FROM THE STRUCTURE OF ANTIBODIES TO THE DIVERSIFICATION OF THE IMMUNE RESPONSE

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by

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> *Cuando se acerca* elfin, escribió Cartaphilus, ***ya no quedan imágenes de1 recuerdo; sólo quedan palabras.*** Palabras, palabras desplazadas y mutiladas, palabras de otros, fué la pobre limosna que le dejaron las horas y los siglos.
>
> ***J. L. Borges***

When an animal is infected, either naturally or by experimental injection, with a bacterium, virus, or other foreign body, the animal recognises this as an invader and acts in such a way as to remove or destroy it. There are millions of different chemical structures that the animal has never seen and yet which it is able to recognise in a specific manner. How is this achieved? Scientists have been fascinated by this question for most of this century, and we continue to be fascinated by the intricacies and complexities that still need to be clarified. Even so, looking back over the years since I myself became involved in this problem, progress in the understanding of the process has been phenomenal. Suffice it to remind our younger colleagues that 20 years ago we were still trying to demonstrate that each antibody differed in its primary amino acid sequence.

What attracted me to immunology was that the whole thing seemed to revolve around a very simple experiment: take two different antibody molecules and compare their primary sequences. The secret of antibody diversity would emerge from that. Fortunately at the time I was sufficiently ignorant of the subject not to realise how naive I was being.

Back in 1962, when I had by accident become the supervisor of Roberto Celis in Argentina, it occurred to me that antibody diversity might arise from the joining by disulphide bridges of a variety of small polypeptides in combinatorial patterns. I don't know whether anybody else had the same idea at that time, but of-all the prevailing theories about antibody diversity that I am aware of, this is one that was widest of the mark. I hold it to my credit that I never put it into print. But it was of great value to me as it provided an intellectual justification to work on disulphide bonds of antibodies. By the time I joined the Laboratory of Molecular Biology in 1963, the model of two heavy and two light

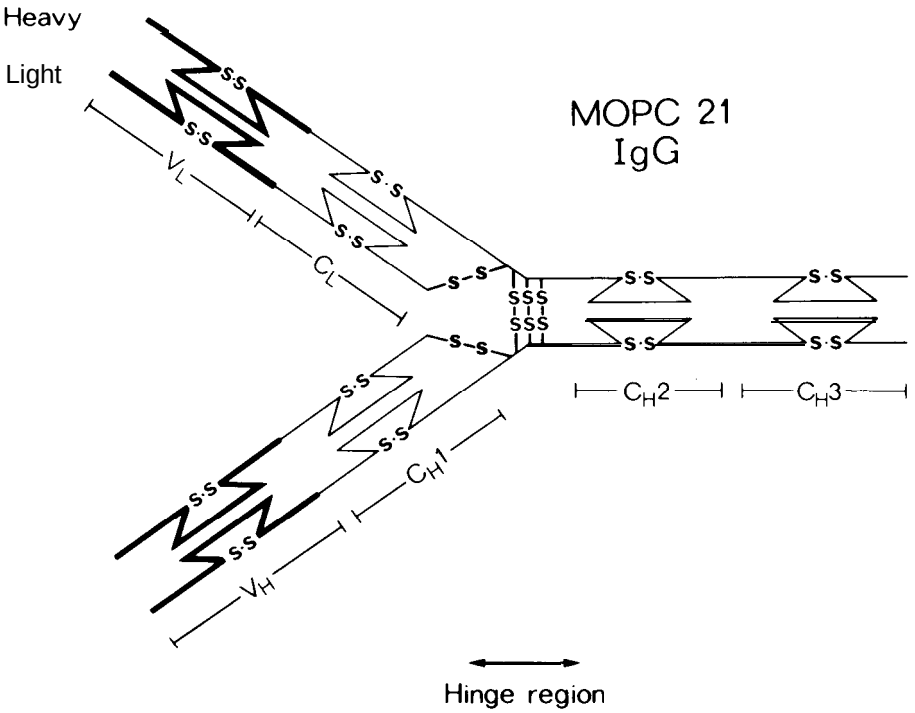

Fig. 1. Antibodies are made of two or more pairs of heavy and light chains joined by disulphide bonds. Each chain has two regions. The variable region differs in structure from one antibody to another and contains the combining site. The antibody combining site is located at the tips of a Y-shaped three-dimensional structure. The constant region is invariant within a given class or subclass, and is responsible for effector functions (complement binding, attachment to and transport across membranes etc). The number and position of the interchain disulphide bonds is characteristic for the different classes and subclasses. In this figure, the structure depicted is the mouse myeloma protein MOPC 21 which was the subject of much research in our laboratory.

chains joined by disulphide bonds (Fig. 1) had been established (l), and I was eager to accept Dr. Sanger's proposal that I should engage in studies of antibody combining sites.

The nature of antibody diversity

At first I looked for differences in fingerprints of digests of iodinated antibodies directed against different antigens. The pattern that emerged from those studies implied that purified antibodies were too complex and differed only in a subtle quantitative way from the totally unfractionated immunoglobulin. I never published those results, which only led me to the conviction that the protein chemistry of antibodies at that level was too difficult to tackle, and that a different approach was needed.

The study of the amino acid sequence around the disulphide bonds of the immunoglobulins was my own short-cut to the understanding of antibody diversity. I soon recognised the existence of what appeared to be a variable

disulphide bridge and a common disulphide bridge (2,3), but the full meaning of that observation only became obvious when Hilschmann and Craig described the variable and constant halves of antibody light chains (4). The variable half contained one disulphide bond, and the constant half the other. This was followed, in later studies with Pink, Frangione, Svasti and others, by the observation of the repeating pattern of similar S-S loops as a distinctive common architectural feature of the different classes and subclasses of immunoglobulin chains. What distinguished them from each other was the diversity of interchain S-S bonds (5).

The period between 1965 and 1970 was full of excitement, both at the experimental and theoretical level. How were these variable and constant regions going to be explained? It was now not only a problem of millions of antibody structures, but that in addition those millions of structures were part of a polypeptide which otherwise had an invariant primary sequence encoded by only one or very few genes. How to solve the puzzle? Dreyer and Bennett (6) suggested that there were thousands of genes in the germline and that the paradox was easy to solve if we postulated a completely unprecedented scheme. This became known as the “two genes-one polypeptide” hypothesis. At the time we did not like that, and proposed a mechanism of hyper-mutation operating on selected segments of a gene (7). There were other ideas at the time to generate antibody diversity. One of them, widely discussed in a Cold Spring Harbor Symposium in 1967, was based on a mechanism of somatic cross-over between gene-pairs (8). It was very exciting for me when soon after the symposium I could show that in the human kappa chains at least three genes must be involved (9). The predicted thousands of V-regions could be grouped into a small number of families or subgroups. The fact that these families were encoded by non-allelic V-genes (10) - coupled to the genetics of the C-region, which indicated a single Mendelian C-gene - provided the experimental evidence that convinced me and many others that the “two genes-one polypeptide” hypothesis was inescapable.

After that, there was a period of consolidation and extension of the results. The concept of V-gene families or subgroups became firmly established, as was the existence of hypervariable residues within the variable segment (9,ll). Crystallographic data showed that such hypervariable residues were near to each other, justifying the idea that they were part of the antibody combining site. This was directly shown with crystals of myeloma protein-antigen complexes (12). The work with myelomas was not only totally vindicated, but also generally accepted. The idea of separate pools of V- and C-genes that were under continuous expansion and contraction was the last element added to the picture. By 1970 we became convinced 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 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” (13).

Light chain mRNA and the signal for secretion

I now began to feel a bit restless. It seemed that protein chemistry alone was not going to get us much further. Furthermore, there was a lot of excitement in the laboratory with the new methods for sequencing RNA being developed by Sanger and his group. Perhaps even more important, one of my closest friends at the laboratory, George Brownlee, was beginning to feel that the time was ripe to attack molecules more complicated than 5S or 6S RNA. So we joined forces in an attempt to isolate immunoglobulin mRNA. This was a difficult problem and when George's new research student, Tim Harrison, joined us we decided to move from solid tumours (14) to cell lines in culture which were kindly provided by colleagues from the Salk Institute (15). The first important breakthrough in the field was a paper reporting *in vitro* synthesis of immunoglobulin light chains (16). We immediately set to work to follow up that approach, and to our delight ran into the unexpected observation of the existence of a biosynthetic precursor of light chains. Further experiments led us to propose that the extra N-terminal sequence was a signal for vectorial transport across membranes during protein synthesis. That was the first evidence which indicated that the signal for secretion was an N-terminal segment, rapidly cleaved during protein synthesis (17,18).

