significance of GALT is more likely related to processes of nutrition and digestion; we shall return to this question in Chapter 3.

In the next stage of development, portions of lymphoid tissue began to gain independence from the gut and to organize themselves into definite lymphoid organs – the thymus, the spleen and lymph nodes. The separation from the gut was gradual, and intermediate forms reflecting this stepwise achievement of independence can still be found among the more primitive vertebrates³⁸.

BLOOD FORMING ORGANS

Invertebrates do not possess any special blood-forming organs or even tissues. Whenever blood cells are present, they arise individually by transformation of certain mesodermal cells. In vertebrates, blood cells form at specific sites in the body at which specialized blood-forming tissues reside; in more advanced vertebrates these tissues organize themselves into two main special organs: spleen (in fish, amphibians and reptiles) and bone marrow (in amphibians, reptiles, birds, mammals). The transition from blood formation primarily in the spleen to blood formation primarily in the bone marrow thus occurs in amphibians. It is probably tied to the more general, drastic changes accompanying the transition from aquatic to terrestrial life. Among other things, terrestrial animals must have efficiently constructed bones, especially long bones, the most effective construction of which involves hollow bones. The newly acquired body space is then used for other functions such as depositing lipids and blood formation³⁸.

SPLEEN

Phylogenetically (as well as ontogenetically) the spleen is derived from the gut. Its ancestor is the mesodermal tissue that envelops the gastrointestinal tract of the most primitive vertebrates, such as the hagfish, lamprey, and dipnoi. In the hagfish, this tissue contains masses of differentiating blood cells, organized into islets and cords around the venous channels³⁸.

THYMUS

The thymus is present in all but the most primitive vertebrates: it has not so far been identified in the hagfish and there is some doubt about its presence in lampreys. When present, it is derived – except in mammals – from the dorsal side of the embryonic gill pouches³⁸.

LYMPH NODES

Although typical lymphatic vessels are present in all vertebrates upward from bony fishes, typical

lymph nodes occur only in mammals³⁸.

THE EVOLUTION OF THE STRUCTURES OF THE VRM SYSTEM

We are now in a position to give a synthetic account of the evolution of the VRM system as this may be reconstructed with fair confidence on the basis of the available evidence. The general evolutionary context has already been summarized in Table 2.1. I shall now present a summary view of lymphocyte evolution supplemented by a consideration of lymphocyte ontogeny. Finally, I shall conclude this chapter by presenting a reconstruction of the evolution of the molecular genetic structures.

LYMPHOCYTE EVOLUTION

On the basis of the evidence we have considered, it is possible to reconstruct the following scenario of lymphocyte evolution.

1. The common ancestor of B cells and T cells probably shared certain characteristics of both cell types. Thus, the lymphocyte-like cells which appear first in coelomates have been considered to represent primitive T cells. In the same class of animals, however, stimulation of coelemocytes by mitogens such as Con A, PHA, and LPS showed that these cells exhibit functional similarities with both T and B mammalian lymphocytes⁵.

EVOLUTION OF CELLS AND ORGANS				
STAGE	CELL-TYPES	HAEMATOPOIESIS	LYMPHOID ORGANS	CIRCULATORY SYSTEM
INVERTEBRATES	COELOMOCYTES ↓ "γδ-LIKE" LYMPHOCYTES	LOCAL TRANSFORMATION OF MESODERMAL CELLS	————— (FREE DIFFUSION THROUGH TISSUES)	COELOM BLOOD ↓ HEART ↓
1ST VERTEBRATES				
BONY FISH	T-CELLS (γδ-LIKE) B-CELLS	MESODERMAL BLOOD ISLETS LIVER ↓ SPLEEN	GALT ↓ SPLEEN ↓ THYMUS ↓	LYMPHATIC VESSELS ↓
AMPHIBIANS	αβ γδ	↓ SPLEEN / BONE MARROW		
REPTILES				
BIRDS				
MAMMALS	SUB-TYPES		LYMPH NODES	

Figure 2.1. A summary of lymphocyte evolution which provides the evolutionary context for the emergence and development of the VRM system. The common ancestor contemporary lymphocytes most closely resembled γδ "T" cells (at this stage there was no thymus). Ig-producing B-cells diverged from this ancestor at an early stage, probably that of the common ancestor to all contemporary vertebrates from hagfish to mammals. Finally, αβ T-cells derived from the γδ-like ancestral line at a much later stage. These cells are unique in absolutely requiring two specific organs for their production: initially formed in the bone marrow, where their commitment to colonize the thymus rather than to produce immunoglobulins occurs, they then migrate to the thymus, an obligatory step for them to complete their maturation. This striking characteristic rules out any interpretation which would attribute them the status of evolutionarily primitive cells. GALT: gut-associated lymphoid tissue.

2. The divergence between B cells and T cells occurred with the appearance of light and heavy-chain immunoglobulins and the emergence of a secretory form in B cells. Just before this divergence, the common ancestor probably resembled $\gamma\delta$ cells more than any other contemporary class of lymphocytes. We may conclude that B cells separated from the $\gamma\delta$ -like ancestral lineage before the appearance of $\alpha\beta$ T cells. At this point, B cells were probably more similar to B-1 cells than to B-2 cells.

3. Finally, $\alpha\beta$ T cells derived from the $\gamma\delta$ -like ancestral line at a later stage. In contrast to $\gamma\delta$ TCR, $\alpha\beta$ TCR are predominantly directed at MHC molecules (either class I or class II) that are expressed by most cells in the body, are highly polymorphic in the species, and are "recognized" by $\alpha\beta$ VRMs in association with CD4 and CD8 molecules. It is thus likely that $\alpha\beta$ T cells, and specific forms of B cell-T cell interaction such as T-helper activity, co-evolved with the polymorphism of those MHC loci in the species; I argue that this was a relatively late pattern in vertebrate evolution. This scenario is graphically represented in Figure 2.1.

LYMPHOCYTE ONTOGENY

As noted in the introduction, although precise parallels between phylogeny and ontogeny are unreliable, the fact that evolution occurs by modifications in the processes of development means that there is always a relationship between phylogeny and ontogeny. The usefulness of viewing ontogeny in the light of phylogeny (and vice versa) is well illustrated by the case of lymphocytes.

In mammals, VRM producing lymphocytes belong to one of three types: Ig-producing B cells; $\alpha\beta$ T cells; and $\gamma\delta$ "T" cells (the reason for the inverted commas on "T" will presently become apparent). We have come to the conclusion that phylogenetically, the common ancestor to these three types of VRM lymphocytes most closely resembled contemporary $\gamma\delta$ "T" cells. Ig-secreting B cells diverged from this ancestor at an early stage, that of the common ancestor to all contemporary vertebrates from sharks to mammals. $\alpha\beta$ T cells diverged from this ancestral line at a later stage. Mammalian lymphocyte ontogeny *cannot* parallel this phylogeny directly, because the definitive commitment to one of these three types depends on which of the three mutually exclusive forms of gene rearrangement (necessary for VRM production) occurs, and this is a single irreversible event in the ontogeny of any cell-line. However, as we shall see, the ontogeny of the three types of VRM producing lymphocytes does reflect their phylogeny.

The ontogeny of metazoans generally (as opposed to that of plants and fungi) is characterized by the migratory propensity of differentiating cells¹. This propensity is particularly marked in the case of hematopoietic cells; thus, even their intra-embryonic or extra-embryonic (yolk-sac) origin has been an object of discussion¹. On the other hand, in the course of phylogeny, hematopoiesis in the adult animal has been progressively localized in specialized organs (mesodermal tissue, gut, liver, spleen, bone marrow, thymus). It is on this background that I shall examine the ontogeny of the three types of VRM producing lymphocytes.

In order of evolutionary appearance, the first type of VRM producing lymphocyte is the $\gamma\delta$ "T" cell. These cells are typically made *outside* the thymus (hence the quotation marks on "T"), and subsequently migrate, notably to the surfaces of the body: digestive and urogenital tracts, lungs and skin. The precise site where $\gamma\delta$ cells are made is not known (it is *not* the bone marrow; they could actually be produced locally): this recalls the diffuse mesodermic origin of hematopoietic cells in evolutionarily primitive animals. It is true that in mammals, some subclasses of $\gamma\delta$ lymphocytes do migrate to the thymus, where they expand; but *this* is almost certainly a relatively recent evolutionary development.

According to my reconstruction, the second type of VRM producing lymphocyte to appear in evolution is the Ig-secreting B cell. In adult mammals, B lymphocytes are made in the bone marrow. However, during ontogeny, the successive waves of B lymphopoiesis occur in the coelom (para-aortic islets and omentum), liver, spleen and finally bone marrow³⁹ – a sequence which is indeed a striking recapitulation of phylogeny. Moreover, if the bone marrow is selectively destroyed by strontium 90, B lymphopoiesis can actually revert to spleen, and upon splenectomy to the liver, which can be

interpreted as another reflection of evolutionary history. It also seems plausible to suggest that at the time of their first appearance in evolution, B-cells resembled the ontogenetically primitive B-1 cells which are characteristically formed in the fetal omentum.

Finally, according to my reconstruction the third type of VRM producing lymphocyte to appear in evolution is the $\alpha\beta$ T cell. These cells are unique in requiring two specific organs for their production: initially formed in the bone marrow, where their commitment to colonize the thymus rather than to produce immunoglobulins occurs, they then migrate to the thymus, an obligatory step for them to complete their maturation. This striking characteristic clearly corresponds to their status as evolutionary late-comers, and is sufficient to virtually rule out any interpretation which would attribute them the status of evolutionarily primitive cells.

THE EVOLUTION OF THE MOLECULAR GENETIC STRUCTURES

The Initial Stages

The basic events, which serve as a foundation for our subsequent discussion, are as follows. At the beginning there was a primordial gene, P, which probably carried information for some 110 amino acids – the present length of a single V or C domain. At an early stage, perhaps when the most advanced invertebrates began to branch off into vertebrates, the P gene gave rise to two genes by tandem duplication. The two genes, separated by a primordial intron, were transcribed as a unit into one messenger RNA. The duplication allowed the two genes to evolve separately: one became the ancestor of all V genes and the other the ancestor of all C genes. The whole complex of V + C loci was still transcribed as a unit.

It is interesting to consider the possibility that VRMs may have antedated the evolutionary eclosion of the somatic recombination process for generating diversity. The Ig and TCR molecules are uniformly made up of unit building blocks with very typical overall folding structures (the so-called Ig domain), with some conservation of critical amino-acid positions. The simplest VRM chains (such as an Ig light chain) are made up of one constant domain together with one variable domain. In turn, the Ig domain is thought to have evolved from membrane-bound one-domain molecules, such as Thy-1, that already show the basic structural features of the Ig superfamily. Therefore, a possible scenario is that, after the original tandem duplication of the primordial one-domain encoding gene, the duplicated gene was set free to evolve and diversify in a "proto-V gene" manner. The products of these two one-domain genes (the original membrane-bound molecule and the "V-gene-like" duplicate) could be assembled as proteins to form a two-domain chain, even before a mechanism of gene rearrangement was developed. Such a mechanism of post-translational assembly of a VRM has just been discovered in the earliest B cell precursors in mouse and human ontogeny. The diversity of such VRMs is exclusively due to the number and diversity of V-gene-like gene segments in the germ-line, since no combinatorial (V-J or V-D-J) possibilities exist and no junctional diversification is possible. Furthermore, it is very likely that all the existing V-like genes were expressed simultaneously in each cell and thus, that such organization also antedated a clonal distribution of VRMs. This would have obvious implications as to the dynamics in such a primitive system, where all VRMs would be produced in a concerted manner. Furthermore, clonality seems advantageous only in the context of the co-evolution of a "resting" state for the cells expressing VRMs, such that the system can "choose" which to produce in its dynamics. If all VRMs are produced by each lymphocyte (all the time), the system dynamics could simply be reduced to a question of total cell numbers rather than, as of today, by activating resting cells into higher production rates of selected VRM shapes. Finally, we may recall that the primordial VRMs were certainly membrane-bound; this would also have a marked impact on the dynamics of the VRM system at this stage.

The Generation of Diversity: Somatic DNA Rearrangement

A handful of molecular mechanisms provide the basis for the extreme diversity of VRMs in mammals. Their analysis allows us to assess the likelihood of their relative order of appearance in evolution.

Contemporary VRMs are typically heterodimers of two V regions, encoded in separate sites of the genome (in mouse and man, actually in different chromosomes). Since each of the two V regions is randomly expressed in a single cell, their combinatorial assortment provides a strategy that greatly amplifies the total number of VRMs (the product of the numbers available in each set). Each of the gene complexes encoding V regions, however, is very similar in structure; I have already suggested that they evolved by duplication. In other words, the most primitive VRMs were probably homodimers.

We have already seen that in all rearranging groups of gene segments known to date and in all species analyzed, the same "recombination sequence signals" are conserved, so that it is virtually certain that this extraordinary process of somatic DNA rearrangement was invented only once in evolution and was thereafter recurrently used for all V regions. This also implies that the first cell type using such mechanisms is the ancestor for all lymphocyte classes known today, and consequently that T cells and B cells evolved from a common evolutionary precursor. Finally, the first VRM cannot have been a heterodimer, but was rather a monomeric or homodimeric molecule.

The next evolutionary step after the initial tandem duplication, therefore, seems to have been the eclosion of the mechanism for somatic DNA recombination, perhaps after the genomic integration of an episome or viral sequence, inserted into the V-like gene and separating it in two segments (V- and J-) that need to be juxtaposed by somatic recombination before expression. This allowed for the eclosion of clonality in VRM expression since the recombination signals only allow for one (V-J) joining at each gene complex. At this stage, VRMs are likely to be homodimers or, possibly, heterodimers of two different V regions of the same chain type. In contemporary higher vertebrates (already in frogs at least), it is known that expression of one locus prevents expression of the corresponding VRM locus of the homologous chromosome or chromosomes, as is the case in polyploid frogs. This inhibition is, at least in part, the result of the protein product of the rearranged locus acting in "trans" upon the rearrangement mechanisms that would complete recombination in the homologous loci. It is impossible to be certain when such regulatory mechanisms evolved; Du Pasquier⁵ has suggested that their eclosion was related to polyploidy.

Duplication and Heterodimer

This primitive complex of V-J-C genes has subsequently duplicated to provide possibilities for combinatorial association of two different VRM chains in the same molecule. Since these gene complexes are now on different chromosomes (as we shall see, in one of their lines of descent they will become the genes for the immunoglobulin light and heavy chains), this is the event which Ohno speculatively ascribed to a polyploid duplication; however, for the reasons I have already indicated, this is perhaps unlikely. Another major step took place immediately: this event is a second insertion of the episomal RSS in the V-gene of one of the V-J-C complexes to generate V- and D-gene segments, thereby increasing significantly the junctional diversity of the VRM molecules. It is interesting that in all mammalian VRMs known today (Igs, $\alpha\beta$ TCR and $\gamma\delta$ TCR) the heterodimer is always composed of one V region made up of V-D-J and another composed of V-J only. This could indicate that the events of this section preceded the divergence between immunoglobulins and T-cell receptors. It is to be noted that the combination of spacer lengths associated with V-, D- or J-gene segments in the different rearranging loci is not the same; but this could equally well correspond to later events after the Ig-TCR divergence.

Divergence between Immunoglobulins and T-Cell Receptors

All contemporary T-cell receptors are dimers of two-domain chains which resemble the V_L - V_L , dimers of immunoglobulin light chains²⁹. It is therefore likely that the divergence between immunoglobulins and T-cell receptors occurred after another duplication at this stage. Not only are the genes for these different VRMs now on different chromosomes, but the duplication in question here concerns *both* the VJC complex *and* the VDJC complex; this is therefore a particularly tempting candidate for Ohno's hypothetical polyploid duplication.

After this duplication, which was at the origin of immunoglobulins and TCRs as distinct heterodimer VRMs, the C-region of one of the complexes coding for immunoglobulins underwent

tandem duplication to form the C_{H1}, C_{H2} and C_{H3} regions of the heavy chain.

The Emergence of a Secretory Form

We have already seen that the overall DNA structure and recombination mechanisms are shared by Igs and TCRs. Because the original Ig domains (such as Thy-1) are membrane-bound, and even today the TCR is also membrane-bound, it is very likely that the acquisition of secretory capacities is a posterior event to the existence of a primordial lymphocyte expressing membrane-bound heterodimeric VRMs encoded by somatically recombined VDJ and VJ genes. The evolution of secretory forms of VRMs requires that the C-termini of the two chains composing the original heterodimer receptor both lose the very hydrophobic stretch of amino acids that assumes alpha-helical conformation across the lipid bilayer and anchors the chain to the cell membrane. These membrane attachment pieces are encoded by a separate exon on the 3' end of the gene segments that code for the constant region of the molecules. In Igs, two different pathways were seemingly followed for the two receptor chains: the light chain lost the membrane-encoding exon altogether and became a molecule that can never be inserted in a membrane; in turn, the heavy chain acquired an extra nucleotide sequence encoding hydrophilic amino acids, separated by an RNA splice site of the 3' coding sequence of the last C-domain, and followed by a poly-A addition site. The same H-chain VRM (VDJC) can therefore be translated either as a membrane molecule, by reading the whole gene to the end of the "membrane exon" and "splicing out" the secretory C-terminus, or as a secretory protein by terminating transcription 5' of the membrane exon or by splicing it out from mRNA. In both cases, covalent association with L-chains allows for the production of either membrane receptors or secreted VRMs at different stages in the differentiation and/or activation of the B cell.

Combinatorial Rearrangement

In vertebrates from the bony fish onwards, there are multiple V segments adjacent to each other, followed by multiple D segments (for the heavy-chain genes) and multiple J segments. One element of each class is combined by the deletion of the intervening DNA regions to create a variable-domain exon which after transcription is spliced to the exons for the unique domains of the constant C-region for the immunoglobulin M heavy chain. There is thus a combinatorial source of variation based on the choice between several hundred Vs, some 20 different Ds and 4 different Js. In sharks, by contrast, the genes are structured as a single V region linked to a single D region linked in turn to a single J region, all of these linked to a single set of C-region exons; this entire pattern is then reiterated hundreds of times. It is thus likely that the eclosion of a combinatorial mechanism for generating VRM diversity was an evolutionary event that occurred after the separation between the cartilaginous fishes and the lineage leading to bony fish and the higher vertebrates.

$\alpha\beta$ and $\gamma\delta$ T-Cell Receptors

In mammals, T cells are differentiated into two classes: $\alpha\beta$ and $\gamma\delta$ cells. I have given above the arguments which lead me to consider that the $\gamma\delta$ cells are the evolutionarily primitive form. I suggest that the appearance of $\alpha\beta$ TCRs, derived from the $\gamma\delta$ cells and geared to MHC recognition, was a much later event which occurred in the context of a redeployment of the VRMs to trigger immune responses against foreign antigens.

Class Switch in Immunoglobulins

Further evolution of immunoglobulins included the duplication and divergence of C-genes (to produce the various Ig isotypes M, G, A, D and E) together with a mechanism to recombine the whole rearranged VDJ-gene to any of the alternative C-region genes. This process of somatic DNA recombination is based on "signals" and enzymatic activities that are different from those operating in VDJ recombination and are thus likely to have evolved independantly. Interestingly, the mechanism of the "class switch", and the simultaneous expression of more than one class (or C-region) of VRM by the same cell, is related to the process involved in the production of a secretory form. The classic example is the expression of membrane-bound IgM and IgD by most mammalian B-lymphocytes. Once

induced to secretion of IgM, however, such B-cells lose IgD expression, because both the membrane-exon of C_μ and the whole of the C_δ are "spliced out" from most of the transcripts.

