Monoclonal Antibodies

Cells that secrete antibodies can be made immortal by fusing them with tumor cells and cloning the hybrids. Each clone is a long-term source of substantial quantities of a single highly specific antibody

by Cesar Milstein

When a foreign substance enters the body of a vertebrate animal or is injected into it, one aspect of the immune response is the secretion by plasma cells of antibodies: immunoglobulin molecules with combining sites that recognize the shape of particular determinants on the surface of the foreign substance, or antigen, and bind to them. The combination of antibody with antigen sets in train processes that can neutralize and eliminate the foreign substance. Quite apart from the natural function of antibodies in the immune response they have long been an important tool for investigators, who capitalize on their specificity to identify or label particular molecules or cells and to separate them from a mixture.

The antibody response to a typical antigen is highly heterogeneous. There are perhaps a million different lines of B lymphocytes, the precursors of plasma cells, in the spleen of a mouse or a man. All are derived from a common stem cell, but each line develops an independent capacity to make an antibody that recognizes a different antigenic determinant. When an animal is injected with an immunizing agent, it responds by making diverse antibodies directed against different antigen molecules on the injected substance and different determinants on a single antigen, and even different antibodies that fit, more or less well, a single determinant. It is next to impossible to separate the various antibodies, and so conventional antisera contain mixtures of antibodies, and the mixtures vary from animal to animal.

Each antibody is made, however, by a different line of lymphocytes and their derived plasma cells. What if one could clutch out one such cell making a single specific antibody and grow it in culture? The single cell’s progeny, or clone, would be a source of large amounts of identical antibody against a single antigenic determinant: a monoclonal antibody. Unfortunately antibody-secreting cells cannot be maintained in a culture medium.

There are malignant tumors of the immune system called myelomas, however, whose rapidly proliferating cells produce large amounts of abnormal immunoglobulins called myeloma proteins. A tumor is itself an immortal clone of cells descended from a single progenitor, and so myeloma cells can be cultured indefinitely, and all the immunoglobulins they secrete are identical in chemical structure. They are in effect monoclonal antibodies, but there is no way to know what antigen they are directed against, nor can one induce myelomas that produce antibody to a specific antigen.

In 1975 my colleagues and I learned how to fuse mouse myeloma cells with lymphocytes from the spleen of mice immunized with a particular antigen. The resulting hybrid-myeloma, or “hybridoma,” cells express both the lymphocyte’s property of specific-antibody production and the immortal character of the myeloma cells. Such hybrid cells can be manipulated by the techniques applicable to animal cells in permanent culture. Individual hybrid cells can be cloned, and each clone produces large amounts of identical antibody to a single antigenic determinant. The individual clones can be maintained indefinitely, and at any time samples can be grown in culture or injected into animals for the large-scale production of monospecific antibody. Highly specific monoclonal antibodies produced by this general method have proved to be a remarkably versatile tool in many areas of biological research and clinical medicine.

Human myelomas have been known to physicians for a long time, but it was not until the early 1960’s that the precise nature of myeloma proteins was elucidated by immunologists. Michael Potter of the National Cancer Institute then induced myelomas in mice, and these too produced large amounts of monoclonal immunoglobulins. In spite of much effort, however, it was not possible to induce tumors that could synthesize antibodies to an injected antigen. Leo Sachs, Kenko Horihata, Edwin S. Lennox and Melvin Cohn did succeed in establishing a line of mouse myeloma cells in tissue culture at the Salk Institute for Biological Studies, but the line was then lost. Eventually Horihata and A. W. Harris were able to establish a number of lines, which they distributed to other laboratories. My group at the Medical Research Council Laboratory of Molecular Biology in Cambridge subjected a line derived from one of Potter’s tumors to intensive study.

At that time we were not thinking about monoclonal antibodies. We were studying how somatic (body) cells diversify in culture and how mutations modify the combining specificity of antibodies, and the mouse myeloma line was for us simply another appropriate tissue-culture line. By 1973 Richard G. Cotton, David S. Secher and I were able for the first time to produce structural mutants of a mouse myeloma protein secreted by a cultured cell line. That work and parallel investigations by Matthew D. Scharff of the Albert Einstein College of Medicine in New York demonstrated spontaneous mutations in cultured cells that affected the structure of the proteins they manufactured, and also told something about the molecular nature of the mutations and their frequency. The search for mutants was laborious, however, because the proteins made by the parental cells lacked recognizable antibody activity, changes in which would be the most effective indication of slight differences caused by mutations. Clearly what was needed was a cell line that secreted an immunoglobulin exhibiting antibody activity that could be easily assayed. No such line existed.

