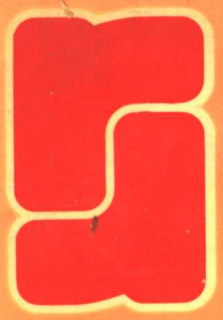

Receptors and Recognition

Series B Volume 16

Genetic Analysis of the Cell Surface

Edited by
P. Goodfellow

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Imperial Cancer Research Fund Laboratories,
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Preface

The cell surface is the barrier between the cell and its environment which regulates the flow of both simple and complex molecules into and out of the cell; it is also the organelle responsible for communication between the cell and its environment. Each cell expresses receptors for a wide variety of hormones, growth factors, growth substrates and other cells. In multicellular organisms communication between cells is required for controlling development, cellular differentiation, morphogenesis and, in a more general sense, integration of myriad cell types into a single organism. The series *Receptors and Recognition* has as its overall aim the dissection of the cell surface to correlate structure and function for this complex organelle. In most of the preceding volumes the approach has been biochemical or physiological. In this volume the mammalian cell surface is analysed by a genetic approach.

Genetic analysis of the cell surface, especially when combined with immunological techniques, has a long history. In 1900 Landsteiner showed that serum from one individual could agglutinate the red cells of another. Besides the practical result of making blood transfusion safe, this was the first demonstration of a human genetic polymorphism and for the next 50 years the red blood cell surface provided most of the genetic markers used to study human populations. The genetics of the surface of nucleated cells can be traced back to early experiments in cancer research and attempts to learn the rules associated with tumour transplantation. The transfer of tumour cells from one mouse to another could be used to define mice which were apparently resistant to tumour killing; this resistance was clearly very complicated and depended both on the tumour transferred and the recipient mouse. The problem was eventually solved by using two new genetic creations: the inbred mouse and the congenic mouse (see Chapter 1). Snell, in one of the most determined series of experiments in modern times (the experiments took several years and had to be started twice because of a catastrophic fire), produced mice which differed at a single region which controlled tumour transplantation: congenic mice which differed at this region rejected tumours exchanged between them; mice which were identical at this region accepted tumours. This was the first genetic demonstration of the mouse major histocompatibility region or complex (MHR or MHC) to which Snell gave the prosaic name *H-2* (histocompatibility locus-2). Following the prescient suggestions of Landsteiner that transplantation and blood transfusion reactions would have a similar basis, Gorer made antisera which could predict the outcome of tumour transplantation experiments. These antibodies recognized products of the *H-2* locus. Thus, the surface of tumour cells contains genetically regulated molecules which are

recognized as foreign by recipient mice. Medawar, studying skin transplantation in humans, demonstrated that normal tissues also expressed genetically controlled histocompatibility antigens and this was the stimulus which eventually led to the definition of the human MHC or *HLA* complex.

Subsequent advances in immunological and genetic techniques have been applied singularly and together for analysis of the cell surface. Particularly important were the introduction of somatic cell genetics (see Chapter 3), monoclonal antibodies (see Chapters 1 and 3) and the new techniques of DNA manipulation (see Chapter 4). Today genetic analysis of the cell surface is applied at many different levels from the structure of populations to the primary DNA sequence. In population studies departures from expected gene frequencies for cell surface markers can give indications of selection which in turn may give intimations of function. Similarly, at the level of the whole organism individuals lacking particular antigens may show increased or decreased abilities to cope with physiological stress or infection. At the cellular level interactions can be investigated by studying communication between cells with different cell surface phenotypes or by blocking interactions with specific antibodies. Also at the cellular level mutants can be selected *in vitro* which lack functional receptors and this can be correlated with changes in cellular behaviour. Gene mapping or genetic analysis at the chromosomal level is a *sine qua non* for all genetic analysis and can provide information about functional and evolutionary relationships between genes in the same and different systems. Finally, in the DNA sequence is the answer to many of the questions we would like to ask, if we but knew how to interpret the information.