Hypermutation in Immunoglobulin Genes

In higher, homeothermic vertebrates at least, yet another mechanism has recently been disclosed which accounts for increased diversity among V regions of Igs. This is a process of somatic hypermutation (10^{-3} /bp/cell generation) of rearranged V(D)J genes, the chemistry of which remains to be investigated. Biologically, it would appear that somatic hypermutation only occurs in some types of immune response and provides for the generation of variants in the VRMs that are rapidly amplified by selection during the course of such responses. It is not implausible to suppose that this mechanism evolved at a time when the VRM system was redeployed as part of the system of defence against infectious microorganisms. Bacteria replicate rapidly enough to generate variants that could readily escape the original immune response; however, the rates of hypermutation of VRM genes are so high that they compensate for the difference in generation time between lymphocytes and bacteria (lymphocytes: 18 hours doubling time, but hypermutation rates of 10^{-3} /bp/cell generation for VRM genes; microorganisms: 20 minutes doubling time, but mutation rates of 10^{-8} /bp/cell generation for structural genes). I shall argue more fully below in Chapter 3 that immune responses to foreign antigens, $\alpha\beta$ TCRs geared to MHC-restricted recognition, the class switch and the phenomenon of hypermutation, are all relatively late evolutionary events that derive from what has been called the "Peripheral Immune System".

This series of events, from the initial tandem duplication to the advent of somatic hypermutation, is graphically summarized in Figure 2.2.

EVOLUTION OF MOLECULAR STRUCTURES

Stage	Genetic event	Schematic structure	Type of molecule
Invertebrates	Tandem duplication		1-domain (110 AAC) Membrane-bound receptor (CF THY-1)
1st vertebrates	1st episome insertion		2-domain receptor 1st VRM
	Duplication		Homodimers
	2 nd episome insertion		Heterodimers
	Duplication (Polyploidy?)		TCR and Ig
	Tandem duplication of C-region		Y-Shaped heterodimers
	Insertion of hydrophilic exon		Secretory Ig
	Deletion of hydrophobic exon		
Fish Amphibians Reptiles (Birds) Mammals	Junctional diversity Duplication Somatic hypermutation		Class-switch

Figure 2.2. A graphic summary of the evolution of VRM genes and molecules. A key point is that in all rearranging groups of gene segments known to date, and in all species analyzed (including sharks, frogs, chicken and mammals), the same "signals" (nucleotide sequences at the joining sites; length of the "spaces" between them) are conserved. Furthermore, mutants have recently been isolated in the mouse that fail to accomplish such rearrangements in all gene complexes, and thus produce no VRMs at all; this shows that the same enzymes and chemical processes are uniformly used for all VRM genes. It follows that in all likelihood, this extraordinary process of somatic DNA rearrangement was invented only once in evolution, and was thereafter

recurrently used for all V-regions.

CONCLUSION

We have now completed our review of the general background and the evolution of the structures which compose the VRM system. The major fact which emerges is that a fully-fledged VRM system, producing circulating immunoglobulins, exists in all vertebrates from sharks to humans, but not in lampreys and hagfish and in no invertebrates. This presents something of an enigma: it is almost as though the VRM system never existed in a primordial form, but sprang miraculously out of nowhere at some point in early vertebrate phylogeny. Quite specifically, there are no less than seven distinct genetic events, between the first tandem duplication of the P gene and the final insertion of a hydrophilic exon leading to a secretory immunoglobulin, which have left no separate traces (see Figure 2.2). Nevertheless, we know on solid theoretical grounds that evolution proceeds only by single steps. The next chapter of this book is a speculative attempt to resolve this enigma by reconstructing the missing links which led to the most primitive VRM system we know, that which exists in sharks. In order to do this, I shall have to build on the basis of this purely structural account, and to embark on a consideration of the possible physiological functions of the VRM system throughout its evolutionary history.

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CHAPTER 3

THE BIOLOGICAL FUNCTIONS OF THE VRM SYSTEM THROUGH PHYLOGENESIS

THE FIRST PHASE: THE EMERGENCE OF A PRIMORDIAL VRM SYSTEM

A PROBLEM TO BE SOLVED

As I have already emphasized, the VRM system of higher vertebrates is of bewildering complexity. At the molecular level it comprises the products of the Ig superfamily: immunoglobulins (Igs), T-cell receptors (TCRs), MHC class I and class II, and a growing number of other cell surface molecules involved in recognition processes (CD4, CD8, Thy-1, etc.). At the cellular level, it comprises lymphocytes which are divided into three main types (B cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells) further divided into many subsets: T-helper, T-suppressor, cytotoxic killer T cells, B-1 and B-2 cells, etc. Conceptually, we have over-riding reasons to think that the evolutionary construction of such a system must have proceeded through the one-by-one acquisition of the various elements in their present form. Going by the appearances from the comparative immunology of extant species, however, it is not easy to visualize things in this way. Rather, we are faced with something of an enigma: sharks already possess a functioning VRM system which at first sight seems to display many if not all the essential features of the contemporary system in higher vertebrates. Since no invertebrates possess even the rudiments of the system, it seems almost as if the VRM system sprang fully-fledged out of nowhere at the time of the first vertebrates. How is this striking finding to be explained?

I have already referred to the recent re-evaluation of evolutionary theory by which Eldredge and Gould¹ have pointed out that evolutionary change is not continuous, gradual and regularly progressive as envisaged by classical neo-Darwinian theory. Rather, evolution proceeds by what they have called "punctuated equilibria". In other words, evolution is characterized by long periods of stability: many species living today have remained virtually unchanged for eons, bearing astonishing resemblance to fossils hundreds of millions of years old. When things do change, however, they tend to change rapidly and dramatically: the periods of stability are "punctuated" by relatively short periods of profound change, often accompanied by "radiation" i.e., the rapid appearance of a diversified range of novel species. Our particular problem, that of the sudden appearance of the VRM system, thus belongs to a more general pattern. However, this does not as yet constitute an explanation.

What we have to explain, then, is firstly the relative rapidity with which the first VRM system appeared; and secondly, why none of the transitional stages, which must have existed, have given rise to living descendants. In general outline, my hypothetical explanation will take the following form. I will suggest that the initial constitution of the VRM system was what I shall call an *endogenous* process: by this I mean that the transitional missing links arose not because of any particular functionality or positive selection, but rather because they could not be avoided and did not fatally compromise the viability of the organisms concerned. The VRM system only acquired a positive function when it reached the stage of contemporary primitive vertebrates, i.e., when circulating VRMs (immunoglobulins) were produced. This function (whatever it was) was thus rather long in coming; but when it did arrive, it was so important that from then on no higher vertebrate could do without it.

THE STARTING POINT: CELL-LINES VERSUS ORGANISMS

What then was this "endogenous process"? I will take as my starting-point the theme developed by Buss² to which I have already alluded. The first multicellular organisms faced an obvious problem: how can it come about that individual cell-lines (which had so recently been independent entities

directly subject to natural selection) effectively sacrifice their own future (for example by undergoing terminal differentiation) in the interests of the whole organism?

This fundamental theme in biological organization has a obvious parallel in the realm of social organization: how does it come about that individuals effectively subordinate their immediate egoistic interests to those of society at large? One of the best-studied examples of ethnological social organization in animals concerns the social insects. It is interesting to note that the mediating signals employed are pheromones, i.e., chemical molecules; the mediating signals in cell-cell interactions are also chemical molecules, and of course the VRM system is centrally concerned with molecular interactions, what I shall call the domain of a "molecular ecology". We recognize again, as we saw in the Prologue, that the immune system is situated at a point of analogy between biology and the social sciences.

To return to our immediate subject, all multicellular organisms, including contemporary higher vertebrates, continue to face the problem of subordinating the interests of cell-lines to those of the whole organism; the only reason that it does not seem to be a major issue at present is that we take for granted the solutions to this problem that present-day organisms have inherited from their ancestors.

The paradigmatic illustration of this problematic theme is the case of cancer. In a multicellular organism, if any cell-line undergoes a cell-heritable modification such that it escapes from negative-feedback regulation, it will implacably proliferate until something intervenes to stop it – if nothing else, the death of the whole organism. Hence the vital importance of regulatory cell-cell interactions such as those giving rise to contact inhibition. The problem becomes particularly acute with the evolutionary appearance of a substantial extracellular space. We may recall here that the development of a circulatory compartment with blood and lymph is a salient feature of the first vertebrates. Cells which are able to proliferate in this extracellular space – lymphocytes being of course the prime example – are particularly liable to go astray and to form cancers because, not being inserted in a cellular matrix, inhibitory cell-to-cell contacts are at best relatively rare. This aspect is underscored by the fact that even today leukemias are a particularly virulent form of cancer.

STAGE ONE: A PRECARIOUS PASSAGE

Let us consider in more detail this problem of cancerous cell-lines that are able to proliferate in extracellular spaces. Since we are postulating that in the case of these early vertebrates the lymphocytes have no particular function, it might seem that the simplest solution would simply be to dispense with them altogether – why run the risk? Against this, however, there is the adage "set a thief to catch a thief"; in other words, it is a sound ecological principle not to leave a space entirely vacant, but to populate it in a balanced manner.

Accepting this, how would the system work? Our hypothesis is that the lymphocytes in blood and lymph would proliferate up to the point where their density is such that cell-to-cell encounters are sufficiently frequent to inhibit further proliferation. In contemporary vertebrates, lymphocytes occupy several percent by volume of the circulatory fluids (blood and lymph). The scheme thus seems feasible, but requires that cell-cell interactions have a reasonably high affinity, and also that the inhibitory effect lasts for some time. There is no positive selection yet, but there will be strong negative selection against organisms where this is not the case. I postulate that the lymphocyte-lymphocyte affinity, and subsequent inhibition, were mediated by a cell-surface receptor molecule, similar to Thy-1, which was the precursor of the subsequent VRMs. The first gene duplication, giving rise to a two-domain molecule, was probably advantageous to the organism because it allowed for higher affinity. It is relevant to note here that apart from Thy-1, all the other prototypes of the products of the Ig-gene superfamily, such as the cell adhesion molecules (CAMs) which play an important role in embryology, are multidomain molecules which as we seen possess the characteristic property of displaying homophilic binding and auto-affinity³. It is therefore plausible to suppose, as is required by our hypothesis, that the primitive precursor of the VRMs also possessed this property.

It is necessary now to pause and to analyze with some care the novel situation which is thus created, for it exemplifies the thematic interplay between the interests of individual cell-lines and those of the organism as a whole. Since cell-cell interactions are inhibitory, it is in the interest of any

particular cell-line to display a variant form of the cell-surface molecule with a *reduced* affinity, since this will allow a greater proliferation of the cell-line in question. However, any such development is constrained by the fact that the overall result should not be such as to increase the total numbers in the lymphocyte population to a point where it becomes a serious burden for the organism. Thus, the different cell-lines (as defined by the specificities of their cell-surface molecules) are effectively competing against each other. I suggest that it is the combination of these contradictory requirements which accounts for the selective advantage of a diversification of the VRMs (by the insertion of an episome and the mechanism of DNA rearrangement described above), subject to the constraint that all the VRMs thus produced should retain auto-affinity and also have a high affinity with at least some of the other VRMs produced by the lymphocyte population. The internal logic of such a process is such that diversification will proceed until the repertoire of VRMs is virtually "complete", i.e., there are no more molecular shapes which do not interact with at least one or other of the VRMs in the system. The advantage of such a situation for the organism as a whole, which would account for the transmission of the necessary genes in the germ-line, is that the "ecological" regulation of the total number of potentially proliferating cells in the body fluids would extend to all cells which interact with the diversified VRM receptors. We may note here that Vertosick and Kelly⁴ have proposed that the "auto-antigen network" provides a basis for immune surveillance against cancers. Perturbations of the network's dynamic equilibrium would manifest themselves as lymphoproliferative disorders and benign or malignant nonlymphoid hyperplasia. This view is obviously very close to the one put forward here.

What then can be said about the "function" of the VRM system at this stage in its phylogenetic development? We have seen that at this point, the ancestral lineage of VRM producing lymphocytes probably resembled contemporary $\gamma\delta$ cells. We have noted above the highly tentative suggestion that $\gamma\delta$ cells may play a role in the immune surveillance of cancers, which is in line with the argument presented here. However, the fact that these are rather nebulous cells, which do not as far as we know perform any strikingly vital function ($\gamma\delta$ knock out mice show no obvious abnormalities), is in accordance with our suggestion that during its initial stages, the VRM system may not have had any particular positive function. As I have portrayed it so far, the VRM system had principally a negative function – that of preventing its own cancerous proliferation in the circulatory body fluids – which (together with its capacity to sustain itself) was nevertheless sufficient to account for its existence.

However, although such a nebulous function (of doubtful value for the organism as a whole) would neatly explain why there are no living representatives of this stage, it is not inconceivable that even this incipient VRM system may have had some positive functionality. As clues, we may note that contemporary $\gamma\delta$ cells are sensitive to heat-shock proteins and the by-products of mitotic growth; that they are typically tissue specific; that $\gamma\delta$ TCRs are related to cell adhesion molecules (CAMs); and that $\gamma\delta$ cells may have some role in immune surveillance against cancer. Putting these four facts together with our postulate that these cells had a capacity to regulate growth in a self-limiting manner, it is not implausible to suggest that this very first VRM system may have functioned as an "anti-cancer tissue organizer". The intimate relationship between cancer and embryonic growth is indeed illustrated, even today, by the teratocarcinomas. If this hypothesis is correct, this function would be so important that it may possibly have existed in the immediate invertebrate ancestors (perhaps even contributing to the very emergence of vertebrates as such). In this case, it may be that invertebrate coelemocytes bear some relation to contemporary NK lymphocytes, which do not exhibit gene rearrangement, but which control hematopoiesis and which certainly do have cell-surface receptors.

STAGE TWO: CROSS-LINKING AND SECRETION

The next stage in the evolutionary process is the (polyploid?) duplication of the genes encoding for the VRMs, and the duplication of the C-regions of one of the copies to form a heavy-chain immunoglobulin. This event had two concomitant consequences of the utmost importance.

Firstly, the new VRMs were tetrameric molecules with two heavy and two light chains, and consequently the characteristic "Y" shape of all contemporary Igs. This creates the possibility that the relevant signal for the cell is not simply receptor occupancy as such (which implies a monotonic inhibitory dose-response curve), but *cross-linking* of receptors, which can give rise to a bell-shaped

stimulatory dose-response curve: at high doses, the receptors are largely occupied by different ligands and hence are no longer cross-linked⁵. In order to bring out the potential significance of this innovation, we may note that at the previous stage, the VRM system functioned solely on the basis of cell-to-cell contacts. In that situation, even if the individual receptors were monomeric, the effect of cell-cell contact would inevitably be to create "cross-linking" of the receptors. In other words, receptor occupancy and cross-linking were indissociable. In the new situation, if the lymphocytes are sensitive to interactions with individual molecules in free solution (such molecules being *secreted* by other lymphocytes in the VRM system – see below), then cross-linking becomes dissociated from receptor occupancy and the phenomenon of a bell-shaped dose-response curve emerges.

The consequence of this is that the meaning of the signal can be radically altered: whereas before the logic of the system absolutely required that the signal be inhibitory, in this new situation the signal can be stimulatory. In other words, the stage is set for the innovation of programmed cell-death: *lymphocytes die unless they are stimulated*. This modification dramatically attenuates the potential conflict between cell-lines and the whole organism: firstly, because the mechanism of programmed cell-death obviously reduces the risk of cancerous variants; secondly, because the bell-shaped dose-response curve guarantees that the system is automatically self-limiting (if the total population starts to expand, this will push the system to the upper portion of the bell-shaped curve where cell-cell interactions, via their secreted VRMs, are inhibitory). At the same time, this new regime is perfectly viable from the point of view of the individual cell-lines: the cooperative nature of the cell-cell interactions at low dose levels means that all cell-lines connected to the system can survive and indeed that they must be connected to the system in order to survive. In fact, it seems intuitively plausible that this new regime will actually consolidate the pressure towards diversification until the repertoire is complete.

The second (intrinsically inseparable) consequence of the emergence of a tetrameric Y-shaped molecule is that it makes possible and gives meaning to the innovation of a secretory form of the VRMs. Secreted molecules in free solution can only produce cross-linking of receptors on a target cell if they have the two-armed Y-shaped form. It can thus hardly be an accident if the twin innovations of a tetrameric Y-shaped molecule and that of the emergence of a secretory form of the molecule (which in terms of purely molecular mechanisms are unrelated events) occurred concomitantly in evolution. In a dynamic system of the sort we have envisaged, the innovation of a secreted form of the VRM molecule, which vastly multiplies the effectiveness of the influence that a cell exerts on other members of the population, is greatly advantageous to any cell that possesses it. This is true regardless of whether the action is taking place at the lower end of the dose-response curve (where the interactions are stimulatory and cooperative), or at the higher end of the curve (in which case the interactions are suppressive and competitive). At the same time, this innovation is also advantageous for the organism as a whole, for it means that the system reaches a dynamic equilibrium with a total lymphocyte population considerably smaller than if the existence of a secretory form had not multiplied the effectiveness of cell-cell interactions.

THE CONCLUSION TO PHASE ONE

I consider that this is the end of the first phase of the evolution of the VRM system. We have arrived at a picture of what I shall call the "*primordial VRM system*" which is in accordance with the essential characteristics of the VRM system in primitive vertebrates, and also with the VRM system in the early stages of development in somewhat more advanced vertebrates such as bony fish and amphibian tadpoles. Specifically, these animals have a repertoire of circulating immunoglobulins which is virtually complete; but it is to be noted that these immunoglobulins are essentially "natural antibodies", i.e., their presence does not depend on specific antigenic stimulation, and their "immune responses" (i.e., the increases in specific antibody levels following immunization), are poor both quantitatively and qualitatively. We have noted above that "natural antibodies" are typically produced by the subset of B-1 cells, and argued that these cells are ontogenetically and phylogenetically "primordial"; we have also noted the suggestion that B-1 lymphocytes constitute the physiological equivalent of the neoplastic elements in various lymphoproliferative disorders. This fits in very well

with our account of the "primordial VRM system" as being concerned with the regulation of proliferation.

It is also worth commenting here on the relationship between the new immunoglobulin-secreting "B cells" and the ancestral lymphocyte lineage at this point in the evolution of the VRM system. We have seen that at this point in evolutionary history, the common ancestral lineage probably resembled $\gamma\delta$ lymphocytes with a somewhat nebulous function. With the appearance of secretory B-cells, this $\gamma\delta$ -like ancestral lineage was probably overshadowed for a time. It must however have survived in parallel with the B-cell lineage, for as we shall see it will later give rise to $\alpha\beta$ T cells.

CONSOLIDATION, EPIGENETIC EMERGENCE AND ENDOGENOUS SELECTION

Three aspects of the evolutionary process to this point are worthy of note. Firstly, at the end of this first phase in its evolution, the VRM system is consolidated for the first time in its history. In general terms, this consolidation is due to the fact that whereas at the outset the situation was precarious – the potential conflict between cell-lines and the organism was liable to erupt violently at any moment, with disastrous consequences for all concerned – by the end of this period these conflicting interests have been reconciled and indeed in many respect are already convergent. Earlier, I raised the problem of the "missing links": why is it that none of the transitional stages have given rise to living descendants representative of those rudimentary forms of the VRM system which must have existed? I suggest that the relative precariousness of the initial and intermediate stages already constitutes a substantial part of the explanation for this awkward fact.

