The hybridoma revolution: an offshoot of basic research

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Summary

In this narrative, I describe how my interest in the nature and origin of antibody diversity led me to tackle the problem by using somatic cell genetic techniques. The first hybridoma (an immortal antibody-secreting cell line derived by fusion of a short-lived lymphocyte and a myeloma cell line) was an offshoot of this approach. Although not intended for such purposes, it soon became obvious that this invention had widespread potential in basic research and industry. Indeed, the technique opened new inroads into the study of complex biological substances and became the method of choice to define new differentiation markers. Hybridomas also allowed us to dissect the immune response to a simple antigen and to demonstrate the critical role of somatic mutations in the generation of high affinity antibodies. Now, monoclonal antibodies can be derived and manipulated in vitro, leading to important new developments in therapeutic applications. *BioEssays* 1999;21:966–973.

Introduction

The production of monoclonal antibodies (MAb) against predefined and, even more importantly, novel antigens has had an enormous impact in biology, medicine, and industry.[a] Indeed, the hybridoma technique has been one of the pillars of the biotechnology revolution. Yet, none of the current applications were the goal of the research that made it possible. With hindsight, it may seem obvious that the invention of a method to immortalize cells that produce specific antibodies should have such potential. At the time, however, these most important applications were neither in our minds nor in the minds of biologists or even immunologists. When we stated in the original study that "Such (monoclonal antibody) cultures could be valuable for medical and industrial use"[1] we were thinking about immunoassays and passive therapy. It was only later that we started to consider seriously other possibilities.

The technology was based on methods of somatic cell genetics, which we were using to analyse the origin of antibody diversity, and arose from a practical need, namely to have an antibody secreting cell line that was suitable for studies of somatic mutation of antibody genes. As it happened, we found that hybridomas represent a nonhypermutating differentiation stage of B-cell development. Yet, the technology eventually became the essential tool to establish the critical role of somatic hypermutation in the affinity maturation of antibodies.

Origin of antibody diversity

My early work was on metal activation, kinetics, and sequence of active sites of enzymes.[2] However, in 1962, as head of a unit at the Instituto Nacional de Microbiologia in Buenos Aires, I inherited a research student who was working with gamma globulins and became fascinated by the antibody diversity puzzle. Thus, when I returned to Cambridge in 1963 to join the recently opened Medical Research Council Laboratory of Molecular Biology, I decided to analyze the disulphide bridges of immunoglobulins[3] and the amino acid sequences of myeloma proteins, to understand the nature of antibody diversity (Fig. 1). Those studies led me to believe in the somatic origin of antibody diversity[4] and to new concepts concerning the evolutionary origin of the subclasses and families of antibody genes. In a theoretical study written in

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[a]I apologize to all those who made major contributions in early and later stages of the "hybridoma revolution" for not giving them the credit they deserve. My only justification is that it would have suggested not only an impossible task but also would have required an amount of space well beyond the limits of my allowance.