The chapters in this volume are designed to illustrate the wide variety of methods available for the genetic analysis of the cell surface. For reasons of space, consideration has been largely limited to mice and men but similar studies have also proved fruitful in other mammals, simpler eukaryotes and prokaryotes.

Contents

1 Immunogenetic Approaches to Cell Surface Molecules in the Mouse

MELANIE J. PALMER and JEFFREY A. FRELINGER

EDITOR'S INTRODUCTION

The genetics of both mice and men have been extensively studied and in both cases cell surface molecules have proved to be amenable to genetic analysis. However, studying mice has one great advantage: mice are experimental animals and mice with specific desired genetic constitutions can be created to solve particular problems. Besides being able to mate mice with required characteristics it is also possible to reduce genetic complexity by using inbred mice and congenic strains of inbred mice.

The combination of immunological and genetic techniques has been termed immunogenetics. As first demonstrated by Gorer, Lyman and Snell for *H-2*, employing antibodies to define cell surface molecules and congenic mice to define the genetic loci involved is a very powerful approach. In recent years the immunogenetic approach has been used to fine map *H-2* and has been extended to many other systems. Particularly fruitful has been the analysis, instituted by Boyse, of the cells which interact to form the immune system. The definition of T lymphocytes, B lymphocytes, subsets of T and B lymphocytes and even lymphocyte precursors and different maturation stages have all been facilitated by immunogenetics. In Chapter 1 Melanie Palmer and Jeffrey Frelinger explain the basic techniques of murine immunogenetics and stress the use of the two relatively new methods of recombinant inbred strains of mice and monoclonal antibodies. The principles involved are illustrated by reference to two antigenic systems: β_2-microglobulin, which is found on all cells, and Ly-5, a differentiation antigen which is restricted to lymphocytes.

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1.1 INTRODUCTION

Our purpose in writing this chapter is to demonstrate the use of two distinct, immunological approaches to the study of cell surface molecules. These approaches are (1) the production and use of genetically standardized inbred mouse strains and (2) the use of antibodies produced either by immunization between genetically defined individuals or by myeloma–B cell fusions (monoclonal antibodies). The inbred mouse strains provide a constant pool of genes coding for cell surface molecules and the antibodies provide tools for direct access to the molecules themselves. These reagents can be employed together to provide structural as well as genetic information about cell surface molecules.

Congenic mouse strains (those differing at only a small number of genes on a given chromosome) simplify antisera production by genetically limiting the possible antigenic differences to those coded by a small section of a single chromosome. Alloantisera are produced by immunization of one strain with tissues of another strain. The use of congenic mouse strains in conjunction with analysis of alloantisera can define new antibodies which can then be used to identify novel cell surface components.

It is interesting that at the time of inception of congenic mouse strains, their utility was not generally appreciated. When George Snell began the production of H-2 congenics he was thought to have developed a tool of little use to any investigators outside of a rather esoteric branch of transplantation biology (Snell, 1958). In the 1970s it became increasingly apparent that such animals were of great utility for the understanding of the interaction of molecules on the cell surface that were involved in regulation of immune responses (Benacerraf and McDevitt, 1972). Although initially used to study major histocompatibility genes, this approach is now used to study other genes such as immunoglobulin and minor histocompatibility genes. The immunogenetic approach has gone from being one which was rather arcane to one which is now a straightforward, accepted approach to solving almost any problem in cell surface biochemistry. This approach has been especially useful for investigating the immune system.

In addition to congenic mice, another genetic approach, again not appreciated at the time of its inception, has been useful. This is the production of recombinant inbred strains of mice (RI strains) (Bailey, 1971). Although RI strains have not yet affected immunobiology as much as the congenics, they have begun to have an impact on the genetics of cell surface molecules.