However, our major concern remained the sequence of the messenger RNA for the light chains. In those days there was no DNA sequencing, only mRNA sequencing via elaborate fingerprints of radioactive mRNA. Every radioactive messenger preparation on which we could do sequence analysis involved the labelling of cells with inorganic ^{32}P-phosphate at levels of 50 mCi. So there we were, dressed up in our new-style laboratory coats (namely heavy lead aprons), behind a thick plastic screen, labelling cells and then frantically working up our messenger purification procedures and performing fingerprinting experiments, before the inexorable radioactive decay. Although we didn't go very far in our sequencing, we could isolate oligonucleotides that corresponded to the protein sequences (19). Among these were oligonucleotides spanning the V- and C-regions, demonstrating that the protein chain was made from a single messenger RNA and that, therefore, integration of the V- and C-genes did not take place during or after protein synthesis (20). At this stage the radioactive approach was stopped and we tested alternative methods for the sequencing of mRNA, using synthetic primers and cDNA synthesis. This approach went on in the background while our main efforts were moving in a different direction. Eventually however, it paid off (21). I will come back to that later, because it forms part of my story.

Spontaneous somatic mutants of a myeloma protein

The introduction of tissue culture methods to our laboratory had a major impact on the direction of our research. With my new research student, D. S. Secher, and soon after with R. G. H. Cotton, we decided to embark on an analysis of the rate and nature of somatic mutation of myeloma cells in culture. We were hoping that we might reveal a high rate of mutation of the hypervaria-

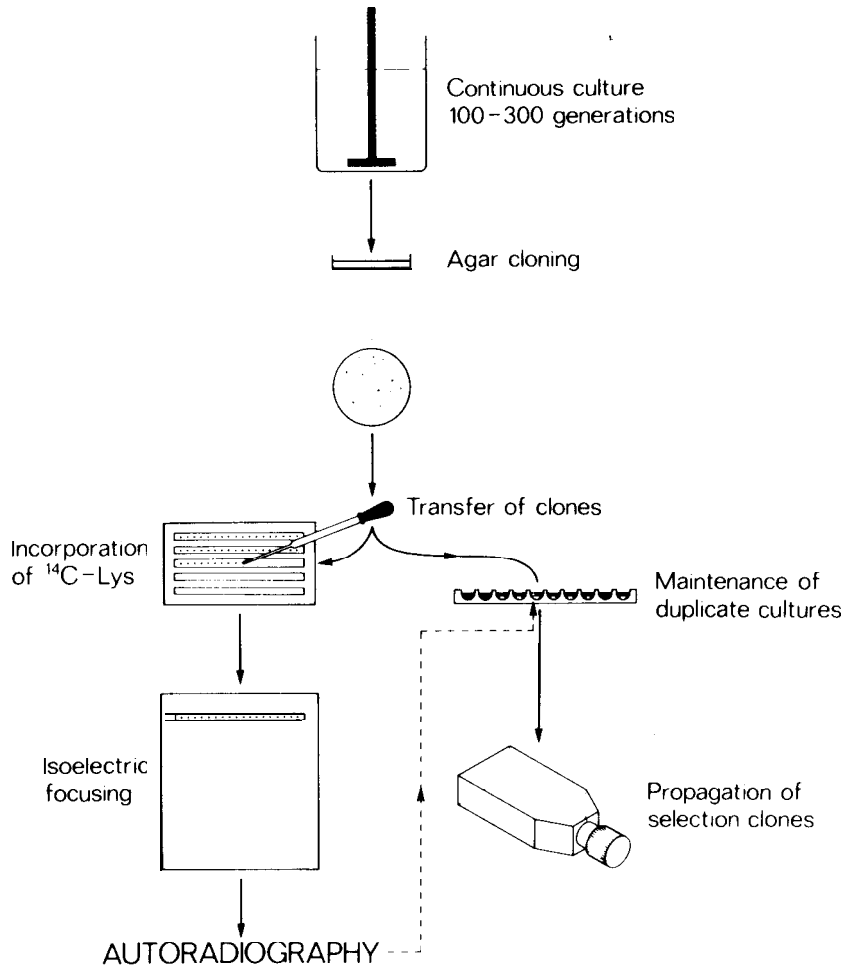

Fig. 2. Protocol used for the screening of the isoelectric focusing pattern of the immunoglobulin secreted by 7,000 clones of P3 myeloma cells. Mutants were detected and their primary defect analysed by amino acid and mRNA sequence analysis. The results are described in Table 1 (taken from Ref. 23).

ble segments. (The protocol is described in Fig. 2.) A continuous culture was grown for a minimum of three months to allow mutants to accumulate, and individual cells were taken and grown as colonies. These were incubated with labelled amino acids and the radioactive immunoglobulin analysed to detect mutants with altered electrophoretic properties. Our first structural mutant appeared after a few thousand clones (22), and the final analysis of 7,000 individual clones gave us a pool of mutants which are described in Table 1. We were believed that this elaborate experiment provided the first evidence at the protein and nucleic acid levels of the existence of somatic mutations of mammalian cells (23). Furthermore, the rate at which these mutations occurred suggested an important role in the generation of diversity (24). But the mutations were not in the variable region, and we were forced to conclude that in the cells

Table 1. Spontaneous structural mutants of MOPC 21 heavy chains

Mutant	Protein defect	Genetic defect
IF1	Last 82 residues of CH3 missing; carbohydrate difference	Ser (387) → Ter small deletion?
IF2	Whole CH1 deleted	5.5 K bases deleted including CH1 exon. Aberrant switch?
IF3	Altered sequence of residues 367–380. Deletion of rest of CH3	Frameshift (−2) Premature "ochre" termination.
IF4	Asparagine 452 to Aspartic acid.	A to G transition. ("mis-sense").
NSI1/1	Deletion of last 67 residues	Trp (406) → Ter G to A transition ("non-sense").

we were studying, there was no evidence for a hypermutable segment. So that in a sense we were back to square one.

Hybrid myelomas

While this work was going on, Cotton was preparing another type of experiment which turned out to be more important than we anticipated (25). This involved the fusion of two myeloma cells in culture (Fig. 3). That fusion demonstrated that the phenomenon of allelic exclusion was not dominant. On the contrary, fusion of two myeloma cells gave rise to a hybrid co-dominantly expressing the antibody chains of both parents. In addition, we proved that the expression of V- and C-regions was *cis*, probably because the V- and C-segments were already integrated at the DNA level by a translocation event in the precursors of plasma cells. This was in contrast to the assembly of heavy and light chains, which combined with each other to give rise to hybrid molecules.

Armed with these results, I went to Basel to give a seminar, and the important consequence was that Georges Köhler came to Cambridge. He joined in our main research project of looking at somatic mutants in immunoglobulin-producing cells, and in the other minor project concerning the phenotypic expression of somatic cell hybrids prepared between myelomas and myeloma mutants. It became increasingly clear that we could not go on looking for mutants by the procedure we had employed before, and the only way ahead was to use a culture of a myeloma cell line capable of expressing an antibody. Mutants from that cell could then be made based on the antibody activity. Although at that time there had been reports in the literature of myeloma cells

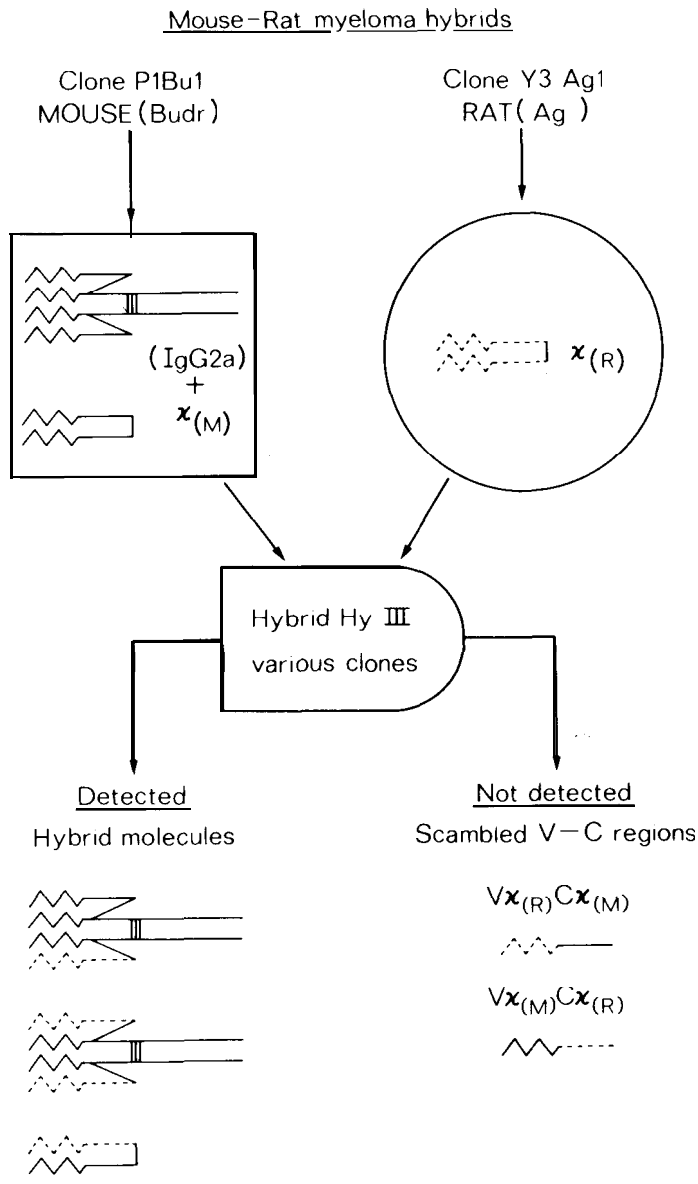

Fig. 3. Co-dominant *cis* expression of antibody genes in hybrids of myeloma cells. The diagram describes data taken from Ref. 25.

capable of fulfilling that role, none proved suitable in our hands. The myeloma cell line P3 (MOPC 21) would have been ideal from a chemical point of view, because at the time sequencing the protein was a major undertaking and we knew how to deal with MOPC 21. But we were unable to find a suitable antigenic binding activity to this myeloma protein. We failed, but others who were pursuing similar types of experiments succeeded. Scharff and his co-workers were the first to demonstrate that one can isolate somatic mutants of a variable region in that way (26).