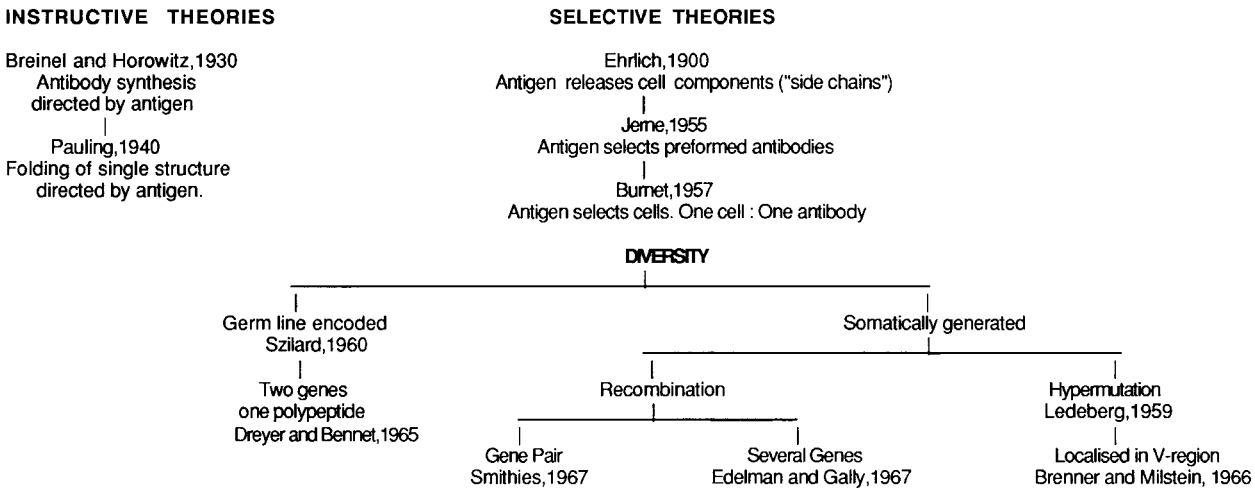

Figure 1. How many genes are required to encode antibodies? Although technically challenging, the puzzle seemed to be solvable by protein chemistry methods. Theories on the genetic bases of antibody diversity prevalent in the 1960s predicted from one to millions of genes.[4,53–62]

1969/1970 with Richard Pink, my first British research student, we suggested that "the section of the genome involved in the coding of immunoglobulin chains undergoes an expansion-contraction evolution; that the number of individual genes coding for the basic sequences is not large, and that it varies in different species and even within species at different stages of its own history. The task of providing for the endless variety of individual chains is left to somatic processes."[5]

At this stage, it was clear to me that protein chemistry alone was no longer sufficient to advance our understanding of the problem. Encouraged by the advances in RNA chemistry spearheaded by F. Sanger, I decided to work on immunoglobulin mRNA and started a very close collaboration with George Brownlee. For our purpose, it was best to use myeloma cells in culture. Thus, we had to introduce tissue culture methods to the laboratory and settled on the P3 cell line that was derived from the MOPC21 mouse myeloma.[6,7] This proved to be a turning point in my career.

Paving the way

To maintain a constant stream of large quantities of P3 cells in logarithmic growth, I constructed a spinner vessel that included an inlet for continuous feeding and an outlet to remove cells at will (Fig. 2). Cells in such spinners were kept in continuous growth for many months and sometimes, either for practical reasons or accidentally at very low or very high cell density. These conditions probably encouraged the growth of very robust cells, an important factor in the success of later experiments.

Quite apart from the mRNA work (e.g., Refs. 8–10), I started thinking about other possibilities offered by tissue culture techniques and with a newly arrived research student (David Secher), decided to attempt to detect and measure the rate of structural somatic mutations in cultured cells. David was to develop isoelectric focusing screening methods (which he did very efficiently), and I was to find conditions for cloning

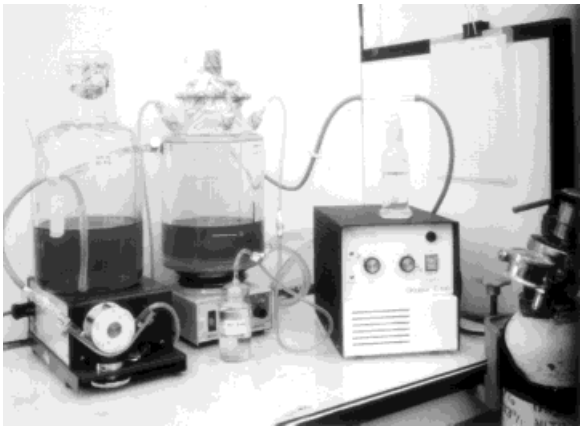

Figure 2. Arrangement for continuous growth of myeloma cells. The spinner culture on the right was drip fed continuously from the container on the left. Thus, cells were kept at optimal density and removed as required. The arrangement was optimized to avoid contamination, allowing long-term cultures to accumulate mutants. The X63/Ag8 clone was isolated from a 3-month-old culture. Some cultures were grown for up to 1 year. A more sophisticated apparatus was constructed for measurement of mRNA half-life by using synchronized cultures.[63]