Secondly, the evolutionary process is largely epigenetic⁶ rather than genetic. In other words, although genes are essential components of the system, and although genetic events such as duplication and mutation undeniably play a significant role, the creation of a dynamic *system* endowed with a coherent, intrinsic logic is an *emergent* phenomenon which is not, as such, "inscribed" in the genome⁷. As a precise illustration of this, consider the controlled proliferation of a particular cell-line, due to the fact that its immunoglobulin (resulting from a unique variant arising in ontogeny by joining of a V and J region) confers on it a set of affinities with other immunoglobulins existing in the system at that time such that its level of the bell-shaped dose-response curve is optimal, neither too high nor too low. This proliferation, although it obviously depends on genes (as well as on many other factors) cannot be transmitted as such in the germ line, and is thus *not*, strictu sensu, a genetic phenomenon.

Thirdly, the evolutionary process as I have described it is the result of a selective process. However, the agent of the "natural selection" for immunoglobulin diversity (one of the principal characteristics of the system) is *not*, as it is in classical neo-Darwinian theory, the external environment. The "agent", here, is none other than an emergent property of the system itself. The process is thus one of "organic" or "endogenous" selection, and this fact confers on the system a relative *autonomy*.

THE FUNCTION OF THE PRIMORDIAL VRM SYSTEM

THE REGULATION OF PHYSIOLOGICAL COMPLEXITY

My speculative hypothesis that the first appearance of the primordial VRM system may have been the result of an autonomous epigenetic process does not, however, mean that the long-term evolutionary survival (and subsequent development) of this system has been, or even could have been, completely unconstrained. In other words, acceptance of my hypothetical explanation of the initial *appearance* of a primordial VRM system does *not* give license to evade the question of the significant biological function of such a system once it did appear. On the contrary, it positively calls for pursuing the question and going on to give (or at least, to attempt to give) a definite answer to this question.

Any serious attempt to answer this question requires that we consider the general biology of the first vertebrates. We may recall that our consideration of this question led us to the following conclusion. In the first vertebrates, the main features of the physiological organization that will be further developed and refined in the course of subsequent evolution are already laid out. On the one hand, there is a localization of discrete functions in specialized organs – heart, gills, kidney, eyes, ears,

nose. On the other hand, and simultaneously, we see the emergence of "central systems" which *coordinate* these differentiated functions: the circulatory system, the central nervous system and the endocrine system.

There can be little doubt that this quantum jump in physiological complexity had some direct relevance to the relationship between the organism and its external environment. In general terms, we may suppose that these innovations were relevant to the active maintenance, by homeostasis, of a constant internal environment in the face of variation in the external environment. There is a fairly obvious selective advantage to such internal homeostasis: it permits an extension of a capacity to tolerate considerable variation in their external environment. In a similar vein, the central coordination of diverse sensory inputs also has a fairly obvious selective advantage, this time in terms of a capacity for locomotion and finely adapted behavior, both in the search for food and in escape from potential predators.

It is essential to realize, however, that such primary constraints from external selection processes must have been multiplied many times over by what I propose to call derivative, endogenous selection processes. Physiological complexity can surely contribute to an increased capacity for homeostasis; but at the same time, it inevitably increases the *fragility* of the organisms, so that it concomitantly creates an increased requirement for effective homeostasis. This is illustrated by the great vulnerability of contemporary Agnatha to sudden changes in temperature: unlike simpler invertebrates, normal methods of fishing represent for them a shock so brutal that thereafter they cannot survive more than a day or so in an aquarium⁸.

The logic of this process means that physiological complexity is, to a very large extent, its own cause: *an initial increase in complexity positively requires further increases in complexity*. This runaway tendency (reminiscent of technological progress in human society!) is reinforced by the Malthusian propensity of living organisms that so impressed Darwin: however great the extension of a habitat, natural populations will generally proliferate until the habitat is saturated, so that they always return to a situation where they are in confrontation with the limits of their habitat. Similarly, innovative behavior has an initial effect of permitting a diversification of ecological niches; but the very act of occupying these new niches itself creates new requirements, and new opportunities, for further innovation. This endogenous creation of self-renewing selection pressures means that complexity, and behavior, are a motor for an ongoing and largely autonomous process of evolution, as Piaget⁹ among others has pointed out.

MOLECULAR SELF-ASSERTION

The upshot of all this is that *the* over-riding, compelling evolutionary problem faced by the first vertebrates was arguably neither more nor less than that of handling and regulating unprecedented physiological complexity. We may recall also the perennity of the theme of coordinating competing populations of cell-lineages, and regulating the potential conflict between cell-lines and the organism as a whole. These considerations may suffice to render plausible the central hypothesis of this book: I suggest that *the principal function of the primordial VRM system was to contribute to the regulation of the internal molecular environment of these animals*. It is worth pointing out that such integration is only partially and inadequately conceptualized as "homeostasis", which implies return to a single fixed-point attractor. What I have in mind is rather a dynamic emerging pattern, which may be locally homeostatic but also oscillatory or chaotic, and which globally corresponds to "drift" within a domain of viability¹⁰. Varela et al¹¹ have proposed to coin the term "self-assertion" to designate processes of this sort, which possess overall coherence without necessarily being homeostatic.

The suggestion that the function of the primordial VRM system was self-assertion is, of course, heterodox in the extreme: as we have already noted, the assumption that the principal function of the "immune system" is defense is so deeply ingrained that it has given its name to the scientific discipline of immunology. This view is not, however, quite without precedent. Recently Scofield¹² has noted that every mammalian immune response begins first with self-recognition events, and has quite explicitly suggested that the truly "primitive" characteristic of vertebrate immunity is self-recognition. Avrameus¹³, commenting on the biological roles of natural autoantibodies, notes that the term "horror

autotoxicus" was originally proposed by Paul Ehrlich at the beginning of the century to express the notion that autoantibodies are prevented from reacting to self constituents. However, misinterpretations have gradually altered this notion, so that nowadays "horror autotoxicus" is generally used to denote the idea that autoantibodies, which are considered to be formed only in pathological situations, are intrinsically and unavoidably deleterious for the organism. In order to combat this sort of misinterpretation, Avrameus proposes that since the VRM system can recognize self constituents, the term "horror autotoxicus" should be replaced by "*gnōthi seautón*" which comes from the ancient Greek and means "know yourself", found as "know thyself" in the early English translations. We may also note that as early as 1983, Vertosick and Kelly⁴ proposed an auto-antigen network model according to which autoreactivity is a universal property of the immune system; self-tolerance results from a dynamic network equilibrium rather than static clonal deletion. Vertosick and Kelly view the immune system as an autoregulatory organ, explicitly compared to the nervous system, which takes an active, essential role in the expression of auto-antigens (tissue differentiation antigens in particular) so that it aids in the morphostasis of large, molecularly diverse organisms. This view has striking similarities to the concept of "self-assertion" presented here. Finally, to my knowledge the very first expression of this alternative hypothesis concerning the primordial function of the vertebrate VRM system was due to Vaz and Varela¹⁴ who pointed out that any cognitive system can only recognize entities that already exist in its own cognitive repertoire and hence that whatever is recognized must, in a fundamental sense, be "self".

There are indeed two major arguments against attributing a "defense" function to the primordial VRM system. The first is that the invertebrate animals which were the immediate ancestors of the first vertebrates must *already* have had an entirely adequate defense system, otherwise they could not have existed. The innovations associated with the emergence of the first vertebrates do not, as far as I can see, create any strikingly novel defense requirements: there is no reason to suppose that the relationship between these animals and potentially pathogenic microorganisms would be in any significant way different from what it had been previously. The second argument is that even if there was a need for a more sophisticated defense system, the primordial VRM system as I have postulated it would not fit the bill. As we have noted, this system is incapable of mounting a significant "immune response" – which is indeed the standard experimental finding in many of the lower vertebrate classes. It is worth examining this point in more detail, because this absence of an aggressive immune response is not an accidental feature. The primordial VRM system, as I have portrayed it, does not yet have the capacity to make a "self/nonself" distinction: its repertoire is "complete" and essentially homogeneous. Hence if this system *did* respond to antigens with aggressive immune responses, it would immediately run into severe problems of autoimmunity.

On the other hand, the primordial VRM system *is* suited, almost ideally, to the role of a self-assertion. The fact that its repertoire is complete (for its own intrinsic reasons) neatly guarantees that it will automatically play a "central system" role, coordinating and integrating the effects of all the molecular events that occur in the body. As Jerne¹⁵ has remarked, the fact of completeness also means that the system will necessarily form a connected network. Since this system, because of its intrinsic structure, is bound to exhibit dynamic behavior such that all changes occur within a domain of viability¹⁰, it is entirely plausible to suggest that it will also naturally play a role of self-assertion in its interaction with other molecular species. The very difficulties experienced by theorists in getting "network" models of the immune system to mount convincing "immune responses" are a neat if largely unintended illustration of this point. In particular, I would like to suggest that the VRM system may contribute to a regulatory coordination between endocrine systems on one hand (hormones are a *prima facie* case of a complexification in the molecular composition of the vertebrate organism), and the nervous system on the other hand.

Warr and Cohen¹⁶ have noted that most immunologists have only recently come to appreciate that although the immune system is capable of functioning in a test tube, in the intact organism it is in continuous contact with the endocrine and nervous systems. Primary and secondary lymphoid organs (of trout and frog as well as mouse and man) are richly innervated by autonomic nerves that terminate adjacent to cells in specific compartments of the immune system, and release neurotransmitter

substances into the local milieu. Lymphocytes and accessory cells have receptors for a wide range of neurotransmitters and neuropeptides and such ligand-receptor interactions can have profound immunomodulatory consequences. Moreover, lymphocytes themselves can synthesize ACTH, endorphins, other neurally related peptides, and classical neuroendocrine hormones. Given these bidirectional lines of communication between the central nervous system and the immune system, it should come as no surprise that the immune response can be modulated by CNS functioning as in "stress" and classical Pavlovian conditioning. It is interesting that this "neuro-endocrine-immune network" appears to be particularly salient in lower vertebrates¹⁷.

It is important to emphasize, however, that coordination results from interactions between *systems* which each have complex emergent properties. The interactions between the immune system, the central nervous system and the endocrine system are mediated by molecules such as neurotransmitters, brain hormones, interleukins and so on which are common to the systems in question but which are so simple that they cannot carry any substantial informational content. This being so, *significant* coordination is quite incomprehensible simply on the basis of mere identification of these mediatory molecules; it is only possible because, as we have said, the interacting entities are *systems* which each have complex emergent properties, so that the mediatory signals can *trigger* (but not determine) the "interpretative" responses of the systems. I shall return to this question in my discussion of the immune system as "cognitive" in Chapter 4.

My suggestion here, then, is that the central nervous system and the endocrine system may have been inter-related, via the VRM system, since their very inception in phylogeny. This is all the more plausible in that the central nervous system and the endocrine system also function on the mode of "self-assertion" rather than homeostasis in the narrow sense. It is even conceivable that the VRM system actually participated in the phylogenetic development of the nervous and endocrine systems – for example, by providing ready-made "stepping stones" which could help to overcome the "chicken and egg" problem in the emergence of a hormone-receptor system, which I have already alluded to in the introduction.

SUBSEQUENT EVOLUTION OF THE VRM SYSTEM

THE RELATION TO NUTRITION

If we accept this account of the primordial VRM system and its principal function, the next question is: where do we go from here? Two observations from comparative immunology may serve to guide us at this point. Let us first consider the fact that the first specifically lymphoid organ to appear is "gut-associated lymphoid tissue" or GALT. Indeed GALT is the one lymphoid tissue which is absolutely ubiquitous in all vertebrates¹⁸; it may be that GALT is the most underestimated component of the immune system, since even in contemporary mammals, under normal conditions, it constitutes a very substantial proportion of all the lymphocytes in the body. The second observation, which points in the same direction as the first, concerns the non-IgM immunoglobulin classes of all non-mammalian vertebrates. Thus, when antibody cross-reaction is used to determine the relation between isotypes from different species, it appears that these are most closely related to mammalian IgA¹⁸, the isotype typically produced by mammalian GALT. Finally, we may also recall that the B-1 cells that we have identified as phylogenetically "primordial" are virtually absent from lymph nodes (which are the site of defense-oriented immune responses), but are very frequent among free-living lymphocytes in the peritoneal cavity, and also among B cells in the lamina propria of the gut.

Upon reflection these observations, far from modifying my initial hypothesis, are not only compatible with it but actually serve to confirm the notion of an integrative homeostatic function by rendering it more precise. The major external source of perturbation in the internal molecular identity of an animal is, without doubt, what it eats. The lymphocytes in GALT are placed in the exact position which enables them to take stock of these incoming influences, and to modulate the self-assertion realized by the VRM system so that they can be harmoniously integrated. As work on the phenomenon of "oral tolerance" shows¹⁹⁻²¹, this is still an important (and arguably much underestimated) function of the immune system in mammals. The intuition that the immune system is fundamentally related to

nutritional processes was shared by Metchnikoff and Ehrlich, each in his own idiosyncratic way, during the early days of immunology²²⁻²⁴. It is admittedly not a conclusive argument, but it would be aesthetically pleasing in the highest degree if this intuition could be substantiated.

METADYNAMICS AND MORPHOGENESIS IN SHAPE-SPACE

In order to discuss the question of the possible functions of the VRM system in the later stages of its evolution, we now need to examine more closely some of its self-organizing properties. In particular, we shall examine the consequences of further sophistication in the mechanisms for generating diversity in the V-region of immunoglobulins. As we have noted, the evidence from comparative immunology is not entirely adequate; but we may speculate that these new mechanisms were, firstly, *combinatorial* joining of V, D, J and C regions; and secondly, non-template amino-acid insertions during the gene-rearrangement process. Whatever the mechanisms, we have seen that VRM diversity increases progressively through vertebrate evolution.

In order to appreciate the significance of this increased diversity, it is necessary to consider more closely the mechanisms which determine the composition of the population of activated B lymphocytes at any one point in time. Throughout the life of an individual animal, new clones are continuously generated (in higher vertebrates, in the bone marrow) and presented as candidates for recruitment into the current population of activated lymphocytes; at the same time, previously existing clones may die and be lost. The composition of the population at any one point in time is thus the result of a dynamic process with a definite history. Stewart and Varela²⁵ have proposed the term "metadynamics" to designate second-order processes of this sort, which are distinct from the first-order dynamic fluctuations in immunoglobulin levels and clonal expansion or contraction, but which are nevertheless of central importance from a biological point of view.

In order to represent these metadynamic processes, we have employed the "shape-space" concept originally introduced by Perelson and Oster²⁶⁻²⁸. According to this concept, the "shape" of the combining site of each immunoglobulin can be defined by p stereo-chemical parameters. The values of these parameters can be taken as coordinates in a p-dimensional "space", so that each molecular shape corresponds to a point in "shape-space". This representation has the virtue that molecular shapes which are similar, and hence have similar profiles of interaction with other shapes, will correspond to points which are close in the shape-space. However, it is also important to represent a relationship of complementarity between any two molecular shapes, since this gives rise to the high-affinity interactions between the shapes in question which will determine the dynamic behavior of the system. We have therefore used a modified version of the shape-space concept, according to which each point in shape-space corresponds not to a single shape, but to a pair of complementary shapes (distinguished as "black" and "white") having high-affinity interactions between them²⁵. This makes it possible to calculate the affinity m_{ij} between any two shapes i and j as

$$m_{ij} = \exp(-d_{ij}^2) \quad \text{[Equation 1]}$$

(where d_{ij} is the distance in shape-space between a black/white point i and a white/black point j).

It should be noted that the shape-space concept, and in particular our modified version, implicitly presupposes certain assumptions which are rather unlikely to be valid²⁹. However, its heuristic value is such that it will nevertheless be employed here.

As a result of the metadynamics, the molecules produced by the VRM system will occupy shape-space in a particular way. In the case of the "primordial VRM system" as I have portrayed it, the whole of shape-space is occupied (i.e., the repertoire is "complete") by a diffuse cloud of points with little internal structure. With the innovations in the generation of the diversity of VRM molecules, a much more fine-grained occupation of "shape-space" becomes possible.

In order to gain some idea of the emergent phenomena that may arise as a result of the metadynamics, we have built a mathematical model of the system^{30,31}. Since the metadynamic processes of recruitment and elimination of lymphocyte clones depend on the constitution of the population resulting from its previous history, the system is characterized by circular causalities and its behavior is highly nonlinear even in the highly simplified form to be described below. Exact analytical

solutions are therefore inaccessible, and so we have performed computer simulations to study its behavior.

The basic constituents of this model are a set of lymphocyte clones, numbered. In the simple form of the mode presented here, there is no proliferation; clones are either present at unit concentration, or absent. The net effect of all the other clones in the network on any given clone i is then defined by the "field" h_i , where

$$h_i = \sum m_{ij} \quad \text{[Equation 2]}$$

(summation over $j=1\dots n$).

On this basis, the model postulates that the criteria for the recruitment of a new clone and for the maintenance of a pre-existing clone are the same: if the field experienced by a clone is within a "window" between a lower threshold and an upper threshold, the clone is recruited/maintained; outside the window, the clone is not recruited/eliminated from the population.

The simulations to be shown here employ a 2-dimensional shape-space, not because this is a particularly realistic assumption (to the extent that the shape-space concept is valid at all, the dimensionality is certainly higher), but because this lends itself to a graphic visualization of the results which contributes greatly to their interpretation. Each simulation commences with a single "black" clone at the center of the shape-space. "Black" and "white" clones are generated at random points in the shape-space and proposed as candidates for recruitment into the network. After each recruitment, the entire population is re-evaluated and clones with a field outside the "window" are eliminated. The result of this process is illustrated in Figure 3.1; schematically, it comprises two phases.