At that point a lucky circumstance led us to the hybrid-myeloma technique. While we were working on somatic mutations Georges Köhler and I were also following a quite different line of research in an attempt to learn more about the genetic control of the synthesis of antibodies. The synthesis of antibodies is controlled by two sets of genes. One set encodes the “variable” region of the antibody molecule’s light and heavy chains, the region that controls antibody specificity; the other set encodes the
"constant" region of the chains, the region that is responsible for such effector functions as the binding of complement (a complex of blood-plasma proteins implicated in the immune response), the transport of the antibody molecule across membranes and the binding of the molecule to membranes. Each lymphocyte synthesizes an antibody encoded by a single pair of V (for variable) and C (for constant) genes out of a large repertory of such genes in the cell, and when there are different alleles, or variant forms, of a V or a C gene on each of the cell's two chromosomes, only the allele on one of the chromosomes is active; the other is excluded.

In 1973 Cotton and I did an experiment to find out if allelic exclusion could be broken and, if it could, what the molecular consequences would be. We fused two myeloma cells, one from a mouse line and one from a rat line. Analysis of the hybrid cells showed that they secreted hybrid molecules consisting of various combinations of the chains synthesized by the parental cells but never a combination of a V region from one animal and a C region from the other. That meant the genes for the V and the C regions must be on the same chromosome. It is now known that the DNA sequences coding for the V and the C regions are separated by introns, or intervening sequences of DNA. The entire stretch of DNA is transcribed

ANTIBODY-SECRETING CLONES of hybrid-myeloma cells were first detected by a test for antibodies to sheep red blood cells. In the standard test (top left) the red cells and antibody-secreting cells are incubated on agar, and complement (a protein complex from blood plasma) is added. Antibody diffusing from each secreting cell binds to antigens on nearby blood cells, initiating a complement reaction that kills blood cells, forming a plaque: a clear area (white spots) around each secreting cell. The author and Georges Köhler fused T.K. L. and K. H. stage we added cells with tumor (myeloma) cells and plated the hybrids (top right). Hybrid-cell colonies developed (black spots). When a layer of sheep red cells was added along with complement, a few hybrid colonies gave rise to plaques (white areas around colonies), indicating that they were secreting specific antibody. Individual cells were picked from a colony of antibody-secreting cells and plated thin (bottom left); most of the clones derived from them proved to be secretors of the anti-red-cell antibody. A photomicrograph of a single secreting clone (bottom right) shows the individual cells of the clone and the area of dead cells around it.
The immune system responds: a line of B lymphocytes proliferates, each secreting an immunoglobulin molecule that fits a single antigenic determinant (or a part of it). A conventional antiserum contains a mixture of these antibodies. Monoclonal antibodies are derived by fusing lymphocytes from the spleen with malignant myeloma cells (b). Individual hybrid cells are cloned, and each of the clones secretes a monoclonal antibody that specifically fits a single antigenic determinant on the antibody molecule.

Antibodies, shown in the illustration at the top of the page as stylized shapes, belong to the family of proteins called immunoglobulins. The basic shape of an immunoglobulin molecule is that of a molecule of the class immunoglobulin G (IgG), a heterogeneous population of molecules sharing a Y-shaped structure composed of two kinds of molecular chains, heavy and light, linked by disulfide bonds. The number and precise position of the disulfide bonds differ and are characteristic of the IgG subclass. Each chain has two regions. In the variable region amino acid sequences that differ from antibody to antibody provide differently shaped combining sites that bind specifically to different antigens. Constant region of the chains, with the same amino acid sequence in all antibodies of a given subclass, is responsible for other functions.
spectacularly successful fusion that produced a series of monoclonal antibodies to rat histocompatibility antigens: the cell-surface markers that establish individual identity and are responsible for the rejection of grafts. Other results began to come in at a rapid pace as we established a standard protocol for the experiments and developed new methods for assaying antibody secretion.

The success of these experiments was enhanced by an unexpected feature. Of the spleen cells we were fusing only perhaps one in 100 was an actively antibody-secreting plasma cell, and yet about one in 10 of our hybrid clones turned out to secrete antibody. That is, we had 10 times as many positive, immortal hybrids as one would expect if immortality were randomly transferred to the heterogeneous spleen-cell population; apparently we were achieving selectivity along with immortality. The explanation for this selectivity is not completely established, but according to recent evidence it probably has two components. On the one hand, secretion seems to be amplified, with lymphocytes that synthesize antibody but do not normally secrete it giving rise to hybrids that both synthesize and secrete antibody. Probably the myeloma parent provides the secreting machinery some antibody producers lack. On the other hand, the conditions under which the fusion takes place apparently make it unlikely that spleen cells other than B lymphocytes will give rise to long-lived hybrids.