the myeloma cell line (which was not so successful). After a couple of failed attempts, I was rescued by a lucky coincidence. This had the shape of an ebullient Australian post-doc (Richard Cotton) attracted by our protein sequencing record. Although, like us, he had no previous experience with somatic cell genetics, he quickly developed appropriate cloning methods and adapted them for large scale screening of secreted immunoglobulin.[11] He also decided to tackle a technically more demanding project. One of the fascinating mysteries of immunology at the time was the genetic origin of allelic exclusion, whereby a committed B-cell expresses only one of the immunoglobulin alleles. To study this, he derived the necessary mutants of two myeloma cell lines (of mouse and rat origin) to select hybrids by using the HAT approach.[12] He found that the derived hybrid cell lines retained the capacity to express and secrete the two immunoglobulin species.[13] A bonus observation was that both light chains of the parental cells were associated with the same heavy chain, the first example of synthesis of hybrid immunoglobulins. It was left to us to confirm and extend the observation to two myeloma cells from mouse origin.

I met Georges Köhler after a seminar I gave at the Basel Institute for Immunology. In that seminar, I described the isolation and characterisation of the earliest examples of structural mutant proteins defined at the amino acid sequence level.[14] Although these were structural mutants, they did not represent those responsible for antibody diversity. Köhler was ready to submit his PhD thesis and was looking for a postdoctoral position. He too was fascinated by the nature and origin of antibody diversity and was convinced that somatic events were of prime importance. He decided to accept my invitation to join my group to look for mutants that altered the antibody combining site. His initial task was to grow one of the cell lines producing a myeloma protein that displayed antigen binding properties. Twenty years later, he recalled: "In my hand they did not grow, so I had to think about another project . . . So, I turned to cell hybrids, because that was the other thing that went on . . ." Indeed, he and my assistant Shirley Howe were soon able to generate a mouse myeloma HPRT$^-$ line. It was designated P3-X63 Ag8 and was to become the most important object produced in my laboratory! He went on to say, "I could generate hybrid myeloma cells . . . so I was happy that I could fuse . . . (cells) . . . but I was unhappy that I could not do the job I wanted to do. So was César . . . and in my view it was this combination of knowing how to fuse cells and wanting to get specific lines that produced specific antibodies which made the idea that maybe we can make our own fused cell-lines which made antibodies with that specificity."[15] These views coincide with my own on how the need for an antibody secreting cell line, and the experience and materials provided by the myeloma fusion experiments, converged to foster the derivation of the first hybridoma.[16]

In retrospect, it seems obvious that the substitution of one of the fusion partners for a primary antibody producing cell should give rise to an antibody producing hybrid cell. Indeed, we later found that others had either thought or even tried such an experiment. Remarkably, our experiment worked the first time. As it happened, we had all the conditions right, although we did not know what those conditions were. We little suspected that our P3-derived myeloma sublines were particularly favourable for these experiments. So much so that, even today, the cell lines of choice for hybridoma production[b] are derived from them. An unexpected bonus was that the proportion of hybrids that secreted specific antibody were higher than expected, because most hybrids derive from B-cell blasts triggered by immunisation.

The manuscript reporting those early experiments did not have the immediate impact that was attributed to it later. The referee reports were positive but cautious. The editors of *Nature* did not consider it of sufficient general interest to publish it as an article, and the original text had to be severely pruned to fit the length of a letter. Although the scientific community at large did not take much notice, there were exceptions. The most remarkable was a single scientific correspondent (J. Newell, BBC World Service). The most useful (see below) was Ruggero Cepellini, who asked one of his collaborators (Giovanni Galfrè) to join us for a training period.