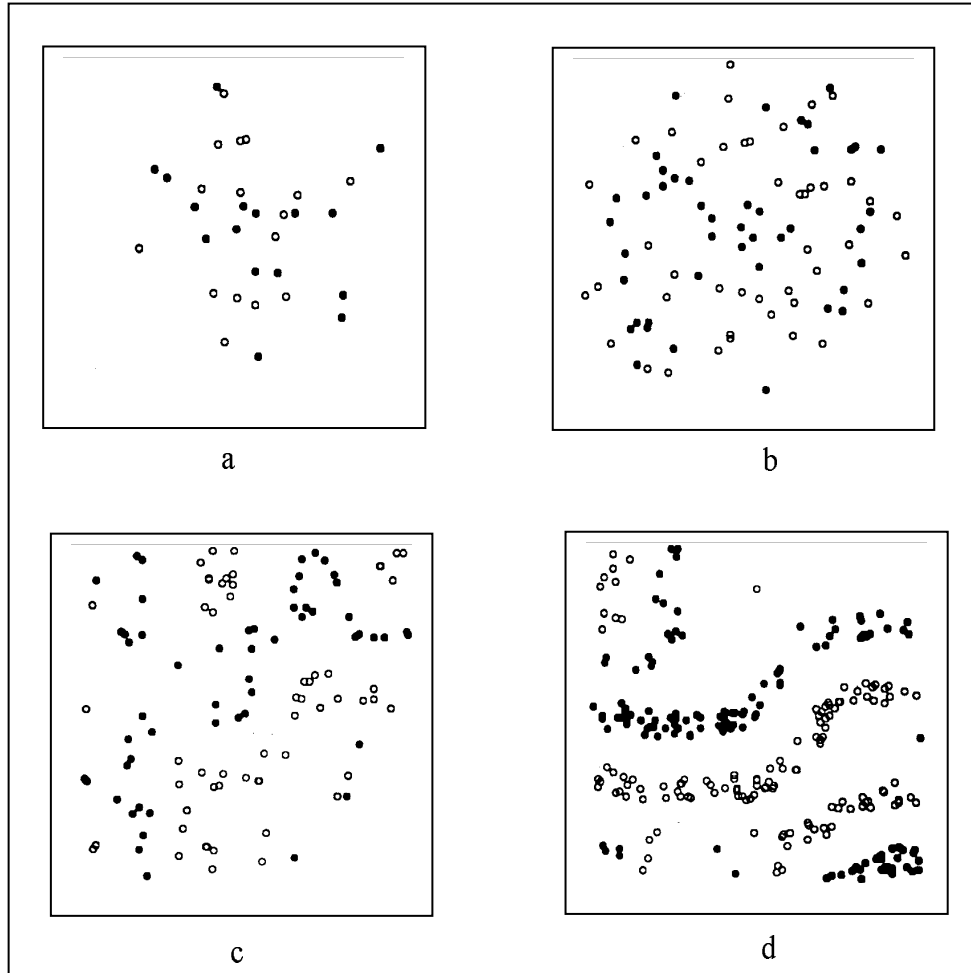


Figure 3.1 . *Four successive stages in the self-organizing constitution of an immune network, as modeled by computer simulation in a 2-dimensional shape-space. After an initial chaotic phase (1a and 1b), chains of parallel black and white clones appear (1c) and stabilize (1d). From Stewart & Varela²⁵.*

Initially, the population grows until the whole of the available shape-space is occupied by black and white clones, but in a rather disorganized manner (Figure 3.1). During this phase, a phenomenon of "local collapse" is frequently observed, due to the following mechanism: the presence of a "black" (or white) clone creates a field permitting the recruitment of a neighboring "white" (or black) clone; this recruitment often has the effect of increasing the field for the first clone above the upper threshold, so that it is eliminated; and this elimination abolishes the field for the second clone, so that it too is eliminated. In other words, the process displays a form of "self-organized criticality" characterized by numerous local "avalanches". The initial phase comes to an end when this perpetual self-reorganization leads to the emergence of certain configurations in shape-space which appear to be quasi-stable (Figures 3.1c and 3.1d). The general nature of these configurations is even clearer if the "edge effects" are eliminated by joining opposite edges of the 2-dimensional square, so that the shape-space has the topology of a torus (Figures 3.2 and 3.3).

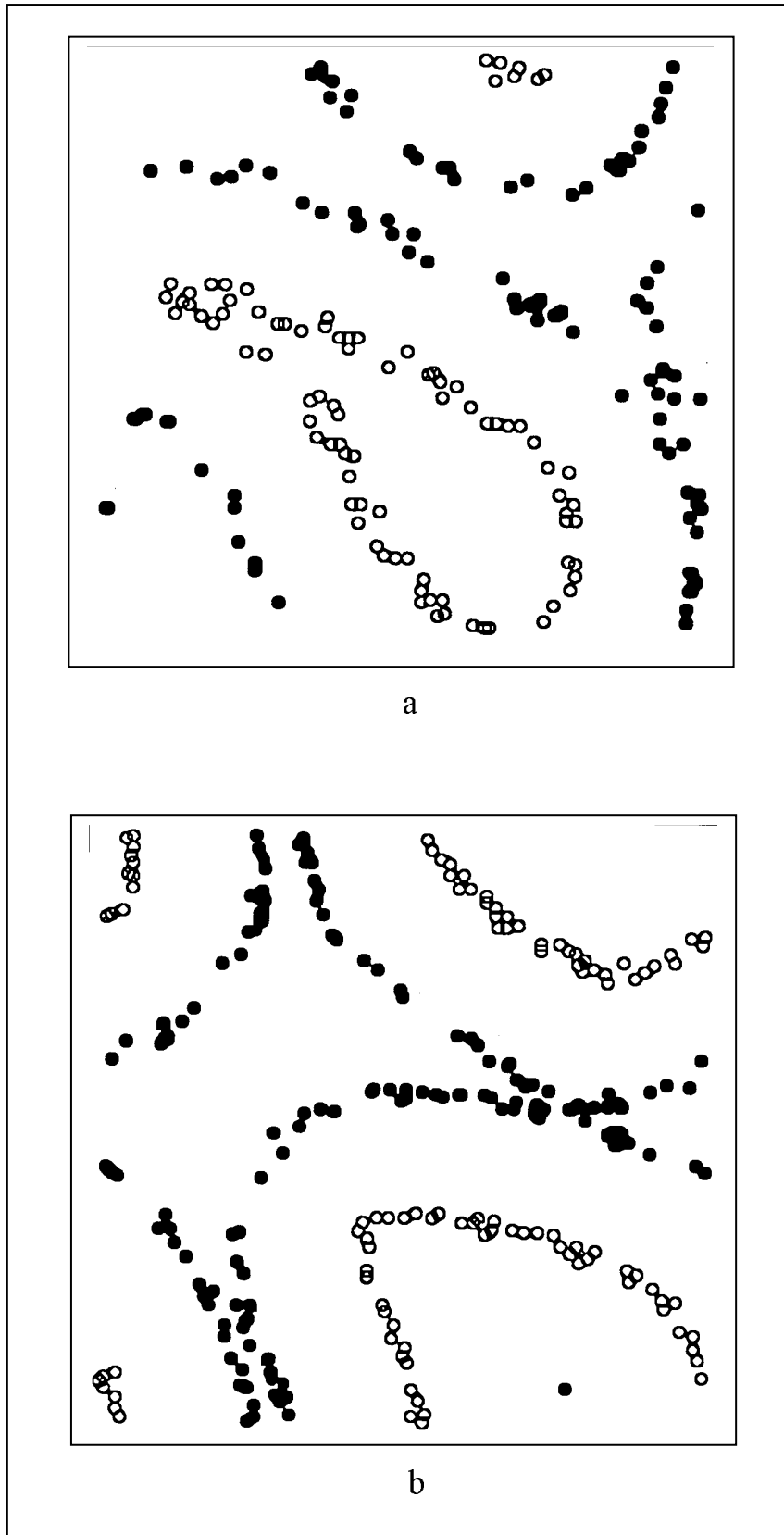


Figure 3.2. Two further examples of the emergent configurations in a simulated immune network, when the shape-space has the topology of a torus. The regularity of the patterns is particularly striking. From Stewart & Varela²⁵.

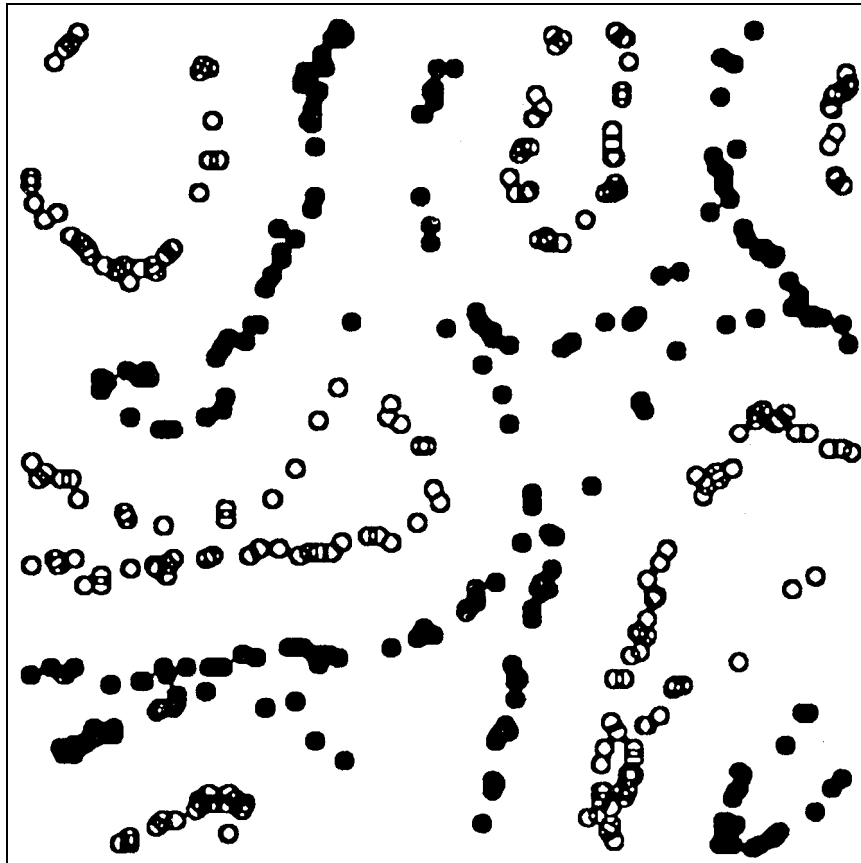


Figure 3.3. Another example of the emergent configurations in a simulated immune network. When the scale of the interactions is reduced, the patterns become more intricate. From Stewart & Varela²⁵.

The quasi-stable configurations in question are based on the formation of linear "chains" of black and white clones. Each black chain runs parallel to a facing white chain (and vice versa). The region "behind" a black (or white) chain is bounded by a second black chain which runs parallel to its own facing white chain, and so on. The quasi-stability of this configuration can be explained as follows. In the region between a pair of facing black and white chains, the field is above the upper threshold, too high for clones of either color to be recruited. In the region "behind" a chain, on the other hand, the field is too low for clones of the same color to be recruited. In other words, the configuration of a pair of parallel facing black and white chains is stable because each chain creates a field (at a distance corresponding to the interval between them) within the window for recruitment/ maintenance of the other. Finally, there is one remaining form of recruitment which could potentially disrupt this overall configuration: that of white clones in the back-to-back region bounded by black chains (or vice versa). Sporadically, this can and does occur (cf. the isolated black clone in Figure 3.2b). However, the isolated clone immediately creates a field leading to the recruitment of neighboring clones of the other color; because of the asymmetry due to the surrounding chains, the field for the isolated clone will systematically exceed the upper threshold, leading to its elimination by a process basically similar to that involved in the phenomenon of "local collapse" discussed above. (As a footnote, we may remark that connoisseurs of the Japanese game of Go will recognize striking and aesthetically pleasing analogies, both in the actual forms of the emergent configurations and in the dynamics of the strategic reasons underlying them).

In conclusion, we can say that certain characteristic configurations emerge precisely because of their relative stability. This process is somewhat akin to natural selection, but with two notable differences compared to the "clonal selection" of the classical theory. Firstly, the entities selected are not individual clones, but entities at a higher level of organization, i.e., global configurations of organized sets of clones. Secondly, the operative agent of selection is not a fixed, pre-given, external entity; rather, the constraints are endogenously generated by the process itself.

THE EMERGENCE OF A CENTRAL IMMUNE SYSTEM

We have seen that if the diversity of VRMs is such that each corresponds to a fine-grained region of shape-space, then the system will self-organize so as to occupy shape-space in a structured manner. Specifically, in certain privileged regions of shape-space, the "shapes" are densely packed in coherent, dynamically quasi-stable configurations. For reasons that will become apparent, we have called a VRM system that self-organizes in this way a "central immune system" (CIS).

In order to gain some clues as to the potential function of the CIS, the next question concerns the interaction of the network with antigens. We have modeled antigens as molecular shapes whose presence is independent of the field they experience. Since antigens can, in principle, have any shape whatsoever, there will be both "black" and "white" antigens. The results of simulations in which the onto-genesis of the network occurs in the presence of such antigens are shown in Figure 3.4; the antigens are represented by square symbols in order to distinguish them from the circular symbols representing lymphocyte clones.

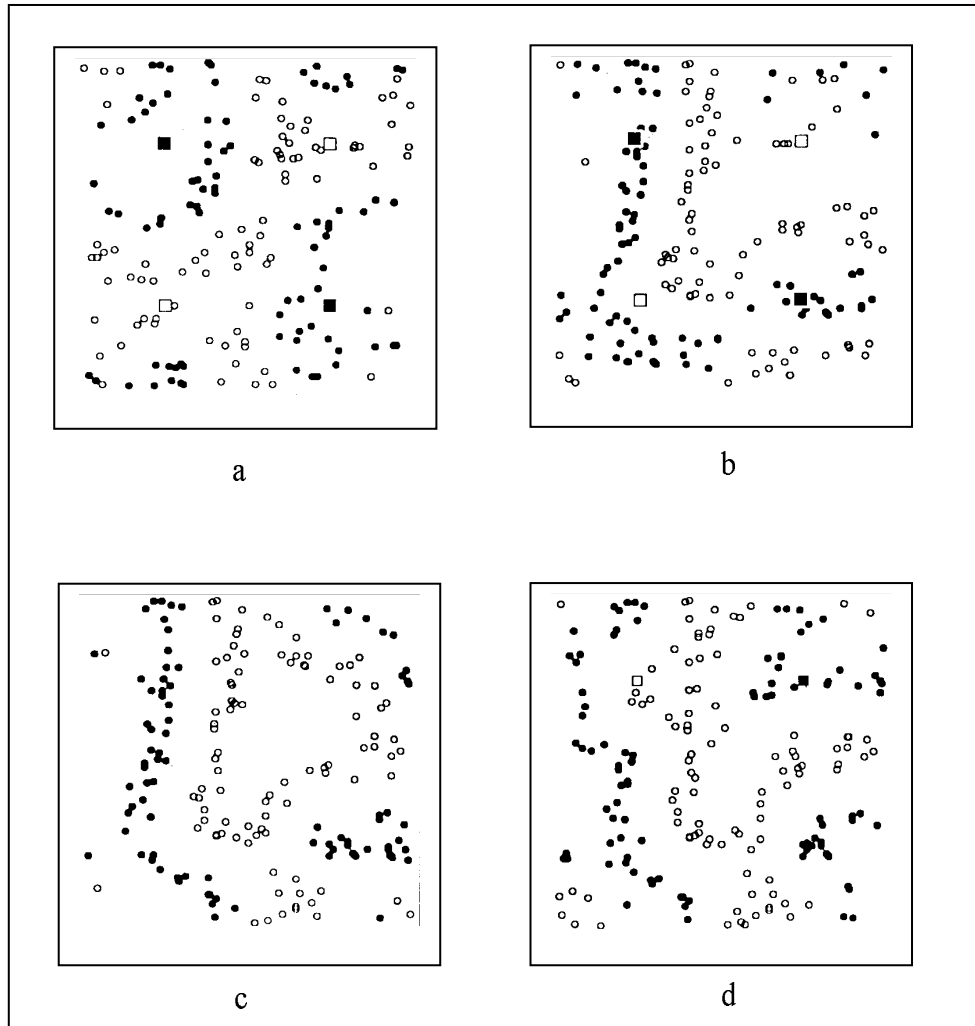


Figure 3.4. *The Biological Functions of the VRM System through Phylogenesis. The effect of coupling an emergent immune network, as in Figures 3.2 and 3.3, with fixed antigens (black and white squares). 3.4a and 3.4b: Two examples showing the incorporation of the antigens into the black and white "chains". 3.4c: The configurations remain quasi-stable even when the antigens are removed, showing "memory". 3.4d: The readjustment of the configurations when discordant antigens are introduced. From Stewart & Varela²⁵.*

What we see is that the network retains its self-organizing capacity, giving rise to the same sort of emergent configurations as previously. The novel feature is that the black and white antigens appear to be systematically incorporated into chains of the same color (Figures 3.4a and 3.4b). In other words, the previously unconstrained disposition of the chains is now constrained by the coupling of the network with the antigens. Figure 3.4c shows that this constrained disposition is maintained even if the constraining antigens are removed. That this is a dynamic feature, rather than the result of static inertia, is shown in Figure 3.4d, the introduction of discordant antigens (black antigen in a white chain, white in a black) results in a realignment of the chains such that they incorporate antigens of like color.

In other words, it seems that if the metadynamic ontogeny of the CIS occurs in the presence of molecular shapes which are continuously present (as is the case, *par excellence*, for the molecules of the somatic self of the whole organism), then these shapes will be harmoniously integrated into the characteristic structures in shape-space. Consequently, the CIS constitutes a "molecular identity" which integrates the somatic self; it thus corresponds to what is familiarly called the "immunological self", which reinforces our notion that the VRM system, and the CIS in particular, constitutes a process of self-assertion. It is to be noted, however, that contrary to what is usually supposed, this "self" is *not* genetically determined, for two reasons. Firstly, the relative stability of the CIS is dynamic, not fixed once and for all nor pre-determined; it can be and normally is continually modulated as a function of molecular events occurring in the body. Thus, the CIS integrates the total history of the molecular composition of the organism, including (without distinction) genetically encoded proteins *and* events that are the result of environmental perturbations (such as the effects of dietary regime). Secondly, and more profoundly, the CIS (even more than the primordial VRM system from which it is derived) is an emergent, *epi* genetic entity, which is not and cannot be transmitted, as such, in the germ line.

THE EMERGENCE OF A PERIPHERAL IMMUNE SYSTEM

It is worth noting that the emergence of a structured CIS is completely dependent on the mutual activation of B-lymphocyte clones by idiotypic interactions. If for any reason this were not to occur, then we would revert to a more conventional view of the VRM system as functioning on the mode of clonal selection by antigens. In this case, clones which are activated by antigens will give rise to a strong immune response which will be limited only by elimination of the antigen in question. Clones which are not activated in this way will die after a few days.

These considerations introduce us to the concept of a "peripheral immune system", or PIS. The peripheral immune system is in the first instance negatively defined: it is simply that part of the immune system which is not integrated or significantly connected to the CIS. The relationship between the CIS and the PIS is well illustrated by considering the fate of newly-formed, diversified lymphocyte clones (in mammals, the B cells that emerge from the bone marrow). If a clone of this sort interacts with the immunoglobulins of the CIS (and this will systematically be the case for all clones which are "self-reactive" in the sense of having affinity for the somatic self of the organism), then this clone will either be integrated into the CIS (in which case it will be homeostatically regulated), or, if that part of the CIS is already "saturated", the clone will be directly "suppressed" by over-stimulation. Alternatively, if a newly-formed clone does not interact significantly with the CIS, it will be stimulated only sporadically or not at all, and hence will die out more or less rapidly. The "PIS" is, initially, nothing more than the collection of these moribund clones: an incidental by-product of the increased diversity and specificity of immunoglobulins which led to the emergence of the CIS.

At the risk of belaboring the general point, I wish to emphasize that the differentiation of the primordial VRM system into a CIS + PIS did not initially occur because of any particular functionality. Of course, this sophistication would rapidly disappear if it were irredeemably dys-teleological, and it would only be retained in the evolutionary long term if it did turn out to have some positive function. But the *appearance* of the "CIS + PIS" system, the very fact that it was there at all to be submitted to the verdict of positive or negative natural selection, was an epigenetic (rather than an genetic) event which occurred not because of any eventual functionality, but merely in accordance with its own intrinsic, ontogenetic logic.