It has been over thirty years since Gorer, Lyman and Snell were able to show that the gene for blood group 2 in the mouse was the gene responsible for tumour rejection between inbred strains of mice (Gorer *et al.*, 1948). This identity provided the first evidence that there were links between genes involved in cell surface phenomena such as graft rejection, and those detected by antibodies. Over the years, serological approaches have been used for the

study of many cell surface-mediated events. In addition, methods such as affinity chromatography and antibody inhibition studies can provide information about the biochemistry and function of cell surface molecules. Antibodies can also be used to interfere with physiological functions. For example, in experimental myasthenia gravis antibodies are produced which block the acetylcholine receptor, resulting in blockage of nerve–muscle signal transmission. In turn, antibodies can be produced which inhibit the antibodies blocking the acetylcholine receptor. These antibodies, since they can block the binding of the first antibody to the acetylcholine receptor, might be used as a therapeutic agent to treat patients with myasthenia gravis. Antibodies have also been used to determine the function of cells involved in the immune response. For instance, antibodies directed at Ia molecules have been used to block immune responses by interfering with antigen presentation on the surface of macrophages. This inhibits the recognition of antigen–Ia complexes by T cells.

The utility of both antibodies and congenic mice is not in their separate use but rather their use in conjunction to define cell surface molecules. Complex antisera can be analysed using congenic mouse strains which express different allelic forms of cell surface molecules. Through the absorption of antibody molecules from the complex antisera with a series of different congenic mouse cells, an antiserum that is specific for one antigen can be produced. Furthermore, by cross-immunizing with congenic mouse strains the complexity of the antibody specificities within the sera can be greatly reduced. By carefully choosing strains which share many known antigens, antibodies can be produced to the small number of antigenic differences between the donor and recipient. The extraordinary usefulness of congenic mice in the production of alloantisera lies in the ability to produce alloantisera specific for a set of allelic cell surface molecules.

We have chosen to discuss two murine examples in which congenic and serological analysis have been used to study cell surface molecules. These are β_2-microglobulin and Ly-5/T200.

The first example, β_2-microglobulin (β_2m), was first described in human urine as a free protein and only later described as a human and mouse cell surface component (Berggard and Bearn, 1968). Initially, the structural resemblance of β_2m to one of the constant domains of immunoglobulins led investigators to speculate that it represented a free immunoglobulin domain (Smithies and Poulik, 1972; Peterson *et al.*, 1972). When it was discovered that β_2m occupied an important role in the expression of major histocompatibility complex (MHC) determinants (Poulik *et al.*, 1974), there was much excitement over β_2m as an evolutionary link between the cellular and humoral arms of the immune system. However, genetics of β_2m and the cell surface functions have remained elusive. Only recently in mice has its genetic location been discovered through the use of congenic and RI strains in combination with antisera produced previously.

In 1975, two groups, searching for T cell differentiation antigens, inadvertently discovered the same molecule by different routes, and named it Ly-5 and T200 (Trowbridge *et al.*, 1975; Komuro *et al.*, 1975). It was the development of a T200-specific monoclonal that finally allowed investigators to demonstrate that Ly-5 and T200 represent the same molecule (Trowbridge, 1978). These molecules are members of a multigene family and members of the same gene family are expressed on most haematopoietic cells. The Ly-5/T200 studies demonstrate the utility of combining the use of congenic mouse strains with serological techniques in characterizing cell surface molecules.

1.2 BACKGROUND TO IMMUNOGENETICS AND THE MAJOR HISTOCOMPATIBILITY COMPLEX

One way of gaining further insight into immunological recognition events is to characterize molecules on the surface of lymphoid cells by biological and chemical means. Another method of dissecting these events at the molecular level is to study the genetics of the molecules involved in mediating immunological phenomena. Immunogenetics involves locating and studying the genes which code for molecules involved in immunological phenomena. One cluster of genes exhaustively studied by immunogenetics is the major histocompatibility complex (see Chapter 4). These gene clusters are designated *HLA* in man and *H-2* in the mouse. The *H-2* cluster consists of a series of linked genes located on mouse chromosome 17 (Fig. 1.1). It is composed of three major regions which are further divided into subregions. The *K* and *D* regions control cytotoxic cellular alloantigens involved in tissue transplantation. They are referred to as class I molecules. Class II genes code for products which are chemically and functionally distinct from class I gene products. The class II