Panic and relief

The first three MAb were against sheep red blood cells (SRBC). This was a convenient antigen because of the simplicity and sensitivity of the sheep red blood cell lytic assays. By using trinitrophenyl-(TNP) coupled SRBC and lytic assays, we derived two hybrid lines that produced anti TNP-antibodies. This gave us confidence that the procedure was not restricted to one antibody specificity.[17] At this stage, our luck ran out and the next 6 months were most depressing. Neither we nor Georges who, by then, had moved to Basel, was able to derive new hybrids. Fortunately, we had the solid evidence of the past successes and were willing to persist. It was Giovanni Galfrè who identified a wrongly prepared stock solution in our laboratory and in the process, introduced polyethylene glycol as a fusing agent. Eventually other variables that contributed to erratic results were identified, leading to a reliable protocol.[18]

Giovanni came to our laboratory to derive new reagents for HLA typing, which might be valuable in organ transplanta-

[b]The term hybridoma was proposed by Len Herzenberg during a sabbatical in my laboratory in 1976/1977. At a high-table conversation at a Cambridge College, Len was told by one of the dons that hybridoma was garbled Greek. By then however, the term was becoming popular among us, and we decided to stick to it.

tion. However, Jonathan Howard (then at the Babraham Institute of Animal Physiology, Cambridge) convinced us that, scientifically, rats were more interesting than humans, because they offered the opportunity of a fresh start on virtually uncharted territory by using MAbs instead of genetics to define the histocompatibility antigens.[19] The choice of rats also allowed us to test the derivation of interspecies, mouse myeloma/rat spleen cell hybrids. Furthermore, rat but not human red blood cells express histocompatibility antigens, so that, as in earlier experiments, supernatants could be tested by haemolytic assays. As we found later, however, haemolysis by MAb is inefficient. Fortunately, by then the efficiency of fusion was extremely high, so that some microcultures were lytic because they were polyclonal. Most of the derived clones were nonlytic but mixing them restored lytic activity, a phenomenon we defined as ''synergistic'' lysis.[19,20] The first of these publications became a citation classic.

A new approach to study differentiation antigens

In the early days, our idea of usefulness for monoclonal antibodies revolved around the production of specific reagents for diagnosis or therapy. The idea that the procedure could be a revolutionary tool to dissect the complexity of the components of the cell surface, however (Fig. 3) acquired momentum during a chance conversation with the late Alan Williams. Alan was interested in differentiation antigens and was a specialist in radioactive assays whereby binding of antibodies to a cell surface antigen was monitored by ^{125}I-labelled anti-antibodies. Would such binding assays be sufficiently sensitive to provide an alternative to the cytotoxic assays we had been using? This was easily tested on red cells, by using supernatants from the anti-SRBC cultures from the earlier experiments. The results (which we never published) were fantastic, and Alan confidently predicted that he should be able to detect as few as 10,000 surface molecules per cell. Thus, we immediately started experiments to hunt for monoclonal antibodies to unknown cell surface antigens expressed during differentiation and to see how we could use them to understand their function. For practical reasons, our system of choice was rat lymphoid cells.

The basic protocol for the collaboration was as follows. We produced the cultures and clones and sent the supernatants by urgent mail. As soon as he got them, Alan proceeded to do the binding assays, and usually within 24 hours. He would phone (no faxes in those days) the results (perhaps 100 assays per round) for us to proceed with clonal selection. Speed turned out to be essential. We discovered that contaminating clones or chain-loss mutants all too often conspired against us. Indeed, literally hundreds and hundreds of failed attempts taught us a lot about the technical problems involved and how to tackle them. Even so, the experiments were a resounding success and yielded three monoclonal