This being said, the question does arise – immediately – as to the possible functionality of the

"CIS + PIS" system. Arguably, this development could have been worthwhile purely in terms of an improved performance of the CIS in the role of molecular self-assertion. Compared to the diffuse, loosely-meshed primordial system, the tightly-knit CIS would be both much more specific, and more robustly integrative within the realm of its self-identity.

However, it transpires that there is more to the "CIS + PIS" system than this. Recalling that the "PIS", as we have defined it, is for the moment only an incidental and somewhat wasteful by-product of the events that led to the emergence of the new CIS, what are the properties of this "PIS" ? Firstly, almost by definition, it is non-reactive with all the components of the molecular self-identity of the organism. Secondly, apart from this non-reactivity to the "self" as defined by the CIS (which now occupies a relatively small, restricted portion of shape-space), the "repertoire" of the clones which make up the PIS is otherwise more or less complete. Thirdly, the clones in the PIS are by and large *under-stimulated*; moreover, they are not "connected", either amongst themselves, or to any other regulatory network. Putting these three properties together, we find that if an antigen with epitopes situated in this unoccupied, unconnected portion of shape-space is introduced into the system, it will predictably provoke a major, unlimited "immune response" on the part of certain clones in the PIS. We may *define* such an antigen, which does not interact with the CIS, as "foreign": i.e., by "foreign" we *mean* neither more nor less than "unrelated to 'self' as asserted by the CIS". Thus, the diversification of VRMs which lead to the differentiation of the VRM system into a CIS and a PIS has as a natural consequence that the VRM system will mount immune responses to foreign substances while being "tolerant" to somatic self-antigens. This emergent property – which as I have portrayed it is an incidental by-product of the emergence of the CIS – means that the PIS is a ready-made candidate ideally suited to the task of triggering defense reactions. We may note here that this view is convergent with that expressed by Vertosick and Kelly⁴, who explicitly proposed that foreign immunity is a by-product of the "auto-antigen paradigm", made possible by the availability of "surplus" V genes having no autoregulatory role.

REDEPLOYMENT OF THE PIS FOR A DEFENSE FUNCTION

We run little risk in postulating that this model candidate for triggering defense reactions was indeed accepted, and that from this time onwards the PIS was progressively integrated into the defense systems of vertebrates. Initially, the role of PIS was probably limited to that of triggering pre-existing defense mechanisms; but as time went on, the PIS was more and more intimately integrated into ever wider aspects of the defense system, until we reach a state in mammals where non-functionality of the PIS actually cripples the whole defense system. To some extent, this means that the view expressed here rejoins the conventional wisdom of classical immunology, according to which the function of the "immune system" is defense against bacteria, viruses and their toxins. This is comforting, but to avoid misunderstanding a few comments may be in order.

Langman³² has argued that when vertebrates moved to land, first with the amphibians and then decisively with the reptiles, this posed a serious challenge to their system of defense against microbial pathogens. The point is that the dry environment of land is hostile for these pathogens, so that the bodies of vertebrates represent an "oasis" that is not just agreeable but a vital necessity; consequently, pathogens will not be easily dissuaded from attempting to colonize vertebrates. This argument is very plausible, and it is prolonged and strengthened when birds and mammals invent homeothermic regulation of their bodies. However, it does *not* follow that, in evolutionary terms, the fundamental raison d'être of the whole VRM system is defense, for three reasons.

Firstly, the first vertebrates were aquatic animals, and they remained in the water for over 100 million years before some of them started emerging on to land. Now, by Langman's own argument, the marine environment does *not* pose the special defense problems that he evokes. Bacteria in the sea are typically very dilute, and at the same time the sea is not particularly hostile compared to the bodies of marine vertebrates. In fact, the defense problems of marine vertebrates do not appear to be notably different from those of marine invertebrates, some of which (octopus or lobsters for example) have life-spans of tens of years which are equal or greater than those of many vertebrates (small fish, frogs, rodents). The invertebrate ancestors of the first vertebrates must already have had adequate defense

systems, and it is difficult to see why these would not also have been adequate for the first vertebrates. Nevertheless, the VRM system *is* unique to vertebrates, and it appears fully-fledged in primitive vertebrates such as sharks. There is thus little reason to suppose that the defense function is primordial for the VRM system.

Secondly, the VRM system, which typically produces circulating immunoglobulins, is not in itself an effector defense system. (Cases in which sera alone are effective, for example as antidotes to snake venom or tetanus toxin, are marginal exceptions). When the "peripheral immune system" (which is only a part of the VRM system) is redeployed and recruited as part of the defense system in higher vertebrates, it functions as a trigger which activates distinct effector mechanisms (complement cascade, macrophages etc.). These other effector mechanisms must have existed in a functional form *prior* to the recruitment of the PIS as a trigger; their origin would appear to be quite independent of the VRM system, and they may well have been inherited from invertebrate ancestors.

Thirdly, the undoubted defense problems faced by terrestrial animals are hardly a sufficient reason to explain the evolutionary creation of a system as complex and specific as that of the "immune system" in higher vertebrates. Insects in particular – many of which occupy ecological niches in decomposing organic matter which is fairly teeming with bacteria – face an equivalent problem; as is to be expected, they are fully capable of reacting when they are invaded by microbes. Hemocytes in the blood have the capacity to identify foreign objects, and to attack them by phagocytosis or encapsulation³³⁻³⁵. Nevertheless, insects have no circulating immunoglobulin-like molecules, and apparently have no need for a VRM system. The insect's "immune" response, i.e., defense response, to bacterial infection is largely based on the induction of a powerful humoral bactericidal system. This consists of several broad-spectrum antibacterial peptides, notably the cecropins, which are rapidly synthesized and released in the hemolymph³⁶. In fact this system, which is based only on RNA and protein synthesis, is actually faster and easier to modulate than the VRM system which depends also on cell proliferation. Since vertebrates are also capable of making cecropins³⁷, it would seem that the involvement of a part of the VRM system in the immune defense system of higher vertebrates is the result of an opportunistic coupling of pre-existing systems, rather than the foundational function of the VRM system as a whole.

This is not to say, however, that the redeployment of the PIS as a trigger for immune effector functions was without feedback consequences for the organization of the VRM system itself. Cohn³⁸ has forcibly made the extremely important point that if a part of the VRM system, which as a whole can and does recognize "self", is used to trigger destructive immune responses, then the question of self-nonsel self discrimination becomes an absolutely central issue. He has also insisted that a single discriminatory mechanism (such that "non-self" is simply negatively defined as that which is not "self", or conversely that "self" is only that which is not "nonsel self") is not enough; "self" and "nonsel self" must *both* be positively identified, which implies that there must be a *dual* recognition system. Whatever the merits or demerits of the detailed mechanisms proposed by Langman³² – and there is room for doubt on this score – x the argument of principle is surely correct. On this background, it is possible to understand the significance of the evolutionary appearance of $\alpha\beta$ T-cells and their interaction with B-cells, notably in the phenomenon of T-help.

T CELLS AND MHC

In my view, the emergence of a substantial T-cell compartment with important functional activity is a relatively late development in vertebrate evolution which can best be viewed on the background of an ever-increasing differentiation between the CIS and the PIS, due to the fact that the PIS has now acquired a functional importance in its own right. We may recall that according to my hypothesis, immunoglobulin-producing B cells derived from a " $\gamma\delta$ like" ancestral line which was not MHC restricted. On the other hand, not only $\alpha\beta$ T cells but also some if not all contemporary $\gamma\delta$ T cells are MHC restricted. It follows that any account of the evolution of T cells as such, i.e., literally, cells which mature in the thymus, is inseparable from an account of the evolution of the MHC. Molecules encoded in the "major histocompatibility complex" are unique in their ability to transport and to "present" at the cell membrane peptide fragments of intracellular proteins. This function of MHC proteins has

seemingly been instrumental in determining the evolution of both that gene complex and of T cells, for it opens intracellular spaces to the universality of VRM recognition. Unfortunately, as we have already noted, the comparative molecular biology of MHC (and its role in peptide presentation) is almost non-existent, particularly in the primitive vertebrates which are crucial for any reconstruction of the origin and early evolution of this system. This means that we cannot discuss this important question in anything like an adequate manner. When and if (as is to be hoped) the empirical evidence does become available, this whole section of the history of the VRM system will need to be rewritten; I willingly recognize, furthermore, that this may well affect my main account to a significant degree. In the meantime, I offer a few fragmentary comments on some aspects of the probable evolution of $\alpha\beta$ and $\gamma\delta$ T cells.

EVOLUTIONARY DIVERGENCE BETWEEN $\alpha\beta$ AND $\gamma\delta$ T CELLS

Since the TCR's of both $\alpha\beta$ T-cells and contemporary $\gamma\delta$ T-cells recognize peptide-presenting MHC molecules on the cell-surface, it is likely that after the divergence of the B-cell evolutionary lineage, the VRM's of the " $\gamma\delta$ -like" ancestral lineage were redeployed for MHC recognition *before* the subsequent separation between $\alpha\beta$ and $\gamma\delta$ T cells. If this is correct, it would follow that the common ancestor to all contemporary T cells recognized MHC molecules that were not polymorphic in the species, but which may already have been organ-specific; a possibility which is in suggestive agreement with my speculation that the " $\gamma\delta$ -like" ancestor may itself already have functioned as a tissue organizer in embryogenesis.

EMERGENCE OF $\alpha\beta$ T-CELLS

It has been argued that the evolution of $\alpha\beta$ T cells, and in particular the much greater diversity of the $\alpha\beta$ TCR compared to the $\gamma\delta$ TCR, is closely related to the evolution of MHC polymorphism in the species³⁹; and it is conventional wisdom that this is in turn related to the defence function of the immune system. According to my hypothesis, this means that the evolutionary emergence of $\alpha\beta$ T cells was indeed a relatively late event, subsequent to the deployment of the PIS for triggering defence reactions.

In contrast with $\gamma\delta$ lymphocytes, $\alpha\beta$ T cells show a very marked alloreactivity. Although it is very difficult to attribute any direct functional significance to this feature of vertebrate immunology, the fact has been abundantly used in the construction of the classical "anti-nonsel" paradigm. We know now, however, that alloreactivity is simply a consequence of MHC allele specific peptide presentation and a by-product of that function of MHC molecules. Not surprisingly, there are no indications that vertebrate alloreactivity is in any way related to the colony-compatibility phenomena observed in invertebrates, which require partial identity but accept differences.

FURTHER EVOLUTION OF $\alpha\beta$ T-CELLS

I suggest that the further evolution of $\alpha\beta$ T cells was geared to their co-operation with other lymphocytes, particularly the development of helper activity with respect to B-cells in the PIS. We may cite here the development of Class II MHC polymorphism and presenting function in B-cells, and their role in the switch of Ig isotypes (cf Langman's "third law of the immune system" concerning class regulation)³². Of particular interest is the essential role of $\alpha\beta$ T cells in promoting somatic hypermutation of Ig genes; T-independent antigens do not induce such mutation. This confirms that the special mechanisms involved are a late evolutionary development. T_H-B cell collaboration in general takes place only in germinal centers; this is consistent with the fact that the appearance of well-organized lymph nodes is also a late evolutionary development.

Overall, it is clear that $\alpha\beta$ T cells in contemporary mammals have become extremely complicated, being divided into many subsets (CD8 recognizing class I MHC; CD4 recognizing class II MHC, themselves further subdivided into Th₁ inflammatory cells, and Th₂ helper cells.) I hold that, in principle, reconstruction of the evolutionary development of these complications would be illuminating. However, at the level of detail what is involved here is the subsequent functional association of *structures* that up until that point were likely to have had independant evolutionary

histories. In other words, a prerequisite for serious interpretation would be an adequate comparative biology (1) of inflammatory processes, complement cascade, and other effector mechanisms shorn of the assumption that they are unique to vertebrates and can only be triggered by immunoglobulins; and (2) of interleukins and other autocrine molecules, again shorn of the indefensible presupposition that *as structures* they have a common evolutionary history with VRMs.

Finally, I would like to note that the activity of $\alpha\beta$ T cells can be regulated by another class of ligands: Ig-variable regions with appropriate affinity to the V-region of TCR. It follows that, even if they have evolved together with PIS differentiation and expansion, not only have $\alpha\beta$ T cells been subordinated to the pre-existing CIS ever since their inception, but that they actually contribute to the establishment of the self-assertive processes that I attribute to the VRM system as a whole.

FURTHER EVOLUTION OF $\gamma\delta$ T CELLS

I have suggested that the ancestral lineage of all contemporary VRM producing lymphocytes was in many respects " $\gamma\delta$ -like". This does not mean, however, that after the divergence of the B-cell lineage, and later that of the $\alpha\beta$ T-cell lineage, this ancestral lineage remained unchanged; if only because it had to adapt to the new situation created by the appearance, first, of a "primordial VRM system", and then later of a "CIS + PIS".

This is indeed a recurrent pattern in evolution: time and time again, a particular life-form has to adapt to the consequences of its own activity and the emergence of its own successful progeny. The classic example is that of anaerobic versus aerobic organisms. The first forms of life were anaerobic cells; some of these invented photosynthesis, which had as a by-product the excretion of oxygen. Over time this led to a substantial "pollution" of the atmosphere, oxygen being toxic for anaerobic organisms which, once ubiquitous, now survive only deep in the soil or as parasites, for example in the digestive tracts of oxygen-breathing animals. In the case of $\gamma\delta$ cells, a process of this sort may explain the fact that whereas (on my hypothesis) cells of the " $\gamma\delta$ -like" ancestral lineage were ubiquitous, and notably populated the body fluids, contemporary $\gamma\delta$ cells have "retreated" rather like anaerobic microorganisms to certain tissues.

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CHAPTER 4

CONCLUSIONS

THREE CONSEQUENCES FOR IMMUNOLOGICAL RESEARCH

What are the consequences for immunological research of the speculative hypothesis that we have put forward in this paper? They may be divided into three complementary kinds:

1. Research in mammals (and possibly birds) investigating the hypothesis that the CIS (which in my view is the direct descendant of the primordial VRM system, the PIS being an associated but collateral development) may even now continue to fulfill a major function of self-assertion which would place it squarely in the mainstream of normal physiology.

2. Comparative immunology in relatively advanced vertebrates, aimed at investigating the fruitfulness of my suggestion that many of the complexities of contemporary mammalian immune systems may best be viewed on the backdrop of a progressive reinforcement of the discrimination between self-reactivity (CIS) and reactivity to non-self (PIS), made necessary by the active deployment of the PIS as part of the organism's defense systems.

3. Research in the comparative immunology of relatively primitive vertebrates, aimed at characterizing the VRM system before, during and immediately after the initial CIS-PIS differentiation.

THE CIS IN MAMMALS

The first branch of research that I have mentioned – investigating the role of the CIS in contemporary mammals – is not entirely original. It was initially proposed, on grounds not explicitly related to evolutionary theory, by the pioneering work of Vaz and Varela¹. This approach is now being adopted by a growing number of immunological researchers²⁻¹⁰. Indeed, the evolutionary account that I have put forward here has been very largely inspired by the theoretical and experimental work already accomplished in the framework of this new paradigm. It will therefore be appropriate to give an account of the original formulation of this new way of conceptualizing the VRM system, before considering some of the current issues and their relationship to the evolutionary story as it has been told here.

SELF AND NON-SELF

Within the community of contemporary immunologists, it is virtually an article of faith that immunology is the science of self-nonself discrimination¹¹. In their landmark article which signaled the birth of a new conceptualization, Vaz and Varela¹ pointed out a fundamental difficulty with this classical view. Any living system can only "perceive" or "recognize" entities which already exist within its own cognitive domain. As a Portuguese poem, cited by Coutinho et al² puts it, "What we see / is not what we see / But what we are". In other words, if an entity were really radically "nonself", it would not be perceived as "foreign" but would simply not be perceived at all. A monkey, for example, will not even notice an objectively dangerous object such as a loaded gun because it is simply beyond his ken; by contrast, he will be scared to death by a familiar object that is deformed but still recognizable, such as a skull, even if that object is "objectively" harmless. In principle the same goes for us of course, although for intrinsic reasons I obviously cannot evoke objects which are "objectively" mortally dangerous for us but are beyond *our* ken.

It was on the basis of considerations of this order that Vaz and Varela were lead to reinterpret Jerne's incipient network theory, thus giving it new and deep significance. The crucial point of departure is to consider that the principal source of stimulation for the production of immunoglobulins by a B-lymphocyte clone is not in the first instance an external "antigen", as in the classical paradigm,

but the immunoglobulins produced by other lymphocytes which have an affinity for the cell-bound immunoglobulin of the clone in question. The VRM system is thus constitutively defined as a self-referential network which does not require any particular interaction with external "antigens" in order to exist or to be active. The fact that the system is self-referential does not mean, of course, that it cannot interact with external "antigens". On the contrary, it can and does; but as we shall now see, the nature of such interactions will serve only to further accentuate the contrast with the classical paradigm.

What are the conditions that a molecule X should interact with the VRM system? According to the classical paradigm, the answer is simple: by the intractable principle of horror autotoxicus, the VRM system will interact with X if the latter is "non-self" but not if it is "self". (Actually this answer is not so "simple" as all that, because it begs the question of how the categorical distinction between "self" and "non-self" is to be established in the first place; but following the usual custom in the classical paradigm, we will defer a proper discussion of this matter).

According to the new view, the answer is a little more complicated, because there are several steps to the argument. The first step is that X will interact with the system if the latter contains immunoglobulins of a type Y which have an affinity with X. The second step is then to ask on what conditions the system will contain immunoglobulins of type Y? The answer is that the system must contain other immunoglobulins of type Z which have an affinity for Y, because it is precisely on this condition that lymphocytes producing immunoglobulins of type Y will have been stimulated to develop. The third step is to remark that from the point of view of the system, this amounts to saying that Z and X must resemble each other. Lingering remnants of the classical mentality, according to which the system is "antigen-driven", have led immunologists, following Jerne, to speak of this situation as one in which Z is an "internal image" of the external reality X. Actually, it is more in accordance with the spirit of the new paradigm to reverse this form of speech and to say that X is an "external image" of a primary internal reality. But in either case, the conclusion is the same: the "network" system will interact with X to the extent that it resembles self, whereas radical "non-self" will have no basis for interaction and will be ignored.

There is thus an evident contrast between the two paradigms: the "classical" VRM system interacts with "non-self" but not with "self"; whereas the "new" system interacts with "self" but not with "non-self". However, in order to deepen our understanding of this contrast, and to avoid misunderstanding and confusion, it is important to point out that the two paradigms give different meanings to the terms "self" and "non-self". In the classical paradigm, "self" is primarily defined, by an external observer, as the total macromolecular composition of the organism to which the VRM system belongs (in practice, a vertebrate animal). It is then a secondary conclusion that the VRM system must somehow manage to ignore this "self", and react only with what the observer calls "non-self". In the network paradigm, on the other hand, macromolecular "self" is primarily defined by the actual operation of the VRM system itself. Indeed, if we wish to attribute a single principal function to the system, on this view it would be neither more nor less than the *constitution* of a "self". What this "self" *is*, will of course be molded by the history of structural coupling between the VRM system and the organism in question; but the cognitive instance that constitutes the category "self" is the VRM system itself, whereas in the classical paradigm this instance was the external scientific observer.