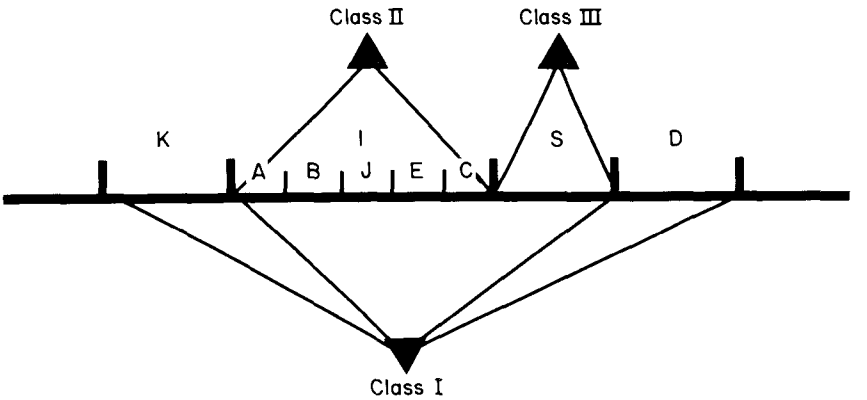

Fig. 1.1 The major histocompatibility complex of mouse.

gene products map to the *I* region. They are designated Ia antigens, and are involved in the control of immune responses. Class III genes located in the *S* region contain the structural gene for C4, a complement component. Each of the regions of the MHC is multiallelic. Polymorphism, or the genetic differences between alleles, provides the basis for such phenomena as graft rejection and mixed lymphocyte responses.

1.3 DEFINITION OF CONGENICS

Congenic mice provide a powerful tool for studying the location and function of both minor and major histocompatibility genes. Two mice which are identical at all but a single locus are said to be co-isogenic. Co-isogenicity is extremely hard to achieve experimentally. It occurs only by a point mutation arising in an inbred strain. However, mice which differ at a small portion of chromosome can be produced by extensive backcrossing. Such pairs of strains are termed congenic. An example of a congenic strain pair would be two strains which differ only at their MHC loci and share otherwise identical (background) genes. The combination of all the alleles at loci of interest on a single chromosome is referred to as the haplotype. The haplotype is denoted with a letter superscript. For instance, strains B10 and B10.A have identical background genes of the B10 strain. Strain B10.A has the haplotype $H\text{-}2^a$ of the A strain MHC, while B10 has the haplotype $H\text{-}2^b$. Congenic mice are important when studying a given gene because the elimination of unlinked genetic factors can substantially decrease experimental error. For example, when cells of B10.A are mixed in tissue culture with irradiated cells of B10 (called stimulators), they will be stimulated to divide and mature into killer cells by foreign antigens (alloantigens) expressed on the surface of B10 cells. Because both strains are identical except at the MHC, the genes coding for the foreign antigens recognized by B10.A must map within the MHC. By using strains which share some MHC determinants by descent, it is possible to assign functions to smaller

Table 1.1 B10 congenic mouse strains*

	K	Aα	Aβ	Eβ	J	Eα	S	D
B10	b	b	b	b	b	b	b	b
B10.A	k	k	k	k	k	k	d	d
B10.K	k	k	k	k	k	k	k	k
B10.D2	d	d	d	d	d	d	d	d
B10.P	p	p	p	p	p	p	p	p

* Each of the above congenic strains was derived from the background strain C57BL/10 (B10).

genetic regions. Strain B10.A shares the *K* end of *H-2* with B.10K and the *D* end with B10.D2. Using B10.A cells in combination with B10.K or B10.D2 cells allows the assignment of genes expressed on the stimulator cells to be mapped to specific regions within the MHC (Table 1.1).