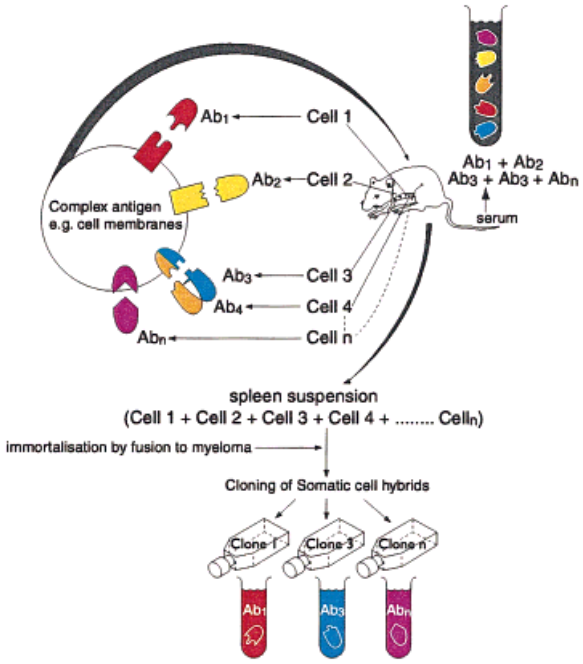

Figure 3. Hybridomas dissect the components of a complex antigen mixture. Immunisation gives rise to diverse antibodies directed against different antigens and epitopes. Each antibody is made by different B-cells, but the products occur as a mixture in the serum. Thus, the serum of a mouse immunized with a complex antigen (like cell membranes) gives rise to an even more complex mixture of antibodies. Immortalization of antibody-producing B-cells and cloning of hybridomas permits the preparation of antibodies specific for individual antigens, which become valuable reagents to identify the unknown components of the complex immunogen.

antibodies with specificities for distinct populations of T-cells.[21] One of them, code named W3/25, was the most exciting. The recently developed FACS (fluorescent activated cell sorter) turned out to be ideal for exploiting the use of monoclonal antibodies.[22] It allowed us to isolate W3/25-positive cells (today we refer to these as CD4-positive cells) and show that they represented the T-helper cell population.[23] I believe that the first of these studies,[21] although not as widely quoted as the others, was the one that most attracted the attention of the scientific community at large and took hybridoma technology beyond the realm of immunologists.

We then decided to try our luck with antigen expressed on human leucocytes. Our first attempt[24] was disappointing. Nevertheless, we derived a pan anti-HLA class I MAb, which became a citation classic reagent, and an anti-blood group A MAb, which although useless at that stage, served as a launching pad for the eventual exploitation of MAbs as blood-typing reagents.[25] The saying was that, if we did not

get what we wanted, we had to learn to love what we got! Our second attempt, in collaboration with Andrew McMichael, was more successful, and we ended up with what later became known as CD1, the first human leukocyte differentiation antigen discovered by using hybridomas.[26] The study describing CD1, and the fact that thymocytes that express CD1 do not express HLA class I and vice versa, was rejected by the editors of the *Journal of Experimental Medicine* with no referee's comments, because they thought it would open the floodgates for studies of a similar nature! This poor reception did not deter us from exploring the approach further and, in particular, exploiting the rat/mouse heterohybridomas to immortalize the rat responses against mouse differentiation antigens.[27,28] This encouraged us to derive rat hybridoma lines better suited for the immortalization or rat responses.[29,30]

The explosion

By then, we were inundated with requests for suitable myeloma lines as the numbers of fusion partners and applications of the hybridoma technique mushroomed.[31] This was particularly so in the search for cell surface differentiation markers. The first International Workshop of Human Leukocyte Differentiation Antigens Detected by Monoclonal Antibodies was organized in 1981/1982, largely through the efforts of Alain Bernard and Laurence Boumsell. The aim of this workshop was to coordinate international efforts to identify new differentiation markers of human leukocytes. Antibodies were exchanged and analysed following established protocols. A subsequent meeting was held in Paris in I982 where the joint reports were presented and discussed. Since then, five more such meetings, summarizing the results of coordinated experiments from hundreds of laboratories in the world, have taken place and the achievements are described in the six volumes of the proceedings under the title Leucocyte Typing.