The conclusion to be drawn from this is that the network "self" is quite simply *not the same entity* as the classical "self". It is for this reason that the striking contrasts between the two paradigms are not direct contradictions, which would of course be amenable to empirical resolution; rather, these two paradigms are, properly speaking, *incommensurable*¹². This incommensurability does not, however, mean that the two paradigms cannot be compared. As we have seen, the classical VRM system interacts destructively with whatever it recognizes; in accordance with the requirement of internal coherence in the paradigm, this system ignores "self" and recognizes only "non-self". By contrast, the network system recognizes only what resembles "self"; by internal coherence, it interacts non-destructively with what it recognizes.

It will be evident that the approach to the evolutionary origins of the VRM system presented in this book has been strongly influenced by these fundamental considerations. I hope and believe that there can be a two-way exchange; that the evolutionary perspective outlined here can, in return,

promote the development of the notion that the mammalian CIS plays an integrative role in normal physiology.

WHAT IS THE VRM SYSTEM FOR?

The pioneering work of Vaz and Varela has led, over the past decade, to the emergence of what Varela and Coutinho¹⁰ have called "second generation" immune network studies. Recent contributions from various laboratories -notably observations in unimmunized mice and humans, the success of novel therapeutics in autoimmune diseases, and theoretical advances- have promoted a new burst of research on immune networks. The time has come to focus on the natural next step that follows from assuming the stance that immune networks are a biological reality, as claimed by second generation studies. In fact, in this perspective the immune response, the bulk of classical immunological lore, is reframed to a more peripheral role than the highly connected immune network. This reorientation is the motivation behind the proposal to speak of a peripheral immune system (PIS), which is concerned mainly with immune responses which can be accounted for by clonal expansion mechanisms, in contrast to the central immune system (CIS) concerned with activated cells tightly arranged in an interacting network.

Now, everybody knows what immune responses are for, and thus how to understand the PIS in functional terms, and we have seen that this defense function fits neatly into the evolutionary perspective presented in this book. The question that immediately follows is: What is the CIS for? If one maintains, as I have argued in this book, that the CIS is the most ancient and primordial dimension of the VRM system, then how are we to understand its function and significance? If not concerned with defense and self-nonsel surveillance functions, then what? So far, the tendency has been to rely on the heuristic expression "self-assertion"; but while suggestive, and adequate for the initial exploratory phase of this new approach to the biology of the VRM system, the time has come to see if it is not possible to give a more concrete identification of the role of the CIS.

The straightforward approach to answering the question "what for?" is simply to take away the CIS and see what happens. The results of this approach, although they must certainly be taken into account, are not immediately helpful because the apparent answer to "what happens" is: not much that one can see. No invertebrates have a VRM system, and yet from octopus to insects they seem able to develop quite sophisticated forms of multicellular animal life. In mammals, several forms of immunodeficient mice with no VRM system at all are known (SCID, rag knockout): if protected from infection, they seem to exist quite happily.

This rather negative result is not conclusive, because biological systems have been put together by evolutionary tinkering and very typically have all sorts of redundancy and fail-safe mechanisms. It is this which renders comprehensible the fact that not just SCIDs and rag-minus, but a wide variety of knock-out experiments are also astonishingly inconclusive. Good examples in immunology itself are the "knock out" mice for MHC or for several interleukins. These molecules have been intensively studied, and would seem to be really important, and yet the knock-outs seem to do very well, apparently as well as normals in some cases. This organizational feature of living organisms is so important that it is worth furnishing another illustration. Ecosystems are constituted by a network of interspecific relations; they can certainly collapse; but more often than not, the removal of any one species can be compensated for. To take an example which concerns us human beings: if an irate God were to remove us all, as a species, tomorrow, the living planet Gaia would get on perfectly well without us. Indeed, probably the only irreplaceable ecological function we fulfill at the present time is to punch holes in the ozone layer and other similar acts of biospherical vandalism. So, what are human beings "for"?

Nevertheless, although this line of argument is valid as far as it goes, it will reduce to mere hand-waving and empty special pleading if we are not able, at some point, to identify some experimentally measurable differences between animals with and without a CIS. To keep myself honest, I must admit that if no such differences exist, the conclusion would indeed be inescapable that even if the CIS exists, it doesn't do much of anything, in which case it could well be argued that we don't really need to know about it. To take up the terms of our analogy, even if human beings are not indispensable to the viability of the global ecosystem of Gaia, they certainly do make a measurable difference.

At this point, the difficulty is to know where to look. Without some sort of indication, the problem is as bad as trying to find a needle in a haystack. The evolutionary principle put forward in this book, according to which the present VRM system *must* have evolved stepwise from simple beginnings, serves to validate the attitude of resolute simplification which has so far guided theoretical attempts to model the CIS^{8,13-16}. In the spirit of the two-way exchange between the evolutionary approach of this book and the theoretical approach, we will now look to the results of computer modeling to suggest possible experiments demonstrating the role of the CIS.

The computer simulations of the metadynamic processes which characterize the CIS have already been described in Chapter 3. We may recall that when the emergent "morphogenesis in shape-space" takes place in the presence of self-antigens, the emergent configurations of the VRM system are such as to *integrate* the self-antigens in a chain of the same "color" (Figure 3.4). In order to use this result to provide clues to our question "What is the CIS for?", we now need to turn the point of view around, and instead of asking how the somatic environment affects the development of the CIS, to ask what is the effect of a CIS on the somatic environment. In terms of the shape-space concept, the somatic molecular environment can be assumed to have a definite morphology of its own. One of the great difficulties here is that the nature of this morphology is unknown at present. One could certainly leave aside extremes such as a small bounded domain (which would go against the diversity of known components), or a complete filling of the entire space (which would go against the finitude of synthetic machinery of cells). However, whether this molecular "homunculus" is regular or fragmented, or whether it has some deep topological or geometrical properties remains a great challenge to immunology. In our state of ignorance, the most reasonable hypothesis is perhaps that the shapes of somatic molecules bear little intrinsic relationship to each other, with one major exception. This is that we can anticipate many pairs of complementary shapes: virtually any biologically active molecule (enzymes, hormones, neurotransmitters, autocrines, interleukins, even toxins, etc...) will have a corresponding receptor with a complementary shape. In terms of our "black-white" shape-space representation, these will appear as pairs of black and white points close to each other. By and large, however, there will be few cross-reactions (for rather obvious functional reasons), so that to a first approximation we may suppose that these pairs are also randomly scattered over shape-space, with no significant relationships between them. What then will be the effect of adding a CIS? The patterns in Figure 3.4 suggest an intriguing possibility: the CIS could give rise to chains in shape-space which link many of these previously unrelated somatic molecules to each other.

Consideration of dynamics and time-scales leads to a further specification of this hypothesis in more precise terms. 1. The constitution of the linking chains, including the determination of which somatic molecules become "linked" to which others, is a metadynamic event on a time-scale of weeks to months. 2. Fluctuations in the strengths of these connections, due to cell proliferation or death, is also relatively slow (days to weeks). 3. For a given set of "chains" in place, changes in molecular concentration at one point will influence immunoglobulin (Ig) concentration and the "sensitivity" or "field" at another point; such influence will run along the lines of *paired* black-white chains. The time-scale of such influence, which is primarily due to molecular interactions, could be rapid (seconds to hours).

The picture which emerges is one which underlines the interdependence between the CIS and its somatic environment, much like the interdependence between an ecological niche and the living species that inhabit it. The phenomenology of the CIS is a two-way affair: the environment is modified while modifying the network of links that interact with it at specific points. Tugging the entire ecosystem (or eco-soma) at one point produces avalanche effects in a distributed mode mediated by the network regulation. The main point, then, is that the somatic "skeleton" and the CIS network are co-defined or mutually determined.

What then could the CIS contribute to the physiology of an animal? At this point, I need to raise the question of the relationship of immunoglobulins (Igs) to effector functions. Even in classical immunology, with quantitatively massive immune responses, Igs per se have little effect: bacteria can live quite happily and undisturbed by a thick coat of Ig. By and large, Igs function biologically by triggering distinct effector mechanisms, e.g. complement cascade, macrophage activity, inflammation

generally, etc. The exceptions (anti-sera capable of directly neutralizing toxins such as tetanus toxin, snake venoms etc., by the very fact of their molecular binding) are just that: exceptions.

So what could be the effector mechanisms for the CIS? As is so often the case in biology, we may turn to human clinical pathology for some clues; in particular, to autoimmune disease which I interpret here as a consequence of CIS dysfunction. By and large, autoimmune pathologies seem to fall into the following categories: hematopoietic disorders; rheumatisms, arthritis, glomerulitis, etc.; endocrine disorders; nervous system disorders.

Of these, hematopoietic disorders may be related to the genesis of the CIS itself; since causes and effects may be intertwined, the situation is difficult to interpret and thus not clearly informative. We may group rheumatisms with allergies and inappropriate inflammation; it may simply be for mechanical reasons that joints and glomeruli are particularly sensitive to a bit of "dust in the works", setting up a vicious cycle where the "cure" (inflammation) is worse than the "ill". We are left, then, with disorders of two systems: endocrine and nervous system. What these systems have notably in common is that neither of them represents a direct effector function: they are both *communication* systems whose function is to link perception and action in a dialectical cycle.

To elaborate this point a little more, think of the nervous system. Certainly a nervous system is not required for an organism to have sensors and effector organs. For instance, coelenterates have pressure receptors on their external surface and secreting glands for digesting their prey. What is new in some coelenterates like Hydra is that these sensors and effectors enter into contact through dynamic links provided by the long axonic profiles of their primitive neurons. The nervous system provides the link between sensory and motor surfaces far distant in the body, allowing the animal to move in physical space as coherent sensorimotor unit. Thus the nervous system does not invent sensoriality or motricity, but it does introduce true behavior, a key evolutionary step in the animal lineage in contrast to plants which correspondingly lack locomotion¹⁷⁻¹⁸.

As for hormones, they do seem to be crucial for the development of higher forms of multicellular life, the evidence being that they have evolved independently in many different branches, not only in vertebrates but also in invertebrates and even in plants. In general, the integration involved is not that between an organism and its external environment, but rather the internal coordination of the various organs and cells.

I have already mentioned the possible evolutionary relations between the origins of the nervous system, the endocrine system and the primordial VRM system. Given that the common point between the nervous system and the endocrine system is that they both employ chemical molecules as signals, and that the CIS could create links in the domain of molecular shapes, it would now seem that a potential functional domain of a more fully developed and elaborated CIS might actually be that of the integration of integrating systems, or "communication about communication". This recalls the germinal idea of Maturana¹⁹ who has proposed a truly theoretical definition of what language is, as a second-order "communication about communication" or a "coordination of coordination of actions". Let us examine in a little more detail some of the consequences of this idea, that the CIS may be analogous to a sort "molecular language of the body".

One attractive consequence is that it fits well with the fact that, as we have mentioned, Igs only exceptionally have direct effects. Indeed, exactly the same is true of words: spoken they are only sound-waves, and written they are only marks on paper, *except* to the extent that they trigger actions that are distinct from language per se. In a similar vein, this also fits well with the otherwise awkward fact that removal of the CIS does not immediately and directly lead to a measurable decrease in the viability of the organism. Again, the same is true of language: a human being deprived of language does not drop dead on the spot or otherwise cease to be biologically viable. Conversely, and more positively, the analogy suggests that the CIS may nevertheless give rise to a whole phenomenological domain of considerable importance, as is manifestly the case for human language.

All this is of course leading up to a crucial question: can this argument, which so far has been essentially heuristic, be translated into concrete, feasible experiments? Recall that on the specific hypothesis under examination here there are two distinct types of event: on a short time-scale, there is "influence at a distance in shape-space" between otherwise unrelated molecular events; on a longer

time-scale, there is the construction of the routes of influence determining which molecular events will be related to which others. Correspondingly, there are two types of experiment, short-term and long-term.

Short-term experiments are attractive because they are easier to carry out. One rather crude experiment of this sort has already been carried out, with a surprising result which bears reflection. The experiment was to inject a dose of insulin sufficient to cause convulsions and coma in normal mice, and to observe the effects of the same dose in SCID mice. The prediction was that the CIS would play a buffering role, conferring overall robustness, so that the SCID mice should withstand the insulin shock less well than the normal mice. The surprising result was that the SCID mice withstood the shock considerably *better* than the controls! (Sunblatt and Coutinho, personal communication). This result is less discouraging than no difference at all. After all, to continue the series of analogies between the VRM system and human society initially suggested by my reading of Durkheim, the fact that modern industrial society has come to rely heavily on communication systems means that it is particularly fragile and vulnerable to violent perturbations such as labor strikes, terrorist sabotage or earthquakes. By analogy, the fact that reliance on a CIS may actually increase vulnerability to certain violent shocks is not a definitive refutation of its functional significance. However, it is clear that in order to argue convincingly for a physiological role of the CIS, it will be necessary to demonstrate experimentally a case in which SCID or rag-minus mice are *subnormal*.

In view of the frustrating results from short-term experiments, it would seem wise to recall that the time-scale of the dynamics and metadynamics of the system is such that significant fluctuations in its dynamic configuration occur over periods of weeks and months rather than minutes and hours^{10,15,16,20}. These considerations direct attention to the regulation of events occurring in post-natal development.

Developmental processes in general are indeed characterized by their great regularity²¹. This phenomenon is dynamic in nature: if development is perturbed, the intrinsic organization of the process is generally such that it has a considerable capacity to return to a normal course. Waddington²² coined the term "chreode" to denote the fact that developmental systems are "canalized" or buffered against perturbations in this way. I conclude that in order to render the regulatory effect of the CIS visible, one approach would be to subject the developmental system to a perturbation that tests its buffering capacity to the limit. In addition, we should recall that the CIS exerts its effects primarily at the level of the molecular identity of the organism.

This rationale leads to the following suggestions. It is known, for example, that excessive levels of dietary sugar in early life can cause chronic diabetes. In order to illustrate the regulatory function of the CIS experimentally, the dose and duration of the perturbation should be such that a substantial minority of normal mice develop diabetes. The prediction is that in mice with an immunodeficiency in the CIS (SCID mice or rag knock outs), the proportion and/or severity of such cases of diabetes should be increased. A similar example would be a form of stress (dietary and/or psychological) leading to chronic hypertension: a functional CIS should protect against this development (supposing that it is indeed dysfunctional) when compared with the immunodeficient animals. A particularly neat way of producing an appropriate challenge to the developmental system would be to use segregation at a major gene locus with limited penetrance and/or variable expression as an "experimental variable". The character affected could be anatomical, physiological or psychological, as long as the molecular ecology of the organism intervenes at some point in the causal chain from genetic difference to phenotypic difference.

COMPARATIVE IMMUNOLOGY IN HIGHER VERTEBRATES

I have suggested that many of the complexities of contemporary mammalian immune systems may best be viewed on the backdrop of a progressive reinforcement of the discrimination between self-reactivity (CIS) and reactivity to what the VRM system itself specifies as "non-self" (PIS), made necessary by the active deployment of the PIS as part of the organism's defense systems. Before evoking the possibilities for comparative immunology in the higher vertebrates (mammals, birds and reptiles) it will therefore be useful to look more closely at the question of the relationship between the

CIS and the PIS. I will also discuss the recent suggestion that immunology can be viewed as a cognitive science.

DEFINING THE RELATION BETWEEN THE CIS AND THE PIS

I have already introduced the concept, first put forward by Coutinho's group²³, according to which the immune system is composed of two distinct compartments, a central immune system (CIS) and a peripheral immune system (PIS). The PIS is composed of lymphocyte clones which do not interact with each other; they remain in a resting state unless they are specifically activated by an antigen. In this case, clonal selection of the reactive lymphocytes gives rise to a classical immune response which triggers destruction of the antigen. The CIS is composed of a "second generation network"¹⁰ of clones which activate each other by idiotypic interactions. Thus, the CIS displays autonomous activity even in the absence of antigenic stimulation. The CIS does of course interact with antigens; typically, however, it does not produce massive immune responses, but rather integrates the antigens into its ongoing regulatory dynamics. Functionally, it is clear that the PIS is appropriate for reactions to external antigens, whereas the CIS is appropriate for self-antigens.

The issue I now wish to discuss in more detail is how the frontier between the CIS and PIS can be defined, and by what mechanisms the two systems and the frontier between them are generated. I shall base this discussion on explicit models of the VRM system; I shall be asking, in effect, if these models are able to account convincingly *both* for tolerance to self-antigens *and* immune responses to foreign antigens. This discussion will be focused, in the first instance, on B-cells because most published models to date have been restricted to this class of lymphocytes. I shall make the assumption, which it is nevertheless useful to render explicit, that any given lymphocyte clone, at a particular point in time, belongs either to the CIS or to the PIS; i.e., in clonal terms, the two systems are mutually exclusive but together make up the totality of all lymphocytes present in the system. On this basis, I shall examine the major computer models of the VRM system that exist in the literature, seeking in each case to characterize the relationship between the CIS and the PIS that they represent.

The first model I shall discuss is that systematically employed by Weisbuch and Neumann, to which de Boer and Perelson have also contributed²³⁻²⁷. In its initial form, this model did not distinguish between free and cell membrane-bound immunoglobulins, but Anderson and Perelson²³ have shown that making this distinction does not modify the essential conclusions. In the simple case of two complementary clones, this model possesses a characteristic fixed point attractor, in which one clone, present in high concentration, is in an "immune" state (further antigenic stimulation leads to expansion), and the other clone, present in low concentration, is present in a "suppressed" state (further antigenic stimulation leads to decrease; see De Boer et al²⁰ for an extensive investigation of the conditions under which this dynamic pattern can be obtained). In order to study an extended network, Weisbuch et al use a Cayley-tree structure of connectivity, and privilege parameter settings which avoid percolation. Depending on the quantitative dynamics of antigen presentation and elimination, two basic patterns arise: "vaccination" (clones complementary to the eliciting antigen are in an immune state), and "tolerance" (clones complementary to the eliciting antigen are in a suppressed state). The network as a whole can demonstrate localized "memory" for a certain number of antigens (in either the vaccinated or tolerant mode), essentially independently of each other.

What happens if we try and fit this model into the "CIS vs PIS" mold? In order to make functional sense, we have to identify "vaccination" with the PIS, and "tolerance" with the CIS. Neumann²⁵ has made an interesting suggestion as to how the appropriate attribution of clones to the CIS and PIS might occur: "At prenatal and neonatal stages, when multispecific clones and odd loops are present, localized tolerance attractors are established in response to early presented (self) antigens. As the immune system develops, the multispecific idiotypes are increasingly suppressed, odd loops vanish from the network, and exposure to antigen now generates immunity rather than tolerance. Those clones that remained virgin during early development are now able to respond to newly presented antigens and immunize the body against them. Nevertheless, the (self) antigens first presented to the network continue to be tolerated because the idiotypes that can react against them are still suppressed by their anti-idiotypes."

It is interesting to compare these results with those obtained recently by Detours²⁸ and Bersini²⁹. They have used the model proposed by Varela et al¹⁴ which distinguishes free and bound immunoglobulins. Usually this model has a cyclic attractor with fluctuating concentrations of immunoglobulins, but with these parameter settings the model always produces strong immune responses to antigens. However, with a sufficiently high source term the basic dynamics of this model has the same asymmetric fixed-point attractor as the single-compartment de Boer and Weisbuch model discussed above²⁰. In order to model the extended network which interests us here, the connectivity structure of the idiotypic interactions is derived from a 2-dimensional shape-space with a single point of symmetry. Under these conditions, percolation cannot be avoided. The presence of a constant antigen stimulates complementary clones, which in turn stimulate idiotypes resembling the antigen; as shown in Figure 4.1, the result is a pattern of spreading "waves" of idiotypes and anti-idiotypes.