When a clone of fused cells has been established, by definition all of the antibody it secretes is genetically derived from a single cell. It is not yet necessarily a monoclonal antibody in the immunological sense of the word, however, because each cell of the hybrid clone has some chromosomes from the myeloma-cell parent and some from the spleen-cell parent and is expressing both sets of chromosomes. Potentially a hybrid cell, instead of producing only the two components of a true monoclonal antibody (one kind of heavy chain and one kind of light chain), can produce two heavy chains and two light ones. We refer to such a cell as HL.3GK because it secretes the heavy and the light chains of the spleen-cell parent and the corresponding gamma and kappa chains of the myeloma-cell parent. It is in the nature of hybrid cells, particularly in the early stages of proliferation, to lose chromosomes rapidly. In this case the detectable loss is not random; heavy chains (H or G) are usually lost first, and then one or the other of the light chains (L or K) is lost. The HL.3GK hybrid therefore gives rise to variants whose secretion pattern is HLK or GLK, and these in turn to such variants as HL, HK, LK, and K.

It is the clone of HL cells, expressing only the heavy chain and the light chain of the specifically immune spleen cell, that one is looking for (although there are reasons for also preserving other variants, notably HK). As one clones it is therefore necessary not only to assay for the specific antibody but also to analyze the immunoglobulin for its type of chain and select a strongly secreting HL (or HK) clone. The selection process can be simplified by choosing a mutant myeloma line that expresses only the light chain (K) and therefore yields HLK hybrids or one that does not express any immunoglobulin and therefore yields an immunologically monoclonal HL hybrid at the outset.

Once the desired clone is selected it can be frozen for long-term storage. At any time a sample of the clone can be

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FIRST SUCCESSFUL FUSION of myeloma cells was between a mouse line that secreted complete IgG molecules and excess light chains and a rat line that secreted only light chains. Mutants of each line, respectively deficient in the enzymes TK and PFR, were cultured together in a selective medium, HAT, in which both enzymes are required for cell survival; only fused cells survived, forming hybrid clones. The clones secreted into the supernatant various hybrid molecules consisting of mouse heavy chains and rat light chains (left) but did not produce scrambled molecules combining variable regions from one animal with constant regions from the other animal (right). These results indicated that the variable and the constant regions are transcribed from DNA into RNA, and the RNA is processed and translated into protein, as is indicated (b), and also that the genetic information of both parental cells is "codominantly" expressed by the hybrids. More recent studies on DNA show that the V and C genes themselves are interrupted by intervening sequences that are excised in the course of processing.
ed the antibody in large amounts. The antibody was attached to carbohydrate beads to prepare an immunoadsorbent column. Passing a totally crude interferon preparation through the column purified it 5,000-fold in a single step. Purification on an industrial scale is now being explored.

Monoclonal antibodies can be prepared that are specific for individual components of any complex mixture, and unlimited amounts of each antibody can be produced for immunoadsorbent columns. This makes it possible to dissect a mixture of completely unknown substances into its components. Animals are immunized with the mixture to be analyzed; hybrid-myloma clones are derived and the antibodies from each clone serve to remove the components from the mixture one by one in cascade fashion.

One of the most exciting investigative applications of monoclonal antibodies is in the area of membrane biology. Membrane proteins are hard to purify. They are present in cells in small amounts; often they have no easily measured biological activity, or else their activity is destroyed when the membranes are solubilized for analysis. One way to overcome these problems is to characterize cell-surface molecules by immunological methods, an approach that has been fruitful in the recognition of surface antigens that characterize particular cell types at different stages of tissue differentiation. Conventional antibodies to surface antigens are usually complex, however, and do not recognize single molecules; elaborate procedures have been required to circumvent this complexity.

In 1977 Gullé and I, along with Alan F. Williams of the University of Oxford, showed how the hybrid-myloma technique could identify individual differentiation antigens. We immunized a mouse with cell membranes from rat thymus. After immortalizing the mouse cells that secreted antibody to thymus lymphocytes we were able to isolate clones producing different specific antibodies. In that one attempt we defined three new antigen specificities, a task that might have required years of sophisticated immunology by conventional methods. Since then a number of other monoclonal antibodies to differentiation antigens of mouse, rat and human cells have been prepared.