The derivation of hybridomas to analyse differentiation antigens went well beyond the analysis of the immune system itself. In embryology, it soon became a major tool to provide markers for stages of biological development, and in oncology, it afforded the opportunity to search for tumour specific antigens.[32,33] However, the hope that monoclonal antibodies might provide the ''magic bullet'' to combat cancer was premature and is only now beginning to pay dividends.

The early use of MAbs in immunohistochemistry, with particular reference to the nervous system,[34] was the result of a lasting and fruitful collaboration with an Argentinean colleague and friend, Claudio Cuello. The initial idea was to make bi-specific monoclonal antibodies as a substitute to the conventional peroxidase antiperoxidase method. The experimental design was simple and obvious: to fuse two appropriate hybridomas and select the hybrid-hybridomas (or quadromas) expressing both specificities. The first bi-specific reagent so derived[35] gave excellent results in histochemistry and also highlighted the all-important problem of how to deal with the combinatorial association of the heterologous pairs of light and heavy chains expressed by the hybrid-hybridomas.[36] Other technical innovations arising from that collaboration included the use of internally labelled MAb and double immunolabelling techniques in EM.[37]

The first commercially valuable MAb to emerge from our laboratory was one specific for interferon.[38] The derivation of this reagent is of particular interest, because the immunogen was not a pure, or even highly enriched, preparation of interferon. The clone producing the specific monoclonal antibody was identified by a functional assay. The derived monoclonal antibody could then be used to purify interferon by passage of crude preparations through immunoadsorbant columns.

Patent controversy

A considerable number of ill-informed opinions (including some from official sources) arose through ''our'' failure to patent the hybridoma technique to produce monoclonal antibodies. This issue (as well as intellectual and environmental aspects related to the invention) has been extensively discussed in a witness seminar organized by the Wellcome Institute for the History of Medicine.[15] The facts are that, at the request of an officer of the Medical Research Council (MRC), I sent him the unpublished paper and he replied saying that the manuscript had been sent to the National Research and Development Corporation (NRDC) to consider its patenting potential. In those days, neither employees of nor the MRC itself, was allowed to register patents. This was reserved for the NRDC, a separate government organization. Leaving aside the puzzling claim by the NRDC that ''the MRC internal procedures for communicating inventions to NRDC failed to work,'' they expressed the view that the ''work as published was not patentable. Considerable further work would have to be carried out in conditions of secrecy to develop the invention to a state when it could have been adequately protected.''[15] So, with hindsight, we may have been extremely lucky that, for one reason or another, we were never asked to work in secrecy or to refrain from sending the X63 myeloma partner to our colleagues all over the world.

Dissecting the immune response

My interest in the genetic origin of antibody diversity remained alive throughout this work. Although major advances in understanding the recombination of gene fragments for the creation of antibody repertoires were taking place through the work of Susumu Tonegawa, Phil Leder, Lee Hood, Terry Rabbitts, and others, the role of somatic point mutations as a source of diversity was not understood. Hybridomas opened a new perspective. Knowing that antibody diversity occurs at various levels, we were in a position to ask how antibodies diversify during an immune response and what is the struc-