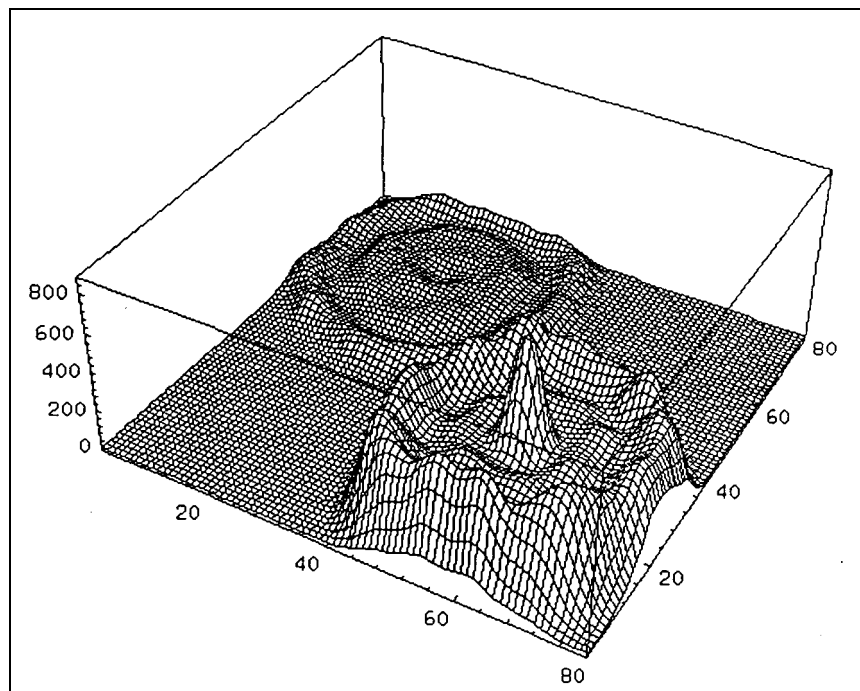


Figure 4.1. *A simulation of the metadynamic process in the presence of a constant antigen. The antigen stimulates complementary clones, which in return stimulate idiotypes resembling the antigen; the result is a pattern of spreading "waves" of idiotypes and anti-idiotypes. From Detours²⁸.*

As shown in Figure 4.2, when the two wave-fronts meet, a quasi-stable "standing wave" of very high field is formed.

This "wall" marks the frontier separating two uniform regions in shape-space, one of which is "tolerant" and the other "vaccinated". As shown in Figure 4.3, the introduction of a new antigen into a tolerant zone leads to a minimal alteration; by contrast, an antigen in the vaccinated zone provokes a strong immune response.

Interestingly, if the ontogenesis of the system from a uniform virgin state occurs in the presence of a fixed antigen, this antigen is reliably incorporated into a tolerant zone. If we fit this model into the CIS/PIS schema, the tolerant zone clearly corresponds to the CIS, the vaccinated zone to the PIS.

This model graphically illustrates a feature which is also characteristic of the Weisbuch model. In both cases, the idiotypic interactions underlying dynamic memory are such that CIS clones are sustained by complementary PIS clones, and vice versa. This implies not only that the CIS and PIS are interdependent with repertoires of essentially equal size, but that tolerance is equivalent to vaccination against molecular forms complementary to self-antigens, and conversely that vaccination is equivalent to tolerance with respect to molecular forms complementary to external antigens. This is somewhat reminiscent of Ehrlich's early model^{30,31} in which the prototypic external antigens were toxins having complementarity to physiological cell-surface receptors. The problem is that this is quite unacceptable nowadays as a picture of the relationship between CIS and PIS: tolerance is simply not equivalent to vaccination for complementary antigens and vice versa.

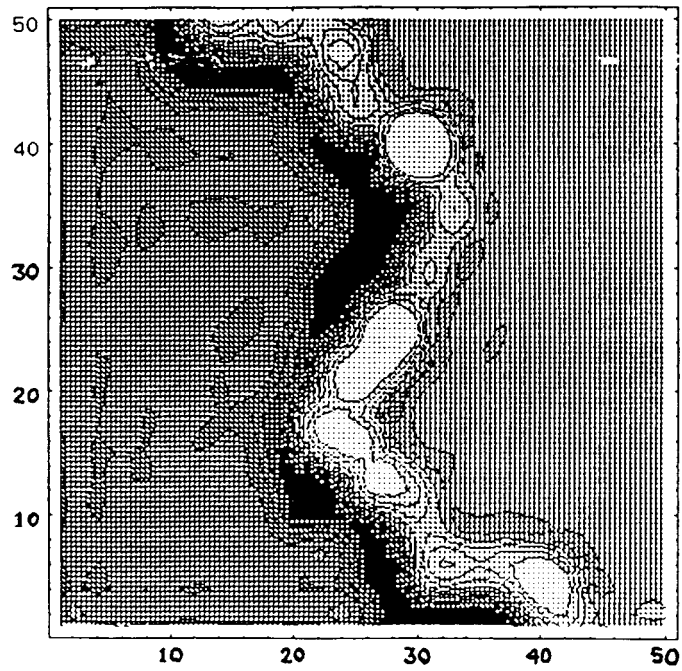
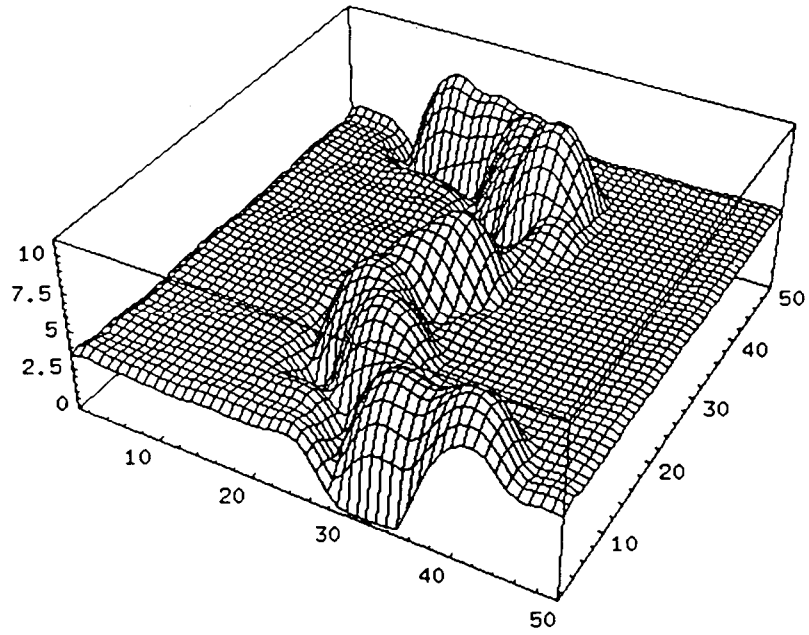


Figure 4.2. When the two wave-fronts of Figure 4.1 meet a quasi-stable "standing wave" of very high field is formed. The upper figure is a three dimensional representation, the lower figure shows the contours. From *Detours*²⁸.

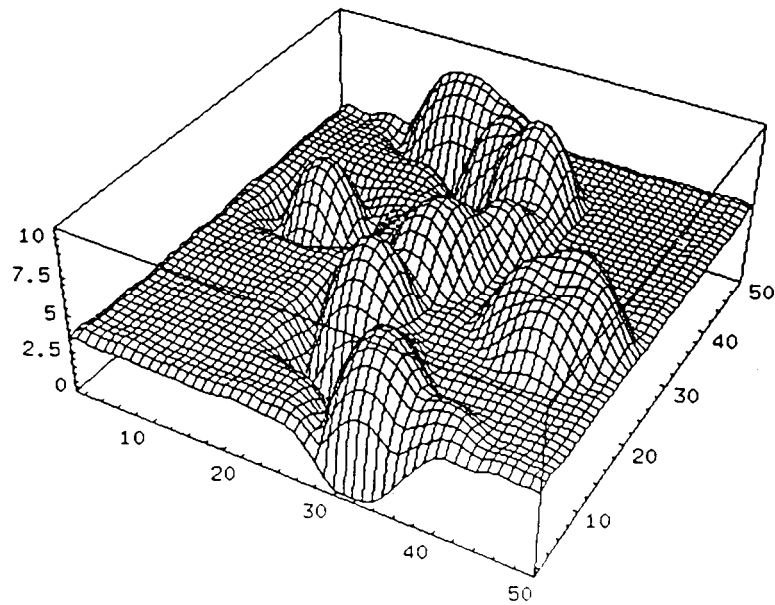
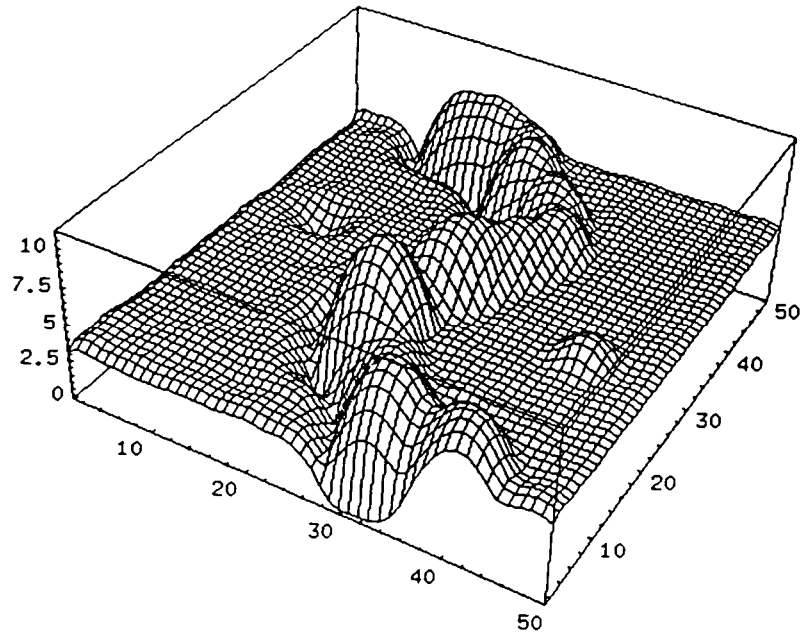


Figure 4.3. The "wall" shown in Figure 4.2 marks the frontier separating two uniform regions in shape-space, one of which is "tolerant" and the other "vaccinated". The introduction of a new antigen into a tolerant zone leads to a minimal alteration (upper figure); by contrast, an antigen in the vaccinated zone provokes a strong immune response (lower figure). From *Detours*²⁸.

A somewhat different picture emerges from the initial attempts to simulate the metadynamic recruitment of clones into an extended network of activated lymphocytes^{16,29,32,33}. Typically, these simulations employ a "double sheet" version of a 2-dimensional shape-space, in which each point corresponds to a pair of complementary clones. A distinguishing feature, compared to the models discussed in the previous paragraph, is that the relationship between mutually sustaining idiotypes is symmetric rather than asymmetric. Under these conditions, the result first obtained by Stewart and Varela¹⁶, which has been confirmed and extended in a more sophisticated version by de Boer et al³³ and has also been reproduced by Bersini²⁹, is that after a rather chaotic initial period, one sees the emergence of characteristic quasi-stable configurations in shape-space comprising parallel chains of complementary clones. Fixed antigens are incorporated into chains of idiotypes that resemble them. This is the model that has already been presented in detail in Chapter 3. Since the field experienced by such antigens is moderate, this corresponds to tolerance. Consequently, in terms of the CIS/PIS distinction, the whole of the self-sustaining network corresponds to the CIS; the PIS is simply the residue of non-activated clones, freshly emerged from the bone marrow, which are not recruited into the network because, being distant in shape-space, they fail to interact with it.

This picture is much more in accordance with the spirit of the CIS/PIS distinction as initially proposed by Coutinho et al, particularly concerning the CIS as an instrument of self-assertion. In its present state, however, there are substantial problems, particularly concerning the PIS. In all the models of this type simulated to date, the CIS network expands to fill the totality of available shape-space. The PIS, being "Totality - CIS", is thus reduced to a few percent; this problem was first raised by de Boer and Perelson³². This is in contradiction both with empirical estimates (in the spleen, at most 20% of lymphocytes are activated in uninfected animals, leaving 80% for the PIS), and with functional requirements: in order that pathological microorganisms should not escape an immune response, the repertoire of the PIS should cover at least 90%, and more likely 99%, of total shape-space (cf calculations relating repertoire size, idiootype specificity and the number of self-antigens: Perelson and de Boer, personal communication; Bhimarao, personal communication). It is just conceivable that this problem could be overcome if the occupation of shape-space is very fine-grained, so that on the ontogenetic time-scale available the percolation does not have time to extend to regions far from the self-antigens (see Figure 4.4).

However, this would not solve another problem concerning the functioning of the PIS which is in all probability related. In the metadynamic models as they stand at present, any antigen will be rather consistently incorporated into the CIS in the tolerant mode. It is true that if reaction to an antigen were to occur by pure clonal selection, without idiootype-idiootype interactions, *then* an immune response would result. However, at present, this is a purely ad hoc supposition, requiring a heteronomous determination of the self-nonsel self distinction; the CIS/PIS differentiation is not an emergent property of the network organization itself.

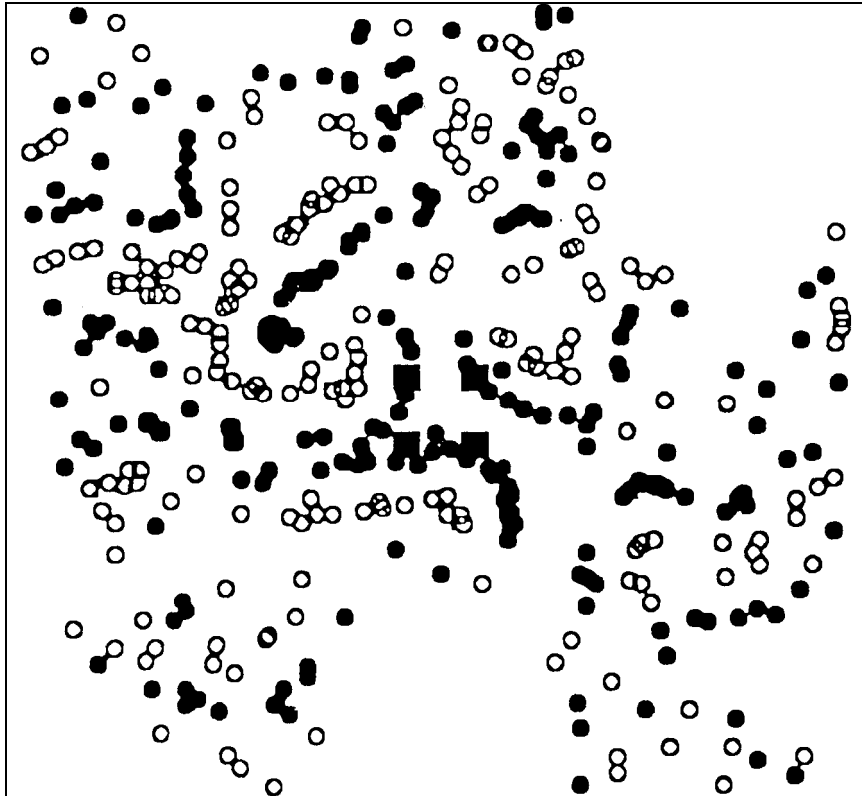


Figure 4.4. *The occupation of shape-space with a very fine-grained range of interactions. The regions close to antigens (the four black squares in the center of the field) are occupied in a structured manner, but peripheral regions are sparsely occupied in a more chaotic manner. The simulations are based on the same model as Figures 3.1-3.4. From Stewart & Varela¹⁶.*

It seems rather likely that the resolution of these problems will require models which explicitly include helper T cells and B-cell/ T-cell interactions. Of course, for the introduction of T cells to help us, they will have to intervene differentially with respect to the CIS and the PIS. The simplest possibility would be that either the CIS is T-independent and the PIS T-dependent, or vice versa. Of these two possibilities, only the first is at all plausible; a great deal of evidence shows that immune responses to most antigens require TCR recognition of MHC presented peptides and consequent T cell help to B cells. This possibility is attractive for several reasons. We have seen that B-1 cells, which produce natural antibodies having the characteristics of the CIS, arise early in development before the advent of a functional thymus. Furthermore, these cells develop in athymic nude mice, so that T cells are not necessary for their development. Nude mice are, by contrast, quite unable to mount immune responses. Finally, this possibility would fit very well with the evolutionary picture drawn in this book. I have postulated that the primordial VRM arose in the absence of helper T cells, and that this continued to be the case when the CIS first made its appearance.

However, it is far from certain that this hypothesis is correct; much evidence suggests that the CIS, in contemporary mammals at any rate, is at least partly produced by T-dependent B cells. We must therefore explore other possibilities, and in this respect the distinction between two classes of T-helper cells, Th1 and Th2, is of particular interest. B-cell activation with Th1 help results in inflammation and destruction of the eliciting antigen; Th2 help is associated with tolerance. The Th1 and Th2 subsets cross-regulate each other, by producing cytokines (IL-2 and gamma interferon for Th1, IL-4 and IL-10 for Th2) which selectively promote their own growth and autocrine profiles, while inhibiting proliferation and cytokine production by the other subset. It is probable that newly-formed resting T cells are not predetermined to one or other cytokine profile, but that the differentiation to Th1 or Th2 is determined by the cross-regulatory dynamics just mentioned. It is interesting to note that the Th2 mode is dominant at the perinatal stage of development, when tolerance to self-antigens is established and the animals are incapable of mounting substantial immune responses; the Th1 mode enters later as the animals become immunocompetent against external antigens. Overall, an obviously attractive hypothesis is to associate Th1 with the PIS, and Th2 with the CIS.

T lymphocytes are activated when their membrane-bound receptor, the TCR, recognizes a peptidic sequence "presented" by the MHC molecule of another cell. B cells themselves are particularly good "presenters", which raises the interesting possibility that the B-cell and T-cell repertoires may mutually determine each other³⁴. Illustrative of the possibilities introduced by including T cells is the following suggestion. If the peptides that are regularly presented when immunoglobulins are internalized, digested and presented by B cells themselves are always recognized by Th2 cells, then we would have a mechanism whereby idio-type-idiotype interactions could be restricted to the CIS. As noted above, this is precisely what is required for the PIS to function on the classical clonal selection mode, without idio-type-idiotype interactions, thus producing strong immune responses limited only by disappearance of the eliciting antigen.

This hypothesis has an interesting corollary: the immune response to an external antigen could be a point of intersection between the CIS and PIS. On the one hand, the response is produced with Th1 help by the PIS. However, on the other hand, the response itself is composed of immunoglobulins, and if these are recognized by other idiotypes they will be integrated into the CIS with Th2 help. This would give rise to a form of immune memory, because B-cell clones complementary to the external antigen would be dynamically maintained, even after disappearance of the antigen, by idiotypic interactions in the CIS network (including "internal images" of the antigen which would not, however, give rise to an immune response because, being immunoglobulins, they would receive Th2 help). When the external antigen is reintroduced, however, these clones would again receive Th1 help and produce a secondary immune response. The mutually suppressive relationship between the Th1 and Th2 subsets would be important here, to avoid down-regulation of the response by idiotypic interactions.

