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Course Manual

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HYBRIDOMA TECHNIQUE AND MONOCLONAL ANTIBODIES FOR MARICULTURE APPLICATIONS

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Introduction

Ever since the beginnings of experimental immunology at the end of the nineteenth century, scientists have exploited the specificity of antibodies to detect, isolate and analyze biological material. The power of antibodies as probes for biological structure underwent a quantum increase in 1975, when Kohler and Milstein developed the technique for production of Hybridomas. Hybridomas are immortal somatic cell hybrids that secrete Monoclonal antibodies (MAbs) of predefined specificity. These antibody-secreting cell lines can be established routinely and maintained *in vitro.* By combining the nuclei of normal antibody-forming cells with those of their malignant counterparts, Kohler and Milstein developed a powerful way of analyzing and purifying individual molecules within the enormously complex mixtures in biological material.

In order to understand the revolutionary impact of monoclonal antibodies, it is necessary to understand the problems and limitations of conventional serology. While the specificity of antibodies provided a way of overcoming the enormous complexity of biological material, the production of highly specific antisera was difficult and unreliable. It required highly purified antigen. However, using hybridoma technology it is now possible to produce unlimited quantities of exquisitely specific antibodies against virtually any molecule, regardless of the purity of the immunizing antigen. The fine specificity, degree of cross-reaction, affinity and physical properties of antibodies may be selected to suit individual needs.

Conventionally, polyclonal antibodies are prepared in rabbits for use in diagnostics, serotyping and vaccine development. Rabbit antiserum contains many different types of antibodies derived from several plasma cell clones that are specific to different epitopes. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques such as background reaction and false positives. The availability of antiserum in limited quantities is also a serious drawback. Therefore the preparation of large quantity of homogenous antibodies with a defined specificity was a long-standing goal of immunochemical research. This was achieved with the development of hybridoma technology for production of MAbs.

Production of Hybridoma

In animals, antibodies are synthesized primarily by plasma cells, which are terminally differentiated B Lymphocytes. Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibodies. For decades, immunologists have sought ways of producing homogenous antibodies of defined specificity. Hybridoma technology allows the growth of clonal populations of cells secreting antibodies with a defined specificity. Here, antibody-secreting cells isolated from an immunized animal is fused with myeloma cell, a type of B cell tumor from BALB/c mice. These hybrid cells or hybridoma can be cloned and maintained *in vitro* to secrete antibodies with a defined specificity, which are known as monoclonal antibodies.

The usefulness of monoclonal antibodies stems from three characteristics – specificity of binding, homogeneity and ability to be produced in unlimited quantities. Another unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single cell cloned prior to use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens. However, it would be wrong to think that monoclonal antibodies will completely replace conventional serology. The production of monoclonal antibodies involves a great deal of work, and a high level of commitment. There will often be occasions when the effort required may not be justified. They are not the best choice for certain immunochemical techniques. In theory, either as single antibody preparations or as pools, monoclonal antibodies can be used for all of the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job.

In a hybridoma, the myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody secreting cells provide the functional immunoglobulin genes. Hybridomas can be prepared by fusing myelomas and antibody secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore fusions are normally done with cells from the same species. All commonly used mouse strains can serve as successful fusion partners with BALB/c myelomas, however immunizations are normally done in BALB/c mice.

Polyethylene glycol (PEG) is the most commonly used agent to fuse cells in hybridoma production. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets or chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy or

light chain gene is lost, production of antibody will stop. In a culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer and will result in unstable lines. If the chromosome that is lost contains a gene used in drug selection, then the growth of the hybridoma will be unstable, and cells will continue to die during selection.

Drug selection for elimination of unfused myeloma cells

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in culture. The cells from the immunized animal (antibody secreting cell) do not continue to grow in tissue culture and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed, which can be achieved by drug selection. Commonly, the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis. For eg. Selection with 8- azaguanine often yields a cell line harbouring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compound that blocks the *de novo* nucleotide synthesis pathway will force cells to use the salvage pathway. Cells containing a non-functional HPRT protein will die in these conditions. Hybrids between myelomas with a nonfunctional HPRT and cells with a functional HPRT will be able to grow. Selections are commonly done with aminopterin, methotrexate or azaserine.

Stages of Hybridoma Production

It is convenient to divide the production of monoclonal antibodies into three stages: (1) immunizing mice, (2) developing the screening procedure, and (3) producing hybridomas.

Animals are injected with an antigen preparation, and once a good humoral response has appeared in the immunized animal, an appropriate screening procedure is developed. The sera from test bleeds are used to develop and validate the screening procedure. After an appropriate screen has been established, the actual production of the hybridomas can begin. Several days prior to the fusion, animals are boosted with a sample of the antigen. For the fusion antibody secreting cells are prepared from the immunized animal, mixed with myeloma cells and fused. After the fusion, cells are diluted in selective medium and plated in multiwell tissue culture dishes. Hybridomas are ready to test beginning about 1 week after the fusion. Cells from positive well are grown and then single-cell cloned. Single-cell cloning ensures that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.

Characterization of Clones

The total characterization of a MAb is a long and complex procedure, which varies widely with the intended use of the antibody. If a single hybridoma has been produced and is intended for a specific function it is unlikely that the antibody produced will have all the required characteristics. To produce MAb superior to conventional sera for most highly specialized functions it is better to make a large panel of cloned hybridomas and further select from these by characterization of the antibodies they produce.