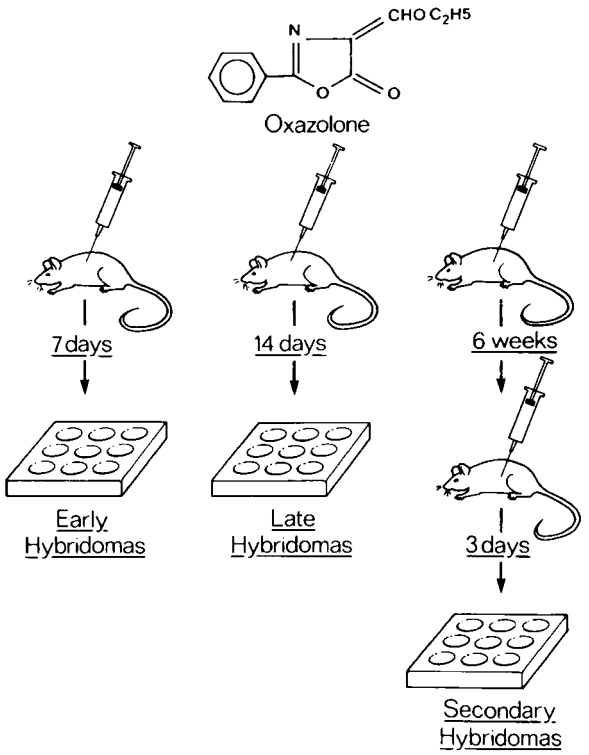

Figure 4. Hybridomas for the analysis of the maturation of the immune response. The hapten 2-phenyloxazolone coupled to serum albumin was injected into mice. Hybridomas were derived at indicated times. mRNA from individual clones were sequenced by using specific primers, reverse transcriptase, and fast DNA sequencing methods (taken from Ref. 64).

tural basis of affinity maturation (Fig. 4). This analysis needed a lot of antibody sequences, and the techniques available at the time were not appropriate for such analysis. Fortunately, in our laboratory, Pamela Hamlyn (later Rabbitts) was adapting the early methods of DNA sequence analysis pioneered by Sanger to sequence the mRNA for the light chain of a myeloma protein.[39] The combination of this fast mRNA sequencing method and the hybridoma technology made the project possible.[40] The sequences of a large number of MAbs, each derived against a single antigen at different times after immunization, could thus be correlated with the increase of affinity for antigen.[41,42] This type of analysis was crucial to our present understanding of the role of hypermutation in the affinity maturation of antibodies.[43,44]

Thus, ironically, we invented a method to derive an antibody-secreting cell line to demonstrate the importance of somatic point mutations but failed initially because the antibody-secreting hybridomas did not hypermutate. As it turned out, the method itself became the tool to show that hypermutation was at the root of the affinity maturation of antibodies.

From hybridomas to man-made antibodies

Even in 1980, it became clear to me that the hybridoma technique was only the beginning of a much more ambitious quest. I stated then that "all that we seem to have acquired is the potential ability to select from an animal any of the antibodies of his repertoire. It is somewhat like selecting individual dishes out of a very elaborate menu: antibodies 'à la carte' . . . A gastronome worth his salt . . . wants to experiment with new ingredients, new combinations. His dream is to invent new dishes and not only to taste what others are doing. I am sure that our next step will be to move from the dining table, where we order and consume our antibodies "à la carte" to the kitchen, where we shall attempt to mess them up."[45]

The increasing understanding of the control of expression of antibody genes brought that dream into the real world. At first the antibody genes were taken from rodent hybridomas, manipulated in bacteria, and reintroduced into myeloma cells to express and secrete the modified antibody structure. The primary aim of these experiments was to "humanize" the mouse antibodies (Fig. 5). Initially, this involved making a chimeric molecule made of the mouse antibody binding domain (the variable region) and the human constant domain.[46] A much more sophisticated and surprisingly successful approach was to graft into a human immunoglobulin framework, the small segments of the mouse antibody (complementary determining regions) that define their specificity.[47] Humanization of mouse antibodies was an essential step toward the use of monoclonal antibodies for therapeutic purposes.

In a lecture I delivered to the Royal Society in 1989,[48] I asked whether the time was ripe to produce antibodies by mimicking, by in vitro manipulations, the strategy of the immune system. The idea was to prepare artificial gene libraries made up of antibody gene fragments, which could then be expressed in cells or bacteria; the main challenge was to find a way to select those clones that expressed antigen-binding properties. From our understanding of the animal models, we expected that, if the libraries were sufficiently large and diverse, they would include binding activities to any conceivable "antigen." As with animals, once initial binding was identified, the relevant genes could then be subjected to further mutation to improve their affinity.[49] A major breakthrough was the derivation of phages expressing antibody binding sites at their tails.[50] In this way, libraries of well over 10^8 variants have been made. Those few phages that bind to the antigen fixed to a solid support can then be easily purified and propagated just like hybridomas.[51] This approach for the derivation of specific antibodies to predefined antigens by-passes animals and, because it is based on human genes, could yield products most suitable for therapy.