More generally, the inclusion of T cells into models of the immune system means that a given epitope, defined as the 3-dimensional molecular shape recognized by an immunoglobulin receptor, could be simultaneously both part of the CIS repertoire (if certain digested peptidic fragments are recognized by Th2 cells) *and* part of the PIS repertoire (if other peptides, possibly from the very same

molecules digested by other presenting cells, are recognized by Th1 cells). Thus, the repertoires *in shape-space* of the CIS and the PIS would not be mutually exclusive, and there would be no problem if the CIS repertoire expands to cover the larger part of this space (although the only positive requirement would be that it should reliably include all shapes in the somatic self). It therefore becomes an important question for empirical investigation as to whether the CIS repertoire is complete in shape-space.

IMMUNOLOGY AS COGNITIVE SCIENCE

The deep theoretical idea that biology and cognitive science are complementary facets of the same phenomenological domain was first expressed in the seminal work of Maturana and Varela^{17,35}. Maturana recounts that this idea came to him when he realized that cognitive perception on the one hand, and the living organization on the other, are both fundamentally characterized by a circular causality. The concept that "Life=Cognition" has recently been proposed as fundamental to the epistemology of the burgeoning new field of artificial life³⁶, and also as a possible basis for the renewal of cognitive science³⁷. Even more recently, the possibility that immunology may fruitfully be viewed as a cognitive science has been taken up explicitly by immunologists themselves³⁸. I now wish to examine this suggestion more closely to see what is involved.

The science of immunology has indeed long been characterized by the fact that immunologists spontaneously attribute "cognitive" capacities to the VRM system: recognition, learning, memory and self/non-self discrimination¹¹. However, a proper examination of the suggestion that the VRM system is "cognitive" requires an adequate scientific definition of what we mean by "cognition". Building on the basic conceptual framework proposed by Maturana and Varela, I propose the following definition:

An entity is "cognitive" if it is capable of both perception and action; moreover, its actions should be guided by its perceptions in such a way that a meaningful constraint is satisfied.

The sort of constraint I have in mind is what may be termed a "proscriptive" constraint rather than a prescriptive one. A prescriptive constraint specifies precisely, in every detail, exactly what the system should do in every possible situation. In "Good Old-fashioned Artificial Intelligence"³⁹ systems are typically subject to prescriptive constraints. In order to cover all possible situations, the specification of such constraints tends to be long, complicated and tedious. Moreover, since a classical system of this sort functions by simply internalizing these external constraints, it is "heteronomous" in the sense of being strictly determined by the outside. In a way, such a system is actually rather stupid, since it does just what it is told to do, neither more nor less.

A proscriptive constraint, on the other hand, is often conveniently expressed in negative terms. Thus, we might require of a robot on a table that it should not stay still, that it should not bump into obstacles, and that it should not fall off the edge of the table. However, although this may sound somewhat negative, the capacity to satisfy a proscriptive constraint is actually a positive achievement. Since the strategy to be employed is not dictated from the outside, a proscriptive constraint not only allows but generally requires a degree of autonomy on the part of the system¹⁷. This creative aspect is particularly clear in what we may take as the prototype of a proscriptive constraint: the capacity of living organisms to remain within their domain of viability⁴⁰. The "negative" constraint (not departing from viability) is, of course, a remarkable, positive achievement: staying alive, or, in the terms of Maturana and Varela³⁵ maintaining a state of autopoiesis. At the same time, it is clearly not a prescriptive constraint, with a single optimal solution; on the contrary, an astounding plurality of qualitatively different strategies are possible, as witnessed by the millions of species and life-forms on the planet Earth.

This definition of cognition is clearly appropriate in the context of biology; it is already satisfied by primitive living organisms (but not by viruses in isolation). Even bacteria, for example, are capable of guiding their actions (their cilia either flow in coherent waves, producing locomotion in a straight line, or wave in chaotic fashion, producing a "tumbling" motion and random reorientation) as a function of their perceptions (their sensors discriminate between a situation in which the environmental sugar concentration is increasing and one in which it is decreasing) in such a way that they tend to move towards a nutritious source of sugar. This performance is minimally "cognitive" in the sense of

the proposed definition.

Armed with this definition of cognition as coupled action-perception subject to a constraint of viability, we can now approach the question of whether the VRM system is cognitive. The first question is this : does the VRM system "perceive" its environment? By "perceive", I mean that the interactions between the system and its environment reliably lead to characteristic modifications in the internal state of the system. The perspective here is not one of transferring "information" from the environment to the system; rather, the interaction triggers a switch from one internal state to another, but what these states are is determined by the self-organizing properties of the system itself.

To answer the question properly, therefore, we need to take into account the fact that the VRM system has two distinct modes of operation: the "classical" mode of clonal selection corresponding to the PIS, and the "network" mode corresponding to the CIS. In the first mode, the system "perceives" an antigen by mounting an immune response, i.e., by producing high concentrations of immunoglobulins with strong affinity for the antigen. In the second mode, coupling with an antigen leads to realignment of the chains of "black" and "white" clones such that they incorporate the antigen in question. In both cases, we can conclude that the VRM system does indeed "perceive" its environment in the sense defined here.

Secondly, is the VRM system capable of "actions" guided by its perceptions? Again, the answer is yes, on condition that we accept to describe its operation in terms that are inhabitual in classical immunology. By the well-known mechanisms of inflammation, macrophage ingestion and the complement cascade, an immune response triggers the destruction of the antigen that provoked it. In the case of the network mode, a positive answer depends on identifying a convincing function for the CIS, a question that we have already discussed at length; we may suppose for the moment that the answer is indeed positive.

Thirdly, does the way in which the actions are guided by the perceptions conform to some non-trivial, meaningful constraint? With this question, we are at the heart of the matter. The classical mode of operation is appropriate in the case of antigens such as bacteria and viruses which, as external observers, we know come from outside the organism and threaten its viability. The destruction of antigens of this sort, triggered by an immune response, defends the organism against infectious disease. The network mode of operation is appropriate in the case of what we, again as external observers, identify as "self-antigens", i.e., molecules which we know to be a part of the organism itself. Conversely, a network response to bacteria and viruses would fail to protect the organism from infection; an immune response to self-antigens would cause autoimmune disease. Thus, the VRM system may be regarded as "cognitive" precisely to the extent that it systematically enters into the appropriate mode of operation. As a matter of empirical fact, this does seem to be the case; but immunology will only fully qualify as a "cognitive science" if the mechanisms by which this comes about can be elucidated. This is the issue conventionally known as "self/non-self discrimination"⁴¹; in view of its central importance, it is worth examining the matter more closely.

To begin with, it is to be noted that the two modes of operation are mutually exclusive: if the system responds in the network mode to a given antigen, that effectively precludes an immune response to the antigen in question. This can be clearly seen by considering the fate of a lymphocyte freshly emerged from the bone marrow. If the lymphocyte is recruited into the network, it will be activated and become part of the CIS¹⁰. If it is not recruited, it will remain as a small resting cell for two or three days before dying. The rate of bone marrow production is extremely high (it is sufficient to reconstitute the entire VRM system in less than a week), so that at any single point in time these inactivated, moribund cells make up fully 80% of the total lymphocyte population; they constitute the PIS. It is to be noted that although normally moribund, the cells in the PIS will be activated if they encounter an antigen having high affinity with their immunoglobulin receptors; in this case, since they do not possess a network organization, they will give rise to a classical immune response.

Recalling our discussion of the relationship between the CIS and the PIS, we may note that it is the CIS which is positively defined: it is composed of cells integrated into a self-activating network. The PIS is negatively defined: it is simply composed of the residue of cells which are not recruited into the network. Since the "repertoire" of lymphocytes produced by the bone marrow is "complete", it

follows that the repertoire of the PIS is "complete minus the repertoire of CIS".

We are now in a position to specify more precisely the conditions under which the distribution of roles between the network mode of operation (instanciated by the CIS) and the classical mode of operation (instanciated by the PIS) will be "appropriate" in the sense defined above. The essential requirements are that the repertoire of the CIS should reliably include all the self-antigens of the body; but that it should not extend too far beyond this, otherwise the repertoire of the PIS will be correspondingly reduced and may not cover the totality of pathogenic microorganisms whose function it is to recognize and destroy. In other words, looking at the VRM system as external observers, we have a fair idea of what might be an "appropriate" definition of the respective repertoires of the CIS and the PIS. The key question is whether the VRM system itself has a way of making this distinction in an appropriate manner. The problem is of course that the VRM system has absolutely no way of getting "outside itself" and perceiving what we consider to be the objective reality. The very categories of "self" and "non-self", as they are usually employed in classical immunology, belong to the cognitive domain of immunologists, not to that of the VRM system. We have already touched on this question in our evocation of Vaz and Varela's discussion of "self and non-sense"¹.

In fact, in order to pose the question correctly, we need to turn the traditional approach upside-down and recognize that the VRM system will actually *specify* what we call "self" but which actually corresponds to the repertoire of the CIS, and concomitantly *specify* what we call "non-self" but which from the point of view of the VRM system itself actually corresponds to the repertoire of the PIS. The real question is by what purely endogenous mechanisms, without any possible recourse to "objective" knowledge of the "real world", the VRM system can appropriately determine the respective repertoires of the CIS and the PIS.

The best clue that we have at present lies in the timing of developmental events in ontogeny. In classical immunological theory, tolerance to self-antigens is achieved by clonal deletion and anergy of self-reactive clones. Coutinho et al⁴² have given decisive reasons why deletion and anergy do not satisfactorily account for natural tolerance; briefly, self-reactive clones are produced throughout life and are present in the adult; in addition, in cell transfer experiments tolerance appears to be a dominant rather than a recessive phenomenon. There is thus a need to explain tolerance in terms of positive recognition by the CIS. The CIS is the first to develop, in the pre-natal embryo, at a time when only self-antigens are present. The dynamic and metadynamic organization of the VRM system should be such as to satisfy three constraints. Firstly, antigens encountered early in its ontogeny should be reliably incorporated into the repertoire of the CIS. Secondly, the repertoire of the CIS should not extend much beyond this. Thirdly, antigens encountered in adult life should come within the repertoire of the PIS, not that of the CIS. We have already examined, in the previous section, some of the constraints on models of the VRM system that incorporate T cells. We now see that these constraints were necessary but not sufficient; these three additional constraints are necessary. It is only on condition that an explicit model satisfying all these constraints can be constructed -and tested against empirical observation- that immunology will fully qualify as a cognitive science.

COMPARATIVE IMMUNOLOGY IN HIGHER VERTEBRATES

The discussion of the two preceding sections makes it possible to bring into focus the questions we can put to the comparative immunology of the higher vertebrates. We may take as the point of departure a situation where there is on the one hand a line of $\gamma\delta$ -like lymphocytes, and on the other a line of B-lymphocytes which are similar to contemporary B-1 cells; they produce a structured CIS but are not dependent on T-cell help. The more or less interrelated events which must have occurred to reach the situation in contemporary mammals are the following. 1. The origin of MHC class I molecules which are not polymorphic in the species but which are expressed by most cells in the body. 2. The activation of $\gamma\delta$ cells becomes dependent on recognition of these MHC class I molecules by its VRM receptors. 3. The origin of MHC class II molecules which present peptidic protein fragments at the cell surface and which are expressed primarily in lymphoid cells, notably the antigen-presenting cells such as macrophages. 4. A duplication of the $\gamma\delta$ line to produce an $\alpha\beta$ line. At the moment of its duplication, the $\alpha\beta$ line presumably resembled the $\gamma\delta$ line, but it became progressively differentiated in

two respects: activation became dependent on recognition of MHC II presented peptides by its VRM receptors, and maturation occurred in the thymus. 5. B lymphocytes became differentiated into B-1 cells on one hand, and on the other B-2 cells which are dependent on cell-cell contact with $\alpha\beta$ T cells for their activation. This is probably related to the expression of MHC II by B lymphocytes, their MHC presented peptides being recognized in clonally specific fashion by VRMs, i.e., $\alpha\beta$ TCRs. 6. The differentiation between Th₁ and Th₂ subsets of $\alpha\beta$ T cells. If I am correct in suggesting that Th₂ cells are associated with the CIS, then the first T-helper cells to arise would be similar to contemporary Th₂ cells; Th₁ cells would then arise subsequently. Finally, 7. there is the origin of a whole host of non-clonal accessory molecules such as CD3, CD40 and CD40 ligand etc., most of which derive from the Ig superfamily. The relative timing of the first evolutionary appearance of these non-clonal molecules is an interesting question. It may have been relatively late, i.e., be strictly limited to the context of B-cell activation being T-help dependent. Alternatively, it may have intervened much earlier. I have postulated that at a crucial point in what I have called "phase one" of the evolution of the primordial VRM system, the meaning of the signal from VRM receptors changed. I argued that initially the signal must have been inhibitory; however, with the appearance of Y-shaped immunoglobulins in solution, the signal could become positive. In such a situation, there will be co-selection for mutual recognition, exactly what is seen with these accessory molecules, so they may have arisen at almost any point after this switch in the meaning of the signal.

The challenge to comparative immunology is, of course, to situate these seven events (which logically must have occurred, but not necessarily in the order I have presented them) in vertebrate phylogeny. After the debacle concerning "immunoglobulins" in Agnatha, it is clear that we must beware of functional characterizations and that we can rely only on results from structural studies and in particular on those obtained with properly constructed DNA probes. As I have mentioned, the fact that MHC molecules and $\alpha\beta$ TCRs exist in both birds and mammals makes it likely that they exist also in reptiles, but this supposition obviously requires positive confirmation. In fact it would be useful to have information on a wider variety of avian species than chickens. More generally, the aim should be to identify the seven events I have mentioned in vertebrates from bony fish through amphibians to reptiles.

From a more functional point of view, it may be that the emergence of the "CIS + PIS" system was more progressive, less "all or nothing", than I have hitherto made it seem (essentially for didactic reasons). In this case, it is just possible that there was a stage in vertebrate evolution when increased structural diversity in immunoglobulins had led to the beginning of a specific, tightly-knit CIS, with the concomitant first timid appearance of a residual PIS, but *before* this nascent PIS was functionally redeployed by recruitment into the defense system. If this should be so, we would have an example, of considerable significance for general evolutionary theory, of *pre-adaptation* – a phenomenon which, if it exists, would quite radically upset classical neo-Darwinian theory.

A corollary of this research in comparative immunology, if it should turn out as I imagine it, would be that what I regard as the second major event in the evolution of the immune system after the initial appearance of the primordial VRM system itself – i.e., the appearance of the CIS-PIS differentiation – would be tied to particular species and to a definite stage in vertebrate evolution. It would then become possible to pose the question of an eventual relation between this innovation and a major change in ecological niche – perhaps the move from water to land, or another less dramatic but nevertheless identifiable modification of habitat or ecological niche. It is here, if anywhere, that novel defense requirements might come in. However, although quite conceivable, in my judgment it is perhaps unlikely that the relationship between internal and external organization was quite so direct. It is true that, on the view put forward in this book, the VRM system contributes significantly to physiological complexity and adaptability; and after the CIS-PIS differentiation is firmly established, that it contributes to defense. Nevertheless, it would be more in accordance with the kind of intelligibility I am seeking to establish that the crucial events should have been endogenous *pre*-adaptations which opened the way for far-reaching subsequent developments, rather than direct responses to clearly defined, inescapable environmental challenges. This is, indeed, the nature of my account of the emergence of the primordial VRM system in the first vertebrates.

COMPARATIVE IMMUNOLOGY IN PRIMITIVE VERTEBRATES

RESEARCH ON THE PRIMORDIAL VRM. SYSTEM

If my central hypothesis is correct, the VRM system of the most primitive vertebrates in which it occurs – in the present state of knowledge, these would seem to be the cartilaginous fishes – should display some of the characteristics I have attributed to the "primordial" system. In other words, the VRM system of these animals should produce a repertoire of circulating "natural antibodies" that is practically complete, and which does not depend on specific antigenic stimulation. This VRM system should be relatively incapable of mounting striking "immune responses", and it should not contribute to a capacity for self-nonsel discrimination significantly greater than that displayed by invertebrates which have no VRM system at all. On the other hand, this "primordial" system should contribute significantly to regulation of the internal environment. These are brave predictions; foolhardy even, for they are eminently open to experimental test. If they should indeed turn out to be substantiated, there would be a further consequence for immunological research, this time methodological: these primitive vertebrates would present a vastly superior animal model for experimental research, because they would present what I believe to be the essential functional characteristics of the VRM system in immensely simplified form, thus rendering them much more amenable to incisive investigation. These animals certainly do not produce very exciting immune responses, which probably explains their relative neglect up until now. However, perhaps the wrong questions have been asked: their study might permit us to give substance to the notion of an internal molecular ecology.

THE MISSING LINKS

Until very recently, it was believed that lampreys and hagfish possessed circulating immunoglobulins. If this were the case, then what I have called the "missing links" – the seven distinct genetic events between the first tandem duplication of the P gene and the final insertion of a hydrophilic exon leading to a secretory immunoglobulin – would be forever lost to investigation by comparative immunology, because the immediate ancestors of the first vertebrates in which they must have occurred would have disappeared without any trace in the form of analogous living descendants. However, as we have seen, it now turns out that the Agnatha do not have circulating immunoglobulins. Logically, these primitive jawless fishes must nevertheless possess at least some ancestral structures, if only the initial P gene – unless of course the living species have lost them in the course of their subsequent evolution. It would therefore be of great interest to make a really determined search aimed at identifying all the members of the Ig superfamily in Agnatha. It is indeed difficult to believe that such structures do not exist; conversely, if a really thorough search failed to find them this would be disappointing from one point of view, but would actually be an important result in its own right.

FINAL REMARKS

My concern in this book has been to argue that faced with the overwhelming complications of the immune systems of contemporary mammals, it is vital to adopt an attitude of resolute simplification. This is of course a risky enterprise; one hopes to make those radical simplifications which correctly grasp the truly fundamental features of the phenomena one wishes to understand, but it is difficult to be sure one has made the right choice. In this difficult situation, the value of appealing to evolutionary origins is first of all heuristic: it serves to validate the very principle of seeking to make a radical simplification by arguing, as Durkheim did in the text presented in the Prologue, that a simple primordial form must have existed.

In this book I have sought to render this purely heuristic argument more concrete by elaborating a speculative hypothesis concerning the evolutionary origins of vertebrate immune systems – or as I prefer to say, for reasons that I have explained, concerning the origins of the VRM systems that exist only in vertebrates. If empirical observations were to substantiate the central hypothesis of this book, then the evolutionary perspective presented here would have more than merely heuristic value for it would indicate positively which are the radical simplifications that do indeed correctly grasp the

fundamental features of the VRM system. This is of course a wild dream; it is far more likely that empirical observations will decisively refute my hypotheses, at the very least on points of detail and maybe on major aspects of its global orientation. Rodney Langman⁴³, in a lively comment on a succinct presentation of my central hypotheses⁴⁴, has pointed out what he considers to be major inconsistencies in my account, but expresses the hope that those who criticize may take the time to build a competing model for immune evolution. Langman's comment loyally expresses the spirit in which I have written this book; I will be more than satisfied if it stimulates the construction of competing models, hoping only that they may be sufficient to replace my hypothesis by an alternative account of these evolutionary processes which will withstand the acid test of scientific credibility. The scientific enterprise is collective, not individual; my best hope is that a successful reconstruction of immune evolution will one day illustrate my profound conviction that in our attempts to interpret to ourselves our present situation⁴⁵, an understanding of where we have come from can provide illumination.

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