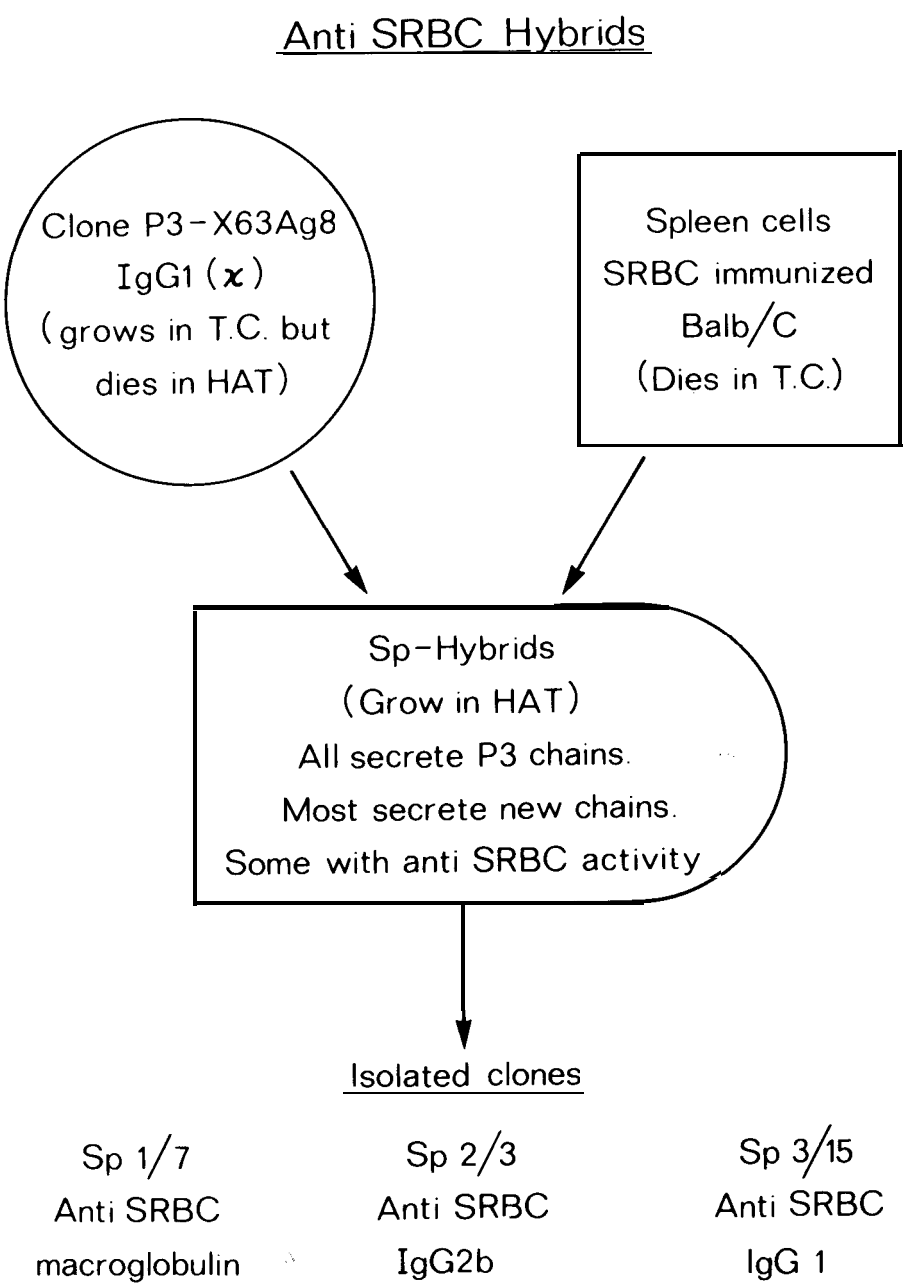

Fig. 4. The first successful hybridoma was prepared from cells from a mouse immunized with sheep red blood cells (SRBC) (56). These were fused to a myeloma cell line producing the IgG protein MOPC 21 (Fig. 1) growing in tissue culture and made resistant to azaguanine. Hybrids were selected by growth in HAT medium (57).

And yet in a funny way our lack of success led to our breakthrough; because, since we could not get a cell line off the shelf doing what we wanted, we were forced to construct it. And the little experiment being done in the background concerning hybridization between myeloma cells developed into a method for

the production of hybridomas. Thus, as illustrated in Fig. 4, instead of hybridizing two myelomas, we hybridized a myeloma and an antibody producing cell. The resultant hybrid was an immortal cell capable of expressing the

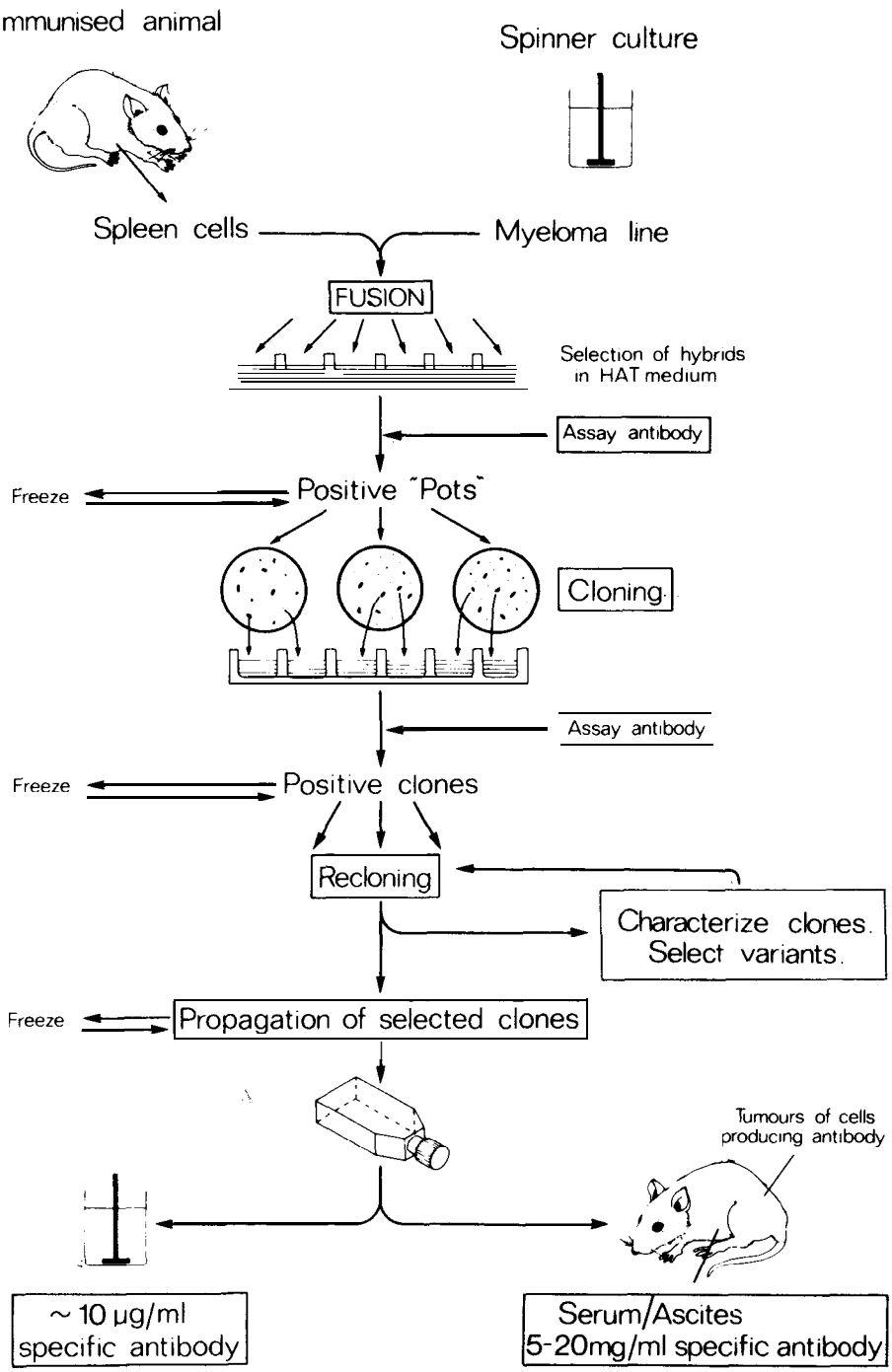

Fig. 5. Most generally used protocol for the derivation of hybridomas (taken from Ref. 58)

antibody activity of the parental antibody-producing cell the immortality being acquired from the myeloma.