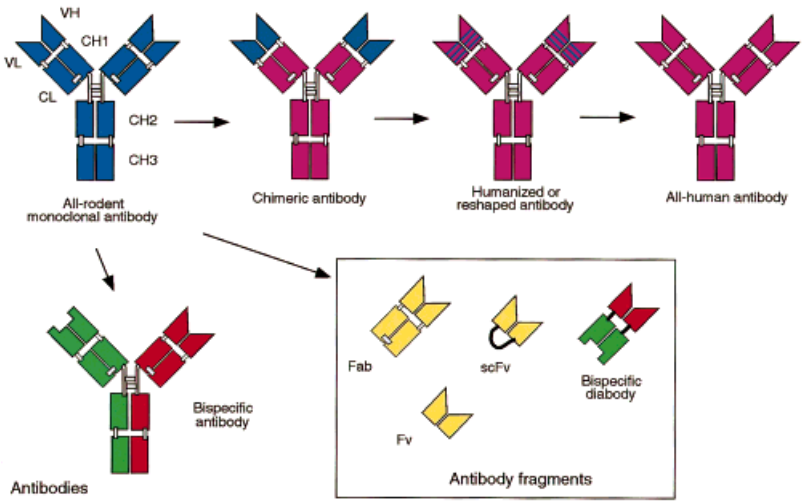

Figure 5. The changing face of antibody therapeutics. In bi-specific antibodies, each half represents a different antibody. Recombinant chimeric antibodies contain rodent variable (V) regions taken from hybridomas, attached to human constant (C) regions. In reshaped antibodies, only the antigen-binding complementary determining region loops derive from rodent hybridomas. All-human antibodies can be constructed by using human V-regions isolated by phage technology or derived as hybridomas from genetically manipulated mice expressing human immunoglobulin transgenes instead of the mouse endogenous loci. Fv and scFv fragments and diabodies comprise only V regions and can be cloned from hybridomas or isolated from phage libraries (taken from Ref. 65).

A more recent approach to making human antibodies is based on the use of transgenic mice that contain very large fragments of human DNA that encode light and heavy chains genes in their germ-line configuration. The mice only express human antibodies, because they have been manipulated to silence their endogenous antibody loci. The advantage here is that one relies on the normal animal strategy for both the onset and the maturation of the antibody response. The hybridomas derived from such animals, secrete human instead of mouse monoclonal antibodies.[52]

Perspectives in therapy

The early notion that MAbs were going to be the magic bullet to cure cancer was over-optimistic and simplistic and led to a lot of disappointment. This was in stark contrast to their use in diagnosis, as exemplified by the blood-group reagents and the introduction of home pregnancy tests. After some spectacular but anecdotal successes, therapy with MAbs went on to be regarded with suspicion by pharmaceutical companies. The required investments were well beyond the possibilities of most academic research laboratories. However, although it lost its front page appeal, this idea was not abandoned. New approaches and, most importantly, a better understanding of the difficulties involved, are slowly helping to bring a more balanced view. Although early attempts gave little consideration to the quality of the therapeutic antibodies, the importance of affinity and the targets involved are now well recognised. Furthermore, compared with rodent, human or humanized therapeutic antibodies are much less prone to elicit an immune response in the treated patient, making the possibility of treatment with multiple injections a realistic option. Optimism has been revived with the recent or impending licensing of MAb-derived products as adjuvants for the treatment of certain cancers, for bone marrow transplantation and for coronary angioplasty. Indeed, it is most likely that therapeutic uses of MAbs will increase considerably in the near future. Yet, many avenues remain to be explored and it is not unrealistic to predict a continuous but, probably, bumpy expansion of antibody-based therapy for cancer and for other medical conditions.

Acknowledgments

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