1.3.1 Production of congenic lines

Mice which are bred randomly without regard to their genetic relationships will be heterozygous at many loci. Inbreeding or mating of mice genetically related to one another (usually brother–sister matings) restricts genetic heterogeneity because as inbreeding continues segregating alleles become fewer. The series of genetic crosses leading to the production of a congenic mouse strain always begins with two strains. One strain, referred to as the background strain, provides the genetic background and must be inbred to provide a uniform genetic constitution. The other strain (donor strain) donates the differential locus or loci and need not be inbred.

While there are several methods for producing congenic mice differing at the

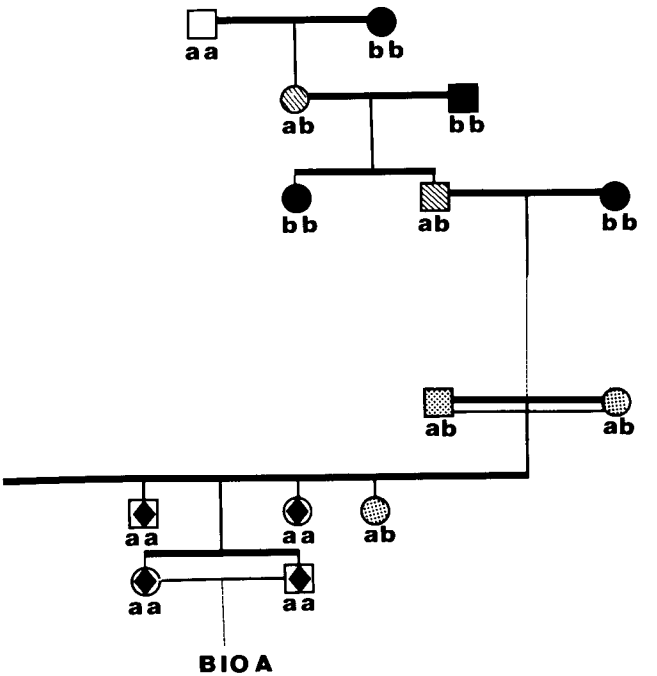

Fig. 1.2 The NX backcross system – explanation in text; aa = strain A; bb = strain B10 (C57BL/10).

H-2 locus (Snell, 1948; Green and Doolittle, 1963; Green, 1966; Snell and Bunker, 1965), the simplest system is the NX backcross system (Fig. 1.2). This system requires that the selected trait is detectable in heterozygotes. Hence this system cannot be used to produce congenics of recessive traits. Fortunately, most genes encoding cell surface molecules are expressed co-dominantly, and are easily detectable in heterozygotes. The production of the congenic line begins with crossing the two strains and 'backcrossing' the hybrid mouse to the parental background strain. The N_1 generation (identical with F_1) is heterozygous, having inherited half of its genetic material from parent A and half from parent B. The N_1 generation is backcrossed to the background parent. The resulting segregating N_2 generation is tested for the desired trait. N_2 animals possessing the desired donor trait (in this case H-2) are again backcrossed to the background parental strain and the N_3 progeny are tested for retention of the donor trait. This process is repeated for a minimum of 12 backcross generations at which time the heterozygous mice are intercrossed and mice which are homozygous for the donor trait are selected and maintained by brother–sister matings as inbred strains. The 12 backcross generations reduce the possibility of an unlinked (and undesired) gene being fixed in the congenic strain to less than 0.01%. To borrow an analogy from J. Klein, producing a congenic strain is like producing the perfect martini. If you start with a martini one part gin to one part vermouth (with an olive) it is not dry enough. Pour out half (save the olive!) and add more gin. If it is still not dry enough repeat 10 more times and the perfect martini results. It is 99.97% gin and 0.03% vermouth and still contains the olive. The olive represents the selected gene, the vermouth represents the donor strains genetic background and the gin represents the congenic partner. The rationale is to replace the donor strain genes of the N_1 heterozygote with background genes while selecting to retain one specific genetic trait derived from the donor strain. An example of congenic lines is illustrated in Table 1.1. All strains listed have identical B10 backgrounds but have been constructed to have different *H-2* genes.