Antibody produced by a clone belongs to one single class, and hence antibody class indicates number of clones in a population. Antibody class is most readily determined by the use of class specific antibodies in an ELISA or by Ouchtherlony assay. Determination of antibody class does not constitute final proof that an antibody is monoclonal. For some of the more sophisticated uses of MAbs this is essential. While it is likely that there is only one cell type if the antibody produced can be shown to be only of the IgM class, it remains possible that there are two or more IgM-secreting hybridomas in the culture. An isoelectric focusing gel is the only final method of proof.

Storage of Hybridoma

Hybridoma and myeloma cell lines can be stored at -80°C or liquid nitrogen by slowly freezing cells in an appropriate solution of nutrients and a cryoprotectant such as dimethylsulphoxide (DMSO). Freezing of cells, which have not been cloned after the primary fusion is not always successful, presumably because of overgrowth by non-producing cells. However, the parent myeloma and established hybridomas can be stored with little difficulty. The cells are centrifuged and resuspended at 5-10 X 10^6 cells/ml in medium containing FCS and DMSO. Aliquots of this suspension are then pipetted into plastic storage ampoules and frozen slowly at -70°C for at least 12 h and then stored at -80°C or transferred to liquid nitrogen

Production of MAbs

MAb production *in vitro*

Mabs can be produced *in vitro* and *in vivo*. For production *in vitro*, hybridomas are best expanded slowly by transfer to 24 well tissue culture plates followed by 25 cm^2 flask and a 75 cm^2 flask containing suitable medium such as Dulbecco's Modification of Eagles Medium, DMEM or Rosewell Park Memorial Institute (RPMI) medium, contàining sera (fetal calf serum, FCS), antibiotics and other required chemicals. The cell density is maintained between 10^5 and 10^6 cells/ml. Typical culture supernatants yield up to 100µg/ml of antibody, the exact amount depending upon the cell density and rate of growth. Culture *in vitro* provides a more pure preparation of antibody. The only contaminants are from the FCS. Contamination may be further reduced by the use of serum-free medium.

MAb production *in vivo*

For producing MAbs in *vivo,* mice are primed by intraperitoneal injection of 0.5 ml pristine (tetramethyl pentadecane) 5 – 10 days before intraperetoneal inoculation with 10^5 - 10^7 hybridoma cells. It is important to use mice, which are histocompatable with the parent cells. The rate of growth of the resulting ascites tumour is in general very variable and can be from less than two or more than five weeks. The ascites fluid can be collected from an anaesthetized mouse. It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping. Ascites cells recover from freezing exceptionally well and can be frozen down in the same way as tissue culture cells and reintroduced into animals without difficulty. Ascites fluid will be contaminated with mouse imunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.

Purification and storage of MAbs

In many cases, purification of antibody is not necessary since all that is required is specificity and each batch of antibody can be tested for titre and used directly. However, if a MAb is required as a standard reagent or for therapeutic purposes it must obviously be purified. In addition, many of the methods used to characterize a MAb involve labelling it with either enzymes or isotopes and a pure antibody is obviously more suitable for this purpose. Before purification it is important to determine the class of antibody. As with all protein-purification techniques, antibodies may be separated according to charge or size. In addition, they may be purified by affinity chromatography utilizing protein A for certain types and species, by anti-immunoglobulin columns, or by antigen affinity columns if large amounts of antigen are available.

Most MAbs are stable and can be frozen and thawed readily. It is, however, advisable to check the titre after freezing and thawing at an early stage. Many IgM antibodies do not retain full antigen-binding capacity after freezing and thawing but many do. In general, an antibody, which does not freeze well, is better discarded unless it is of considerable importance in which case it should be stored in 0.1% sodium azide at 4°C. MAbs like serum should not be repeatedly frozen and thawed and it is best to freeze 4 or 5 small aliquots from each tissue culture flask or ascites fluid, from the bulk for general use or for testing before purification on large scale.

Applications of Monoclonal Antibodies in Mariculture

Hybridoma technology for production of monoclonal antibodies has contributed significantly to aquaculture. Monoclonal antibodies are being employed in disease diagnosis, pathogen classification, epidemiological analysis and development of vaccines.

Outbreak of disease problems of the cultured fish/shellfish is a major bottleneck faced by aquaculturists. Generally we go for routine diagnostic procedures like microbiological examination and histopathology, which are time consuming. Development

of rapid immunodiagnostic techniques based on polyclonal antisera is faced by problems of cross-reactions and inconsistency in results. If MAb based diagnostic kits (which are more specific) are available for important microbial pathogens, it will help us in adopting more scientific health management measures. MAbs can be used to confirm the presence of pathogens fixed in tissue sections or tissue imprints by immunohistochemistry, particularly useful for detecting low-level infections, which would be overlooked by traditional methods. MAb based immunodiagnostic kits such as ELISA (Enzyme Linked Immunosrbant Assay) and Immunodot can be simplified to the field level for use by farmers. Monoclonal antibodies to several viral and bacterial pathogens of fish and shellfish have been developed. It has been possible to develop rapid, simple, cheap, specific and sensitive MAb based immunodiagnostic kits for several microbial pathogens. In India, College of Fisheries, Mangalore has developed MAbs to *Aeromonas hydrophila*, EUS fungus *Aphanomyces invadens* and white spot virus of shrimp.

Furthermore, detection of minute serological difference among bacterial and viral variants of fish and shellfish is possible by MAb based epitope analysis. This has helped immensely in serological and epidemiological studies. Monoclonal antibodies have also been used in detection of epitopes involved in pathogenesis for development of subunit vaccines.

A serious bottleneck in developing standardized diagnostic assays for most important fish diseases is the lack of antifish immunoglobulin reagents. Hence there is also immense scope for developing Anti-fish immunoglobulin monoclonals using hybridoma technology.