So finally, we were able to obtain a continuously-growing cell-line expressing a specific antibody and use it to search for mutants of the hypervariable region. This was undertaken by my research student, Deborah Wilde. While she got more and more discouraged by her lack of success in what she called "looking for a needle in a haystack", it dawned on me that it was up to us to demonstrate that the exploitation of our newly-acquired ability to produce monoclonal antibodies "á la carte" was of more importance than our original purpose. After our early success we ran into technical difficulties and could not get our

Table 2. Selected list of monoclonal antibodies derived in our laboratory

Hybridoma	Antigen	Purpose and use	Selected references
R3/13 R2/10P R2/10S	Rat MHC	Reagents for tissue typing Synergistic effects	Galfré *et al.*, 1977, (27) Howard *et al.*, 1979, (60)
W3/13 W3/25	Rat T cell markers	Analysis of cell surface antigens	Williams *et al.*, 1977, (29)
H6/31	Mouse IgD allotype	Standard allotype reagent	Pearson *et al.*, 1977, (61)
W6/32 W6/1 W6/34 and others	HLA-A,B,C Blood group A Controlled by chromosome 11	Tools for genetic analysis and biochemical studies	Barnstable *et al.*, 1978, (62)
M1/69, M1/70	Mac-1 and other mouse leukocyte surface antigens	Novel mouse leukocyte differentiation antigens.	Springer *et al.*, 1978, 1979, (63,64)
M1/22	Forssman	Embryonic development	Stern *et al.*, 1978, (30)
H9/25	Alloantigen on killer and plaque forming cells		Takei *et al.*, 1980, (65)
NA1/34	Subpopulation of human thymocytes (CD1)	Define subpopulations of human lymphoid cells	McMichael *et al.*, 1979, (66)
NC1/34	Substance P	Radioimmunoassay Immunocytochemical localization of neurotransmitters. Internally labelled antibodies.	Cuello *et al.*, 1979, (32,67)
YC5/45	Serotonin	Dual localization at the EM level	
6D4 NB1/19	Blood group A Blood group B	Standard blood group reagents	Voak *et al.*, 1980, (33,68)
NK2	Human anti-interferon	Large scale protein purification	Secher and Burke, 1980, (34)

fusion experiments to work for quite some time. Then Giovanni Galfré, who had recently joined us, got us out of the deadlock when he discovered that one of our stock solutions had become contaminated with a toxic substance. After this an improved reliable protocol was developed (Fig. 5) and quick progress made towards the first practical applications of the technology. For several years I shelved the antibody diversity problem to demonstrate the practical importance of monoclonal antibodies in other areas of basic research and in clinical diagnosis (Table 2). We were able to show that the hybrid myelomas were capable of being used for the production of standard reagents such as anti-histocompatibility antigens (27) and anti-Ig-allotypes (28). The procedure was ideally suited to the study of cell surface and tumour antigens and to providing reagents for cell fractionation (29-3 1). Monoclonal antibodies produced in this way were suitable for radioimmunoassays and for neuropharmacology (32), as blood group reagents (33) and for large scale purification of natural products (34). We also extended the hybrid myeloma technology to a second species-the rat (35) and to the production of bi-specific immunoglobulins (hybrid-hybridomas) (36).

Genetic origin of antibody diversity

In the period 1970- 1975, a considerable effort was being made to measure the number of germline genes coding for the variable regions of immunoglobulin chains. Our own contributions started when we persuaded Terry Rabbitts to join us. After considerable effort and a lot more radioactivity we obtained results indicating that the number of germ line genes was not much higher than would be predicted from our understanding of subgroups, and this view was shared and reinforced by parallel work being conducted by others (37,38). By 1976 this view was gaining general support (39). But then the impact of the recombinant DNA revolution began to be very strongly felt. Within a few years, and largely through the work of Tonegawa, Leder, Rabbitts, Hood, Baltimore, and others, a coherent picture of the arrangement and rearrange-

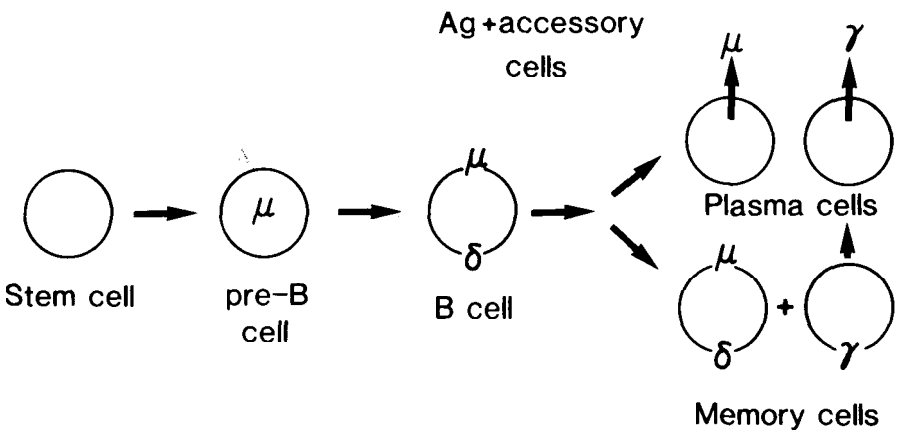

Fig. 6. Differentiation of B cells

ment of immunoglobulin genes and their involvement in the generation of diversity began to emerge (40).

The precursors of the antibody producing cells do not express an immunoglobulin, but during their differentiation into pre-B cells and B cells, they express first the heavy chain and then the light chain (Fig. 6). The first antibody produced is membrane bound and functions as a receptor molecule, which receives antigenic signals. Triggered cells divide and differentiate to antibody producing cells and memory cells. These events at the cellular level are correlated with changes in the DNA structure (Fig. 7). The germline DNA contains the V- and C-genes on different DNA fragments, as predicted. But in addition, there are further fragmentations, and only some of them are shown in the figure. Light and heavy chains can only be transcribed and translated when certain fragments (one of the V and J in light chains, V, D and J in heavy chains) are integrated by a deletion mechanism. During this process of integration enormous diversity is generated.

To theorize about the genetic origin of antibody diversity was a "must" among molecular immunologists for quite a number of years. How do those theories contrast with the reality of today? The hard experimental facts made possible by the methodological advances in molecular biology show that, while none of them was right, most of them contained at least a grain of truth. There were two major currents of opinion. One consisted of germline theories whereby all the diversity was inherited as genes present in the germline. The other included somatic diversification theories, whereby somatic processes were responsible for the generation of diversity, starting from a small number of germline genes. As it turns out, the genetic mechanisms responsible for the

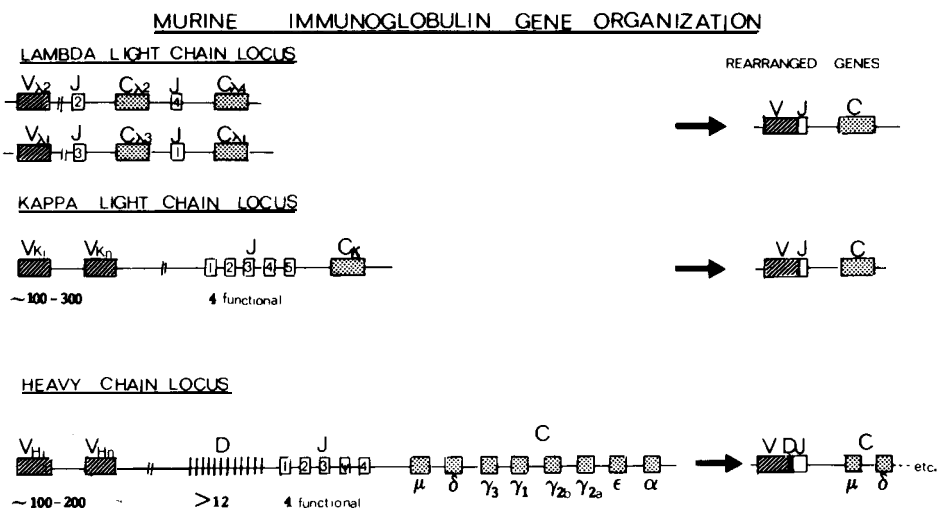

Fig. 7. Genetic arrangement of immunoglobulin genes in the germline. During differentiation into pre B cells and B cells large deletions of DNA lead to the integration of fragments (rearranged genes). Further proliferation leads to somatic mutation of the integrated gene and this is of major importance in the maturation of the response.