Another genetic approach to studying the location of the genes coding for cell surface components is the use of recombinant inbred (RI) strains (Bailey, 1971). RI strains are a group of inbred strains derived from a cross between two different inbred strains. The F_1 mice from the first cross are paired at random and in subsequent brother–sister matings there is a unique pattern of fixation of alleles in each strain. Each genetic locus will have a particular pattern of fixation among the strains of the RI panel. Each locus in which the two parental strains are distinguishable will have a characteristic distribution pattern of alleles in the RI strains. This pattern is called a Strain Distribution Pattern (SDP). RI strains are useful in large panels where the distribution of a particular allele among the panel of strains provides a way to study segregation and interaction among genes.

1.4 SEROLOGICAL ANALYSIS

A heterogeneous population that consists of genetically unrelated individuals will share some antigens and not others. Immunization between two individuals leads to a polyclonal antibody response in which many different antibodies are elicited. The antiserum is composed of antibodies each specific for a particular antigen. The serum can be tested against a panel (random sample) of individuals to examine the distribution of those antigens recognized by that serum. Because the antiserum is a heterogeneous mixture of antibodies, sometimes it is first necessary to reduce the complexity by adsorption or by adsorption and elution. Adsorption consists of removing unwanted antibodies by reacting the antiserum with a cell. If the cell expresses antigens reactive with some of the antibodies in the serum, these antibodies will bind to the cells. The cells together with the bound antibody can then be removed. This leaves only the unreactive antibodies left in the serum. Alternatively, if an antibody of a particular specificity is wanted, it can be adsorbed on to the antigen. Antigen–antibody complexes can be separated from the non-reactive antibodies. The complex can be dissociated and the positively selected antibody eluted.

One of the best ways to reduce the heterogeneity of the antisera is to use inbred and/or congenic mice. Animals of one inbred strain are immunized with cells from a congenic strain. The resulting antiserum is tested against cells from all available mouse strains. If the antiserum reacts with cells from many strains, adsorption is performed. For example, if B10 mice are immunized with B10.A cells, a serum reactive with B10.A, B10.K and B10.D2 cells is produced (Table 1.2). If this serum is adsorbed with B10.K cells, reactivity remains for B10.D2 and B10.A cells. If the serum were adsorbed with B10.D2 cells instead of B10.K, reactivity would remain for B10.A and B10.K cells. As expected, adsorption with B10.A cells completely removes all activity not only to B10.A cells but also to B10.K and B10.D2 cells. B10 cell adsorption has no effect (Table 1.3) as only antigens present on B10.A were used to elicit the immune response. If the B10 anti-(B10.A) serum was first adsorbed with B10.K

Table 1.2 Primary adsorption analysis of a typical alloantiserum: adsorption of B10 anti-(B10.A) serum

	Serum adsorbed with				
Test cell	None	B10.A	B10.K	B10.D2	B10
B10.A	+	0	+	+	+
B10.K	+	0	0	+	+
B10.D2	+	0	+	0	+
B10	0	0	0	0	0

Table 1.3 Secondary adsorption analysis of a typical alloantiserum: adsorption of B10 anti-(B10.A [B10.K]) serum

	Serum adsorbed with				
Test cell	None	B10.A	B10.K	B10.D2	B10
B10.A	+	0	+	0	+
B10.K	0	0	0	0	0
B10.D2	+	0	+	0	+
B10	0	0	0	0	0

(designated B10 and B10.A [B10.K]) and then adsorbed with B10.D2 before testing on B10.A cells, further insight into the relationship between B10.A, B10.K and B10.D2 could be obtained. Here we see that adsorption with B10.D2 not only removed activity for itself but also for B10.A. This serum {B10 anti-(B10.A [B10.K])} is now operationally monospecific, since we cannot define any more specificities, given the strains available. We know from this experiment that antigens present on B10.K cells are also present on B10.A but not B10.D2, while antigens present on B10.D2 are present on B10.A but not B10.K cells. Further, all specificities found on B10.A are present either on B10.K or B10.D2. We can now define two specificities, and four phenotypes. B10.A has specificities 1 and 2. B10.K expresses only specificity 1, and B10.D2 specificity 2. B10 is a null phenotype with respect to these specificities.