The antigenic structure of a cell's surface establishes the cell's lineage and defines subsets of cells. For example, B lymphocytes can be distinguished from T lymphocytes (which take part in cellular immune reactions rather than in the secretion of antibody) largely because the former have immunoglobulin molecules on their surface and the latter have characteristic markers such as the Thy-1 antigen. Most surface markers, how-

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STANDARD PROCEDURE for deriving monoclonal antibodies begins with the fusion, mediated by polystyrene glycol, of spleen cells from an immunized mouse (or rat) with mouse (or rat) myeloma cells. Hybrids are selected in HAT. The medium is assayed for antibody secretion, and a portion of each positive culture is frozen as a precaution. Positive cultures are cloned and the clones are assayed. The positive ones are then frozen, recloned and assayed for the presence of immunoglobulin variants (see illustration on the next page). The clones finally selected can be stored frozen. When the samples are thawed, they can be either grown in culture to produce the antibody or injected into animals to induce myelomas that secrete the antibody.
HYBRID-MYELOMA CELL produced by the usual fusion process (a) synthesizes only the heavy (H) and light (L) chains of its spleen cell parent along with the gamma (G) heavy and the kappa (K) light chains of its myeloma parent: it is thus designated HLGK. Hybrids tend to lose chromosomes, so that as the clone grows some chains are lost as is indicated here. Cultures are assayed to select desired variants (unshaded cells) until a stable H variant secreting only specific antibody (or for some purposes an HK variant) is isolated. Fusion with mutant myeloma cells that make only a K chain (b) or no immunoglobulin at all (c) makes it easier to derive the desired HL clone.

However, are not specific for a single subset of cells. Even the B cell's characteristic immunoglobulin may be present in several functionally different members of the B cell lineage, from the so-called memory cells (which respond to reimmunization with an antigen the organism was previously been exposed) to plasma cells (which secrete antibody). What characterizes a particular differentiated state is the presence of a particular ensemble of surface antigens and their qualitative expression.

To establish a unique antigenic profile for each of many cell types will require a vast collection of monoclonal antibodies and will take a long time. A good start has been made with the few reagents already available and with the help of cytofluorometers and fluorescence-activated cell sorters: instruments that can quickly measure both the size and the fluorescent intensity of large numbers of cells to which monoclonal antibodies, tagged with a fluorescent dye, have been attached. A large cell population can thus be fractionated into subpopulations on the basis of their size and surface antigen pattern, and then the function of each subpopulation can be studied. Monoclonal antibodies, in other words, are standard reagents that can identify new surface molecules and at the same time distinguish among cell populations. So far the best results have been reported with various blood-forming and lymphoid cells, one directly practical application has been the differential diagnosis of various leukemias and related disorders.

The pattern of reactivity of monoclonal antibodies against subpopulations of cells is sometimes consistent with a given cell line's pattern of maturation, but not always. One monoclonal antibody seems to recognize an antigen characteristic of certain bone marrow cells in the rat, whereas in the peripheral lymphoid organs it recognizes lymphocytes and in nervous tissue it recognizes some component that is as yet unidentified. Among the peripheral lymphocytes the antigen is present on T cells but not on B cells; yet it reappears on plasma cells, which are derived from B cells. We say this monoclonal antibody recognizes a kind of “jumping” antigenic determinant.

The monoclonal approach to characterizing differentiation antigens thus makes it possible to probe for the particular stage at which an antigen is expressed as well as for the line of cells that expresses it. The cascade purification method I described above can be applied not only to characterize the antigenic complexity of the cell surface but also to dissect functional as well as structural components of other biological materials such as cell organelles and pharmacologically active cell extracts.

The “monospecificity” of antibodies from hybrid-myeoma clones has thrown new light on some well-known phenomena of antigen-antibody reactions. One indication of the binding of antigen to conventional antibodies in a test tube, for example, is the formation of a precipitate. The effect is not generally observed when the antibody is monoclonal. This is perhaps the first formal proof of the theory, advanced more than 40 years ago, that the precipitate is a three-dimensional lattice of antigens and antibodies. A monoclonal antibody binds only to a single antigenic determinant on an antibody molecule, so that no such lattice can be formed by a monoclonal antibody and most antibodies. It can be formed only if the antigen is a polymer composed of repeated identical structural elements.