Table 3. Mechanisms that generate antibody diversity

I. GERMLINE: multiple V-gene segments
2. COMBINATORIAL: a) Different combinations of V-(D)-J
b) Different combinations Of V_H and V_L
3. JUNCTIONAL: variation at at V-J, V-D, and D-J boundaries
4. SOMATIC POINT MUTATION: nucleotide substitutions throughout the V region

generation of diversity include a little bit of everything (Table 3). There are between 50 and 300 gene fragments in the germline encoding the light or the heavy chains. The number varies from species to species. So there is a considerable germline contribution. Recombination and gene conversion arc probably important genetic events in the evolution and maintenance of that germline gene pool. We still do not know whether these events are significant as somatic generators of diversity (41). As shown in Fig. 7, the V-region is encoded by V, D and J segments (heavy chain) and V and J segments (light chain). Their combinatorial integration into a single gene, although an important component of the generation of diversity, is not the critical mechanism predicted by the mini gene hypothesis (42). Also important is the diversity generated during the joining process, and this contains an element of the errors and aberrations during repair predicted by other theories (7,43). And then there are the somatic point mutations for which a mechanism remains to be elucidated. It may involve error-prone repair enzymes (7), genetic hot-spots (24), appropriate selection either by antigen (44) or by other network elements (45), or quite possibly by a mixture of all or some of these. The instructional theories were largely forgotten as soon as the chemical diversity of antibodies was established (46). Yet they also may contain a grain of truth. It has recently been proposed that peptide segments of the antigen which appear to be mobile are better immunogens, presumably because they adapt their structure to a predefined antibody structure (47,48). It is also possible that to some extent the antibody combining site itself has a certain degree of mobility, which has a limited capacity to accommodate its own structure to that of the antigen. Of course dynamic adaptation has a price to pay in terms of affinity. Adaptability should not be confused with the generation of specificity. As I discuss below, an improved fit of binding to the ligand is the result of somatic mutation and antigenic selection.

Molecular analysis of an immune response using monoclonal antibodies and mRNA sequencing

Let us return to an animal that is being immunized with a certain substance. The immune system recognizes the substance as foreign, and the B cells are triggered to produce antibody (Fig. 8). The different antibodies are secreted and mixed in the serum. The individual antibody molecules are extremely similar and once mixed cannot be separated from each other. For this reason, and until the advent of the hybridoma technology, it was impossible to study the diversity of the antibody response to a given immunogen. The derivation of immortal cell hybrids solved this problem, because it affords individual anti-

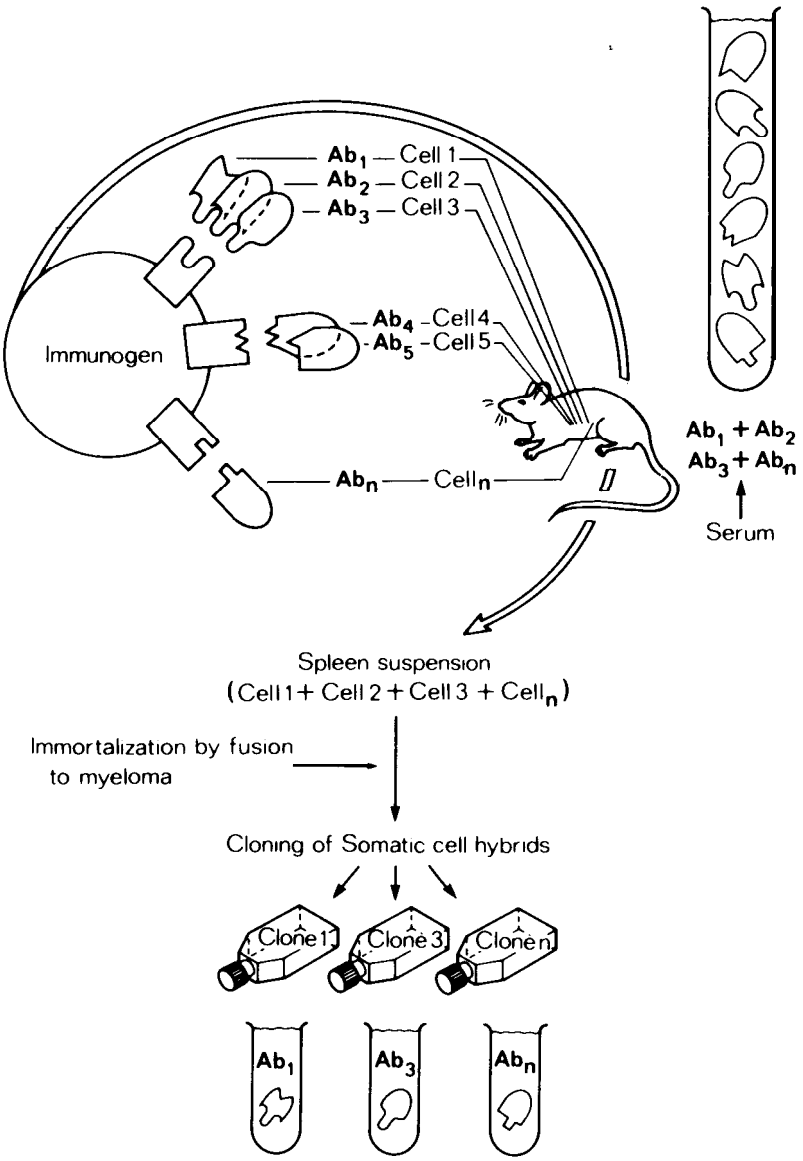

Fig. 8. The dissection of the immune response by the hybridoma techniquc. When an animal is injected with an immunogen the animal responds by producing an enormous diversity ofantibody structures directed against different antigens, different determinants of a single antigen, and even different antibody structures directed against the same determinant. Once these are produced they are released into the circulation and it is next to impossible to separate all the individual components present in the serum. But each antibody is made by individual cells. The immortalization of specific antibody-producing cells by somatic cell fusion followed by cloning of the appropriate hybrid derivative allows permanent production of each of the antibodies in separate culture vessels. The cells can be injected into animals to develop myeloma-like tumours. The serum of the tumour-bearing animals contains large amounts of monoclonal antibody.

bodies separately produced, in culture vessels and as mouse myelomas. This permits dissection of the individual components of the antigen. Monoclonal antibodies prepared against hitherto undefined cellular components can themselves be used to identify the chemical nature of those components, to probe for their function, and later for use as reagents for diagnostic and therapeutic purposes. These are the fundamental properties behind the most important of the general applications of monoclonal antibodies. When we started to explore these applications, and until some years ago, it was possible to some extent to summarize the main results obtained (49). In recent years their application to basic research, clinical biochemistry, medical therapy, and in industry has been so widespread that I do not intend even to attempt to discuss it any further here.

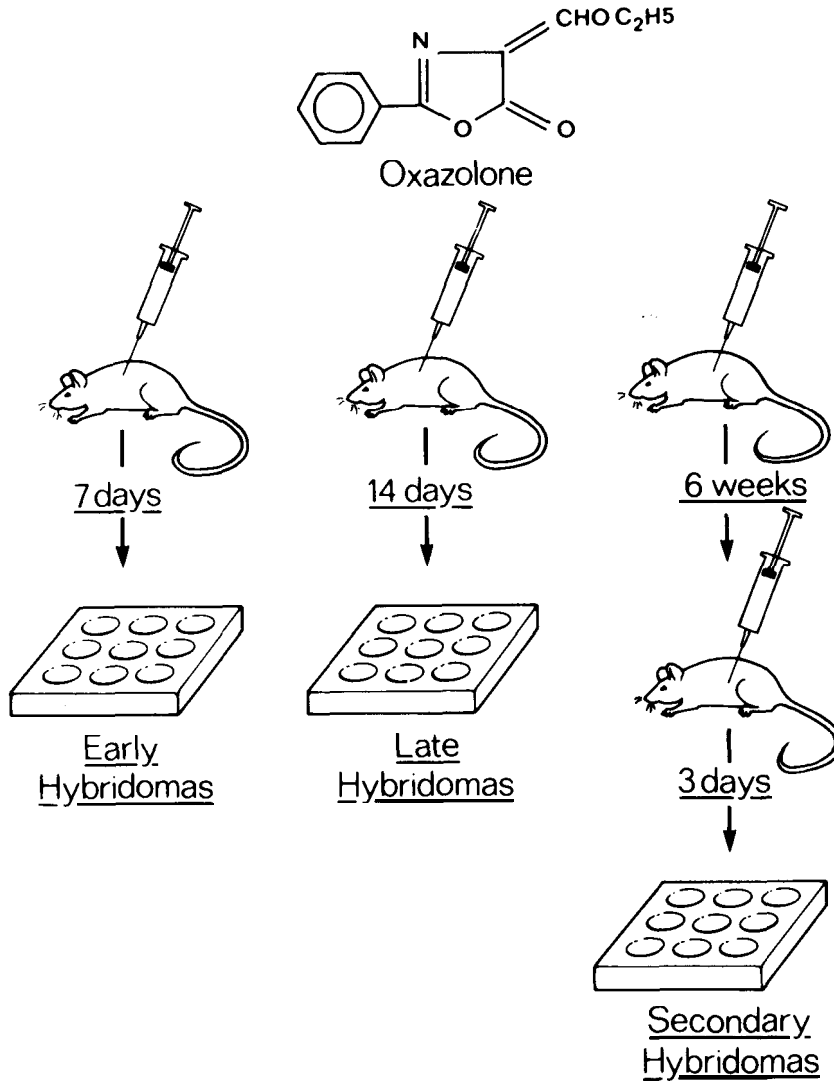

Fig. 9. Derivation of monoclonal antibodies at the onset and during the maturation of the response to oxazolone.