This method of adsorption analysis defines antigens in different strains. Classical genetic segregation analysis allows the determination of the relative genetic locations of the genes encoding these antigens. Unfortunately, genetic analysis tells us nothing about the biochemical or physical properties of a given antigen or its relationship to other antigens. Obviously, a detailed serological analysis depends upon the knowledge of the mouse strains used. Therefore, antigens or differences between strains can only be defined within the limits of the number of inbred strains available for study.

Another potential source for heterogeneity in a serological reaction lies in the antibodies and antigens themselves. For instance, although antibodies possess unique antigen-combining sites, they often cross-react with antigens which either share identical antigenic determinants or determinants which are stereochemically similar. A given antigen may possess more than one antigenic determinant per molecule and thus provoke the response of several distinct antibody families. Therefore, although each single antibody is derived from an individual B cell clone and is specific for one antigenic determinant, it is very difficult to produce a truly monospecific antiserum by a classical immunization protocol. This problem can be circumvented by a technique developed by

Kohler and Milstein (1975, 1976) which results in the production of monoclonal antibodies.

1.5 HYBRIDOMAS AND MONOCLONAL ANTIBODIES

Antibodies arise from clonal populations of B cells which produce antibodies each with unique antigen-combining sites. Each B cell carries a membrane-bound antibody on its cell surface which, when triggered by antigen, causes the B cell to undergo a process of clonal expansion in which the B cell is stimulated to divide extensively. Most of the B cells then mature and secrete antibodies. These mature antibody-secreting cells are called plasma cells. The remaining B cells are reserved in a state of readiness to respond to antigen if it is encountered again and are called memory cells. Although each antibody arises from a clonal population of B cells, most responses are polyclonal. In most immunizations the immunogen is a mixture of different antigens each with multiple determinants. Kohler and Milstein (1976) were able to fuse a B cell with a myeloma cell to establish a cell line (hybridoma) which produces a monoclonal antibody which is specific for a single antigenic determinant.

Normally activated B cells which mature and secrete antibody do not divide. Therefore they cannot be propagated indefinitely in tissue culture. However, myeloma cells which are plasma cell tumours have infinite lifespans and are easily propagated in culture. The two cell types are fused by an agent such as polyethylene glycol or Sendai virus. After fusion both B cell myeloma hybrids and the unfused myeloma cells can potentially survive and propagate in culture. However, since the myeloma cells are selected to be defective in the purine salvage pathway, the addition of hypoxanthine, aminopterin and thymidine (HAT) to the growth medium kills them (see HAT selection, Chapter 3). The resultant hybridoma line retains the antigen specificity of the B cell and the immortality of the myeloma cell. The hybrids can be propagated in tissue culture or passaged in a syngeneic (genetically identical) mouse as an ascites tumour. Ascites tumours grow as single cell suspensions in the peritoneal cavity. Peritoneal ascites fluid containing the monoclonal antibodies secreted by the tumour can be harvested from the peritoneal cavity.

The hybridomas are screened by collecting culture fluid supernatants or ascites which contain monoclonal antibodies and testing them for binding activity to the antigen. Although a monoclonal antibody does not define physical or biochemical properties of an antigen or its relationship to other antigens better than conventional antisera, it does arise from a single B cell and thus has a unique antigen combining site to one antigenic determinant. Thus, it will bind to only a single determinant on an antigen. For instance, when testing several monoclonal antibodies which arise from an immunization with a pure protein antigen, one may find that adsorption to the antigen with one