Monospecificity has also revealed some hitherto unsuspected phenomena that call for new interpretations of antigen-antibody reactions. To take just one example, it appears that the binding of different antibodies to neighboring sites on the same antigen is an important factor in the rupture of a cell membrane by complement. This synergistic effect was discovered as we were isolating the rat antibodies to histocompatibility antigens. We assayed for the presence of antibody-secreting hybrid myelomas by measuring the cytotoxic, or cell-killing, activity of their culture mediums. The supernatants of the uncloned cultures were consistently cytotoxic, but once we had cloned individual cells their supernatants showed no such activity. It occurred to Howard to measure the activity of a mixture of the supernatants of these apparently negative clones. To our delight the mixture was active, and then it was easy to purify two complementary components.

Once the synergistic effect was understood the “silent” activity of the isolated components could be exploited in a special way. Test cells could be “sensitized” by exposure to one monoclonal antibody and then exposed to the antibodies from other clones, thus releasing entire repertoires of antibodies that act synergistically. Clearly there are cases where mixtures of monoclonal antibodies will be essential to produce a desired effect. In each case a decision will have to be made whether the advantages of blending monoclonals in specific proportion...
Monoclonal antibodies are slowly beginning to replace conventional antisera in standard kits for such procedures as the radioimmunoassay; many commercial companies are marketing them. Because they can be produced in large quantities they will make possible widespread use of kits of diagnostic reagents that until now were either not available at all or were considered too highly specialized for general application; one example is an antibody to the neurotransmitter called Substance P, derived recently by A. Claudio Cuello and us. The impact of monoclonals in virology, parasitology and bacteriology is only beginning to be felt. Great hopes are placed on their application to organ transplantation, just one aspect of which should be the worldwide standardization of tissue typing. In basic research the possibilities are even wider, with applications already reported in embryology and pharmacology and in the study of receptors for hormones and neurotransmitters.

Possible roles for monoclonal antibodies in direct therapy are under serious investigation. The most obvious role is in passive immunization (the injection of an antibody into a patient, as opposed to active immunization with an antigen that stimulates the patient's own antibody response). Given the impurity of conventional antibodies, passive immunization is not a common method of treatment, but it may prove to be effective when a purified antibody can be administered. In tumor therapy two kinds of role are foreseen for monoclonal.

**CRUDE INTERFERON** was purified by immunoabsorption. Spleen cells of mice immunized with a somewhat enriched preparation of interferon were fused with myeloma cells. A hybrid-myeloma clone selected for anti-interferon activity and immunoglobulin secretion was injected to induce myelomas; purified antibody from the serum of tumor-bearing mice was attached to carbohydrate beads to prepare immunoabsorbent columns. When crude interferon was passed through such a column, it bound to the antibody and was retained when other components of the crude mixture were washed out; then the interferon was eluted. One passage through a column increased the preparation's interferon activity about 5,000-fold. Electrophoresis of the proteins (radioactively labeled to increase their visibility) made the results visible (bottom). The partially enriched preparation showed a single band (A), apparently albumin; overexposure of the same gel (B) showed minor bands, one of them (arrow) at a position corresponding to the molecular weight of interferon. After the passage of crude interferon through a column (C) there was less contaminant and a strong band at the interferon position (arrow). A second passage (D) produced material with a single strong interferon band.
al antibodies. One role is the targeting of toxic drugs: antibodies to the tissues of a particular organ or to specific tumor antigens could be attached to drug molecules to concentrate the drug's effect. Alternatively it may be possible to produce antitumor antibodies that will themselves find and attack tumor cells.

For therapeutic applications antibodies derived from human lymphocytes rather than from the mouse or the rat would be desirable. Contrary to early hopes, this has proved to be difficult; attempts to immortalize antibody-producing human cells by fusing them with mouse or rat myeloma cells have so far been disappointing. The problem is that when human cells are fused with animal cells, there is a rapid preferential loss of human chromosomes from the resulting interspecific hybrid cells. And so far the search for a suitable human myeloma line that can be cultured and fused to make an interspecific hybrid has not borne fruit.

In this overview of the uses of hybrid myeloma antibodies I have referred only superficially to their obvious applications in basic immunological research. I have preferred to emphasize the fact that, although the technique originated in our effort to understand the genetic organization and expression of immunoglobulins, there has already been an impressive "spin-off" into many other areas. It is always hard to define

The depleted mixture, now enriched in the remaining antigens, is injected to produce more antibody, and so on in cascade fashion. Hybrid myeloma cells therefore provide a tool for characterizing the components and at the same time for separating and purifying them.

DIFFERENTIATION ANTIGENS are cell-surface antigenic determinants that are either specific for individual cell types or common to sets or subsets of cell types. Here hypothetical antigens are shown for four white blood cells. The best definition of cell lineage and subsets is the pattern of expression of such markers. Monoclonal antibodies are an ideal tool for establishing such patterns of expression.