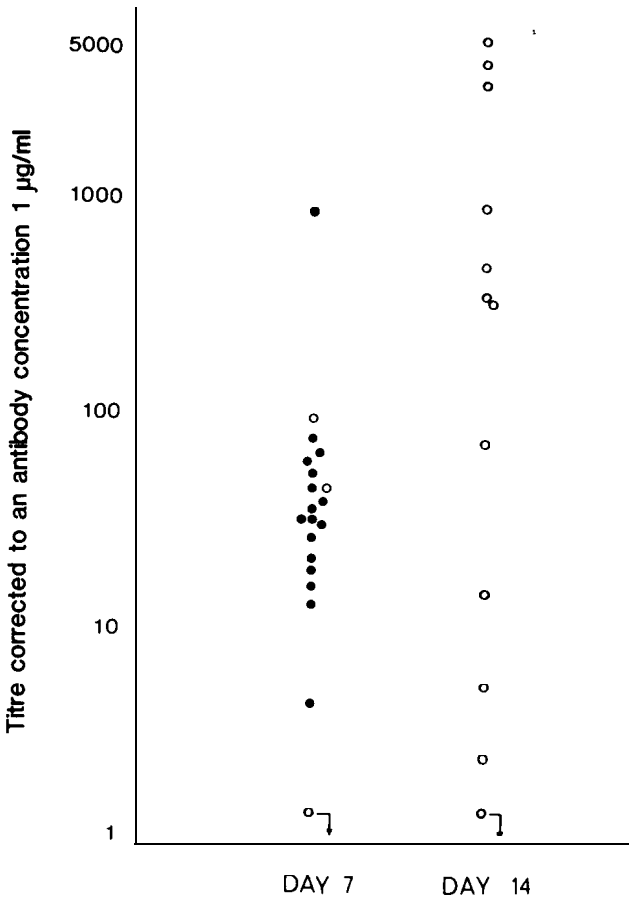

Fig. IO. Avidity of monoclonal antibodies 7 and 14 days after immunization. Haptenated phage inhibition (HPI) per µg of anti-phlOx immunoglobulin from supernatants of IgG-secreting hybridomas. Those on the left were from day 7 and those on the right from day I4 fusions. Black circles represent oxazolone idiotype-positive IgG and open circles represent idiotype-negative IgG (taken from Ref. 50).

Different antibodies recognize different antigenic determinants of the immunogen, and the recognition of each determinant is complex in itself (Fig. 8). It has been known for a long time that even the simplest antigenic determinants are recognized by an unknown variety of antibody molecules. Monoclonal antibodies can be made pure and used to answer the old questions of how complex the collection of antibody molecules produced by the animal as a response to a particular antigen is, and how the individual molecules differ from each other. This brings me back to sequencing messenger RNA.

While in the late ’70s the excitement about monoclonal antibodies and DNA recombinant methods was simmering, Pamela Hamlyn was quietly adapting Sanger’s fast DNA sequencing methods to the sequencing of light chain mRNA. Her eventual success (21) added to our capacity to derive cell lines secreting monoclonal antibodies to a predefined antigen, and to our ability to

sequence quickly the messenger RNA of the antibody molecule they produce. So, instead of asking the question "What is the nature of antibody diversity?", we were now in a position to ask the question "How do antibodies diversify during an immune response?" In other words, how, in real life in the animal, are all those genetic events capable of producing antibody diversity actually operate in response to an antigenic stimulus?

In collaboration with Matti Kaartinen, Gillian Griffiths and Claudia Berek, we have been conducting a study of the response to the hapten phenyl oxazolone (50,51). The essence of the experiment is described in Fig. 9. The hapten conjugated to chicken serum albumin as carrier is injected into mice, and 7 days and 14 days later animals are sacrificed, hybridomas are prepared and a number of random clones isolated in each case. Other animals are left for a couple of months, and hybridomas of the secondary response arc prepared.

Hybridomas prepared 7 days and 14 days after primary immunization are compared in Fig. 10. Each point on the figure represents the avidity of each one of 32 monoclonal antibodies. The mixture of antibodies at each stage, as a first approximation, represents a cross-section of the complexity of a typical antiserum. The average titres of the antibodies at both stages are not very different, although the day 14 average is slightly higher. This is as expected. The antibody titre of an antiserum, as well as its average avidity, increases during the course of an immunization. It is what we refer to as the maturation of the response. What distinguishes the results of the day 7 and day 14 is that while the day 7 results cluster around the average, the scatter at day 14 is much wider.

Since each monoclonal antibody was the product of an immortal hybridoma, we could go one step further and study the total amino acid sequence of each one of these monoclonal antibodies. Better still, we could study the sequence of

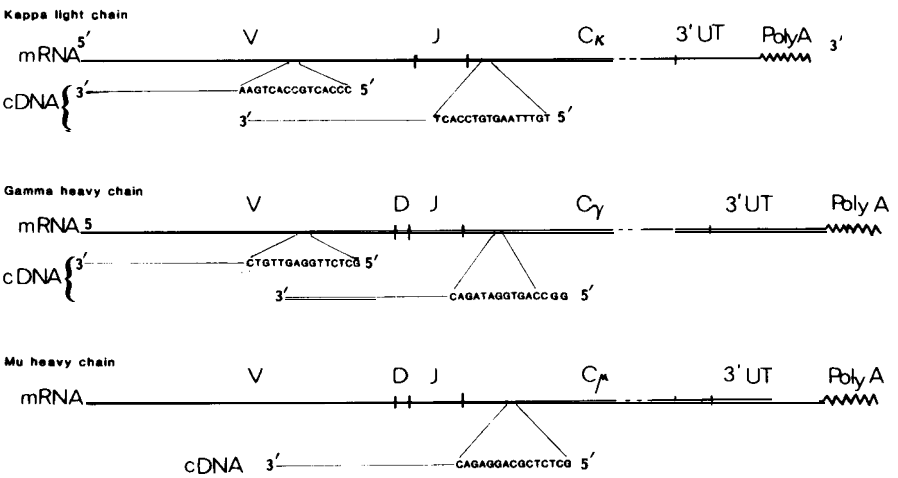

Fig. 11. mRNA sequencing strategy. Synthetic oligonucleotide primers designed to pair with defined bases within segments of mRNAs were used to initiate reverse transcription. Using dideoxynucleotides, specific stops in the cDNA can be generated and the nucleotide sequence determined by gel methods (taken from Ref. 59).

the mRNA coding for each amino acid sequence. This not only provided more information, but was also technically simpler. To do so, RNA was prepared from the hybridoma cells and direct sequencing done on the impure messenger preparations, as shown in Fig. 11. In this way, sequences of antibodies at different stages of the immune response could be compared.

What we have learned from this is that the majority of antioxazolone antibodies at day 7 express a single set of germline V-genes taken from the total pool of over 100 for each of the two chains (Fig. 12). This pair of germline genes (which we refer to as V_H-Ox1 and V_k-Ox1) are at this stage expressed in their unmutated form. The few differences between them arise by junctional diversity - that is the variations introduced during integration of the DNA fragments V, D and J which make up the variable region of the antibodies. At day 14 the same germ line genes V_H-Ox1 and V_k-Ox1 still seem to dominate the response.

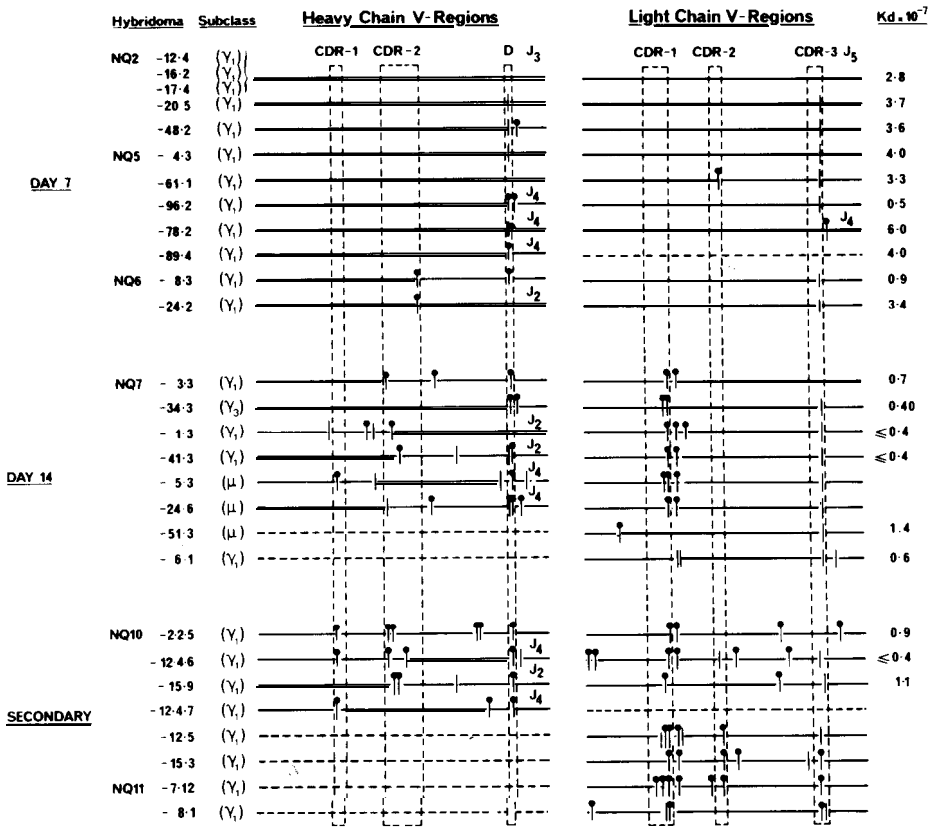

Fig. 12. Diagrammatic comparison of the mRNA sequences from anti-phOx-secreting hybridomas derived at different stages after immunization with Ox-CSA. Only sequences closely related to the prototype are shown. The variable region sequences of each hybridoma have been compared with the sequences of V_H-Ox1 and V_k-Ox1 respectively. Unbroken horizontal lines denote identical sequences, broken lines represent extensive sequence differences. A black circle indicates that these changes predict an amino acid difference at this position. Complementarity determining regions (CDR-1, -2, -3) have been marked as have the D and J regions. Where different J segments are observed these are represented accordingly. Dissociation constants determined by fluorescence quenching (Kd in moles/litre) are shown on the right (taken from Ref. 51).

However, in sharp contrast to day 7, the day 14 antibodies express a small number of point mutations which are responsible for a significant increase in affinity for the same hapten. In other words, as the response matures, new somatic mutants appear in a seemingly endless variety.

The antibodies obtained during the secondary response, expressing the germline gene combination characteristic of the primary response, show a further small increase in point mutations (Fig. 12). However, the most important feature of the secondary response is a shift towards other germline genes (see Table 4).

Table 4. Expression of germ line V genes in the maturation of the response to oxazolone

	V_HOX_1-V_kOX_1 combination	Other combinations	% V_HOX_1-V_kOX_1
DAY 7	11	4	73
DAY 14	6	5	54
SECONDARY	4	18	18

It appears therefore that the development and maturation of the immune response to oxazolone - which we take as a model system - proceeds basically in three stages. In the first the majority of the antibody reflects a very restricted choice from a vast repertoire of germline gene combinations, self-selected for their capacity to bind the antigen. In the second stage, cells expressing these combinations proliferate, and during this proliferation mutants arise which improve the affinity of the antibody for the antigen. In the third stage, as the first type of germline gene combinations and their mutants reach a certain limit of dissociation constants, new germline gene combinations and somatic mutants arc selected for further improvements. Of course the three stages are not absolutely separate and all three processes overlap to a certain extent. In many ways, the system behaves as a Darwinian system, where adaptation is an improvement in antigen binding. It remains to be seen to what extent other regulatory constraints are critical to the process.

From monoclonal antibodies to antibody engineering

The immortalization of antibody-producing cells not only allows the permanent supply of an antibody of a constant chemical structure but, more important, affords all the advantages that can be derived from the techniques of cell culture and somatic cell genetics. The most obvious is cell cloning, and this has been at the root of the explosion in the use of this technology. And yet the derivation of cell lines producing specific antibodies cannot go beyond the immortalization of what already exists. We select hybrids producing monoclonal antibodies of desired properties, but if the immunized animal does not make it, there is no way of immortalizing it. Fortunately we can go further.

Hybridomas are established cell lines and are therefore capable of other ***"in vitro"*** manipulations using somatic cell genetic and molecular engineering techniques. We are at the beginning of a new era of immunochemistry, namely the production of "antibody based" molecules. The derivation of hybrid hybri-

domas is one example of the utilization of such methods for the biosynthesis of bi-specific antibodies (36). Another example is the derivation of class switch mutant antibodies (52).

Some years ago, I discussed the eventual use of recombinant DNA techniques to make more drastic changes (53). Recent developments have shown the feasibility and potential of the approach. Antibody genes have been put into suitable vectors, propagated, modified and re-introduced into myeloma cells which will then secrete recombinant antibodies possessing novel properties. For instance, in my laboratory Neuberger has developed a cell line which secretes a mouse-human antibody molecule with a mouse anti-nitrophenacetyl variable region and a human epsilon heavy chain constant region (54). In another example, the Fc portion of the mouse antibody was replaced by staphylococcal nuclease (55). A novel antibody was thus made which contains an antigen specific Fab portion joined to an enzymatic effector function replacing the normal Fc portion.

More elaborate modifications will be made possible by the fast-developing techniques of site-directed mutagenesis. These will allow well-planned specific modifications of antibody combining sites. In this way we will be able to test the contribution of individual point mutations to the generation of high affinity antibody during the process of the maturation of the response. This brings us back to the problems of the diversity of molecular recognition and the maturation of the immune response.

Exciting as these prospects are, they still require the basic starting genes taken from a hybridoma line. With them, we can introduce changes at the amino acid sequence level but with the exception of simple changes, the ultimate folding pattern and their effect on protein-ligand interaction cannot yet be reliably predicted. This will remain so for the time being. Total construction of antibody molecules to suit specific needs depends on a much better understanding of protein folding.

While selection is the strategy of the antibody response of an animal, the immunochemistry of the future will revert to an instructional approach where the antigen will tell us what antibody structure we should construct. Although this is not science fiction, we need to overcome the theoretical problems involved in the translation of one-dimensional reality into a valid three-dimensional prediction. Although the way ahead is full of pitfalls and difficulties, this is indeed an exhilarating prospect. There is no danger of a shortage of forthcoming excitement in the subject. Yet, as always, the highlights of tomorrow are the unpredictabilities of today.

Acknowledgements

The hybridoma technology was a by-product of basic research. Its success in practical applications is to a large extent the result of unexpected and unpredictable properties of the method. It thus represents another clear-cut example of the enormous practical impact of an investment in research which might not have been considered commercially worthwhile, or of immediate medical relevance. It resulted from esoteric speculations, for curiosity's sake, only motivat-

ed by a desire to understand nature. It is to the credit of the Medical Research Council in Britain to have fully appreciated the importance of basic research to advances in medicine. We are delighted to belong to the small, lucky group of those who are at the window-dressing end of the justification for the wisdom of that policy.

I learned what research was all about as a research student of Stoppani in Argentina, and then with Sanger in the Department of Biochemistry at Cambridge. I owe an enormous debt to the atmosphere of the Laboratory of Molecular Biology, where all the work I have described here was done, mostly under the Chairmanship of Max Perutz, and within the Division of Protein and Nucleic Acid Chemistry, of which Fred Sanger was the Head. From them, I always received an unspoken message which in my imagination I translated as "Do good experiments, and don't worry about the rest".

During my lecture I have tried to acknowledge those of my collaborators whose contributions were critical at specific stages of the work. In addition, so far unmentioned, is John Jarvis, my personal assistant for well over 20 years - only months less than my involvement with immunology. Since this prize mentions the discovery of the principles of the hybridoma technology, I would like to acknowledge specifically the importance of the contributions of Dick Cotton and David Secher, with whom preliminary work leading to that discovcry was made, and my tissue culture assistant, Shirley Howe, who was directly involved not only in the preliminary work but also in some of the specific experiments conducted with Georges Köhler. I would also like to acknowledge other collaborators who were concerned in our own contributions to the demonstration of the practical potential of the hybridoma technology in a variety of fields, particularly G. Galfré, A. F. Williams and A. C. Cuello, and the technical assistance of Mr. B. W. Wright. The list of acknowledgements is certainly much longer, but I wouldn't like to end it without recording my indebtedness to my secretaries, Margaret Dowding, and Judith Firth. Their handling of the press in the immediate period after the announcement of this prize ranks high among my memories of that exciting moment.

REFERENCES

1. Edelman, G. M. and Porter, R. R. (1972) Nobel lectures.
2. Milstein, C. (1964) *J. Mol. Biol. 9,* 836.
3. Milstein, *C.* (1966) *Biochem. J. 101,* 352-368.
4. Hilschmann, N. and Craig, L. C. (1965) *Proc. Natl. Acad. Sci. USA 53,* 1403-1409.
5. Frangione, B., Milstein, C. and Pink, J. R. L. (1969) *Nature 221,* 145-148.
   Svasti, J. and Milstein, C. (1970) Nature *228,* 932.
6. Dreyer, W. J. and Bennett, C. J. (1965) *Proc. Natl. Acad. Sci. USA 54,* 864-869.
7. Brenner, S. and Milstein, C. (1966) *Nature 211,* 242-243.
8. Smithies, O. (1967) Science *157,* 267.
9. Milstein, C. (1967) *Nature 216,* 330-332.
10. Milstein, C., Milstein, C. P. and Feinstein, A. (1969) *Nature 221,* 151-154.
11. Wu, T.T. and Kabat, E.A. (1970) *J. Exp. Med. 132,* 211-250.
12. Rcviewed in: Amzel. L. M. and Poljak, R. S. *(1979) Ann. Rev. Biochem. 48,* 961-997.
13. Milstein, C. and Pink, J. R. L. (1970) In: *"Progress in Bioph. and Molec. Biol."* Vol. *21,* pp. 209-263. Pergamon Press: Oxford and New York.
14. Potter, M. (1972) *Physiological Reviews 52,* 631-719.
15. Horibata, K. and Harris, A. W. (1970) *Exp. Cell Res. 60,* 61-70.
16. Stavnezer, J. and Huang, R.-C. C. (1971) *Nature New Biol. 230,* 172.
17. Milstein, C., Brownlee, G. G., Harrison, T. M. and Mathews, M. B. (1972) *Nature New Biol. 239,* 117-120.
18. Harrison, T. M., Brownlee, G. G. and Milstein, C. (1974) *Eur. J. Biochem. 47,* 613-620.
19. Brownlee, G. G., Cartwright, E. M., Cowan, N. J., J arvis, J. M. and Milstein, C. (1973) *Nature 244,* 236-240.
20. Milstcin, C., Brownlee, G. G., Cartwright, E. M., J arvis. J. M. and Proudfoot, N. J. (1974) *Nature 252,* 354-359.
21. Hamlyn, P. H., Gait, M. J. and Milstein, C. (1981) *Nucleic Acids Res. 9,* 4485-4494.
22. Cotton, R. G. H., Sechcr, D. S. and Milstein, C. (1973) *Eur. J. Immunol. 3,* 136-140.
23. Adetugbo, K., Milstein, C. and Secher, D. S. (1977) *Nature 265,* 299-304.
24. Secher, D. S., Milstein, C. and Adetugbo, K. (1977) Immunol. *Rev. 36,* 51-72.
25. Cotton, R. G. H. and Milstein, C. (1973) *Nature 244,* 42-43.
26. Rudikoff, S., Giusti, A. M., Cook, W. D. and Scharff. M. D. (1982) *Proc. Natl. Acad. Sci. USA 79,* 1979-1983.
27. Galfré, G., Howe, S. C.. Milstein, C., Butcher, G. W. and Howard, J. C. (1977) *Nature 266,* 550-552.
28. Pearson, T., Galfré, G., Ziegler, A. and Milstein, C. (1977) *Eur. J. Immunol. 7,* 684-690.
29. Williams, A. F., Galfré, G. and Milstein, C. (1977) *Cell 12.* 663-673.
30. Stern, P. L., Willison. K. R., Lennox, E. S., Galfré, G., Milstein, C., Secher, D. S. and Ziegler, A. (1978) *Cell 14,* 775-783.
31. Milstein, C., Galfré, G., Sechcr, D. *S.* and Springer, T. (1979) *Ciba Foundation Symp. No. 66,* "Human Biology: Possibilities and realities." Excerpta Medica: Amsterdam, pp. 251-266.
32. Cuello, A. C., Galfré, G. and Milstein, C. (1979) *Proc. Natl. Acad. Sci. USA 76,* 3532-3536.
33. Voak, D., Sacks, S., Alderson, T., Takei, F., Lennox, E. S., Jarvis, J. M., Milstein, C. and Darnborough, J. (1980) *Vox Sang. 39,* 134-140.
34. Sechcr, D. S. and Burke. D. C. (1980) *Nature 285,* 446-450.
35. Galfré, G., Milstein, C. and Wright, B. W. (1979) *Nature 277,* 131-133.
36. Milstein, C. and Cuello, A. C. (1983) *Nature 305,* 537-540.
37. Rabbitts, T. H. and Milstein, C. (1977) *In: "Contemporary Topics in Molecular Immunology"* Vol. *6,* Eds. R. R. Porter and G. Ada. Plenum Press: New York and London, pp. 117-143.
38. Tonegawa, S., Hozumi, N., Matthyssens, G. and Schuller, R. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41,* 877-889.
39. *Cold Spring Harbor Symp. Quant. Biol.* (1976) *41* (various papers).
40. Reviewed in: Tonegawa, S. (1983) *Nature 302,* 57l-581.
41. Edelman, G. M. and Gally, J. A. (1967) *Proc. Natl. Acad. Sci. USA 57,* 353.
42. Kabat, E. A., Wu, T. T. and Bilofsky, H. (1978) *Proc. Natl. Acad. Sci. USA 75,* 2429-2433.

43. Alt, F. W. and Baltimore, D. (1982) *Proc. Natl. Acad. Sci. USA 79,* 4118-4122.
44. Cohn, M. (1971) *Ann. N.Y. Acad. Sci. 190,* 529.
45. Jerne, N. K. (1971) Eur. J. Immunol. I, l-9.
46. Haurowitz, F. (1967) *Symp. Quant. Biol. 32,* 559-567.
47. Westhof, E., Altschuh, D., Moras, D., Bloomer, A.C., Mondragon, A., Klug, A. and Van Regenmortel, M. H. V. (1984) *Nature 311,* 123-127.
48. Tainer, J. A., Getsoff, E. D., Alexander, H., Houghton, R. A., Olson, A. J., Lerner, R. A. and Hendrickson, W. A. (1984) *Nature 312,* 127-134.
49. For example: Waldmann, H. and Milstein, C. (1982) In: "Clinical *Aspects of Immunology"* 4th ed., Vol. 1. Eds. P. J. Lachmann and D. K. Peters, Blackwells Scientific Publications, Oxford, pp. 476-503.
50. Kaartinen, M., Griffiths, G. M., Hamlyn, P. H., Markham, A. F., Karjalainen, K., Pelkonen, J. L. T., Makela, O. and Milstein, C. (1983) *J. Immunol. 130,* 937-945.
51, Griffiths, G. M., Berek, C., Kaartinen, M. and .Milstein, C. (1984) *Nature 322,* 271-275.
52. Preud'homme, J. L., Birshtein, B. K. and Scharff, M. D. (1975) *Proc. Natl. Acad. Sci. USA 72,* 1427-1430.
53. Milstein, C. Wellcome Foundation Lecture. (1980) *Proc. Roy. Soc. Land. B* (1981) *211,* 393-412.
54. Neuberger, M. S., Williams, G. T., Mitchell, E. R., Jouhal, S. S., Flanagan, J. G. and Rabbitts, T. H. (1985) *Nature 324,* 268.
55. Neuberger, M. S., Williams, G. T. and Fox, R. O. (1984) *Nature, 312,* 604.
56. Köhler, G. and Milstein, C. (1975) *Nature 256,* 495-497.
57. Szybalski, W., Szybalska, E. H. and Ragni, G. (1962) *Natl. Cancer Inst. Monogr. 7,* 75-89.
58. Galfré, G. and Milstein, C. (1981) In: *Methods in Enzymol.* Vol. *73.* Eds. S. P. Colowick, N. O. Kaplan and J. J. Langone, H. Van Vunakis, Academic Press: New York and London, pp. 3-46.
59. Kaartinen, M., Griffiths, G. M., Markham, A. F. and Milstein, C. (1983) *Nature 304,* 320-324.
60. Howard, J. C., Butcher, G. W., Galfré, G., Milstein, C. and Milstein, C. P. (1979) In: *Immunological* Reviews Vol. *47.* Ed. G. Möller, Munksgaard: Copenhagen, pp. 139-174.
61. Pearson, T., Galfré, G., Ziegler, A. and Milstein, *C.* (1977) *Eur. J. Immunol. 7,* 684-690.
62. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfré, G., Milstein, C., Williams, A. F. and Ziegler, A. (1978) *Cell 14,* 9-20.
63. Springer, T., Galfré, G., Secher, D.S. and Milstein, C. (1978) *Eur. J. Immunol. 8,* 539-551.
64. Springer, T., Galfré, G., Secher, D.S. and Milstein, C. (1979) *Eur. J. Immunol. 9,* 301-306.
65. Takei, F., Waldmann, H., Lennox, E.S. and Milstein, C. (1980) *Eur. J. Immunol. 10,* 503-509.
66. McMichael, A. J., Pilch, J. R., Galfré, G., Mason, D. Y., Fabre, J. W. and Milstein, C. (1979) *Eur. J. Immunol. 9,* 206-210.
67. Cuello, A. C., Priestley, J. V. and Milstein, C. (1982) *Proc. Natl. Acad. Sci. USA 79,* 665-669.
68. Voak, D., Lennox, E. S., Sacks, S., Milstein, C. and Darnborough, J. (1982) *Med. Lab. Sci. 39,* 109-122.