CELL BIOLOGY OVERVIEW

More than 15 years experience in cell biology and cell culture enables our staff assist your needs for your projects. We can recommend strategies and provide contract services for cell-based assays, tissue culture, cell culture optimization, and bioassays.

CELL-BASED ASSAY, BIOASSAY DEVELOPMENT

Cell-based assay or bioassay development can range from cytotoxic assays including apoptosis to cell proliferation and metabolic assays. Cell-based assay development can also include high throughput screening assays and other custom bioassays. Mechanisms of action, such as receptor binding, receptor activation, cell signaling, drug internalization and subcellular localization can be delineated in cell-based assays following treatment with your pharmaceuticals. Bioassay development can encompass testing of conditioned medium, cell lysates or whole cells in a variety of formats including ELISA, Luminex and immunohistochemical methods such as flow cytometry.

CUSTOM CELL CULTURE

Custom cell culture can be performed for your cells, whether it is primary explants, cloning and optimizing expression of a cell line or developing the optimum medium, to include serum-free medium. Large scale cell cultures using bioreactors can be implemented for production of your specific protein. Additionally purification of your protein can be performed from either the cells themselves (eg. baculovirus) or from the conditioned media.

Cellular Assays

High Throughput Screening Assays (HTS)

High throughput screening assays is often used for screening to identify candidate drug leads. A frozen stock of the cell line is generated at the onset of any high throughput screening assay development to maintain reproducibility of the desired bioactivity. Initial design of the assay will be performed with a 96 well plate and the read out could be fluorescence, luminescence, colorimetric or radioactivity depending upon the variable to be measured. This enables microscopic visualization of the cells as the assay is being developed. Morphologic information on the status of the culture and individual cells can be useful in assay development and often cannot be obtained from biochemical readouts. We can develop and transfer the high throughput screening assay or perform them for you.

Cell Proliferation, Cell Death, Necrosis & Apoptosis

For most studies, cell growth is measured by a homogeneous, vital dye method in which one of several choices of dye is added to cells in a 96 well plate at the conclusion of the study, incubated for increasing hours, and read directly in a plate reader. The dye is enzymatically changed in healthy cells so that development of color or fluorescence is measured using a different wavelength than the unaltered dye. Addition of a growth factor, an inhibitor or a cytotoxic factor to cells is easily read. This procedure has very few steps, has minimal manipulation of cells, and allows good reproducibility.

Death occurs by lysis, necrosis, or apoptosis. Lysis is the destruction of the cell surface membrane such as by the action of an antibody and complement that makes holes in the membrane. Necrosis occurs through the action of toxic factors that act within the cell, such as irreversible inhibitors of protein, RNA or DNA synthesis, or mitotic poisons. Apoptosis is a programmed cell death used by the body to remove damaged or unwanted cells, and occurs during cytotoxic T cell killing and with some cancer chemotherapies. Apoptosis is characterized by early events such as expression of phosphotidylserine on the cell surface and fragmentation of the DNA, followed by loss of membrane integrity and mitochondrial function.

Cell death is assessed microscopically by uptake of trypan blue dye that is excluded by live cells. The percentage of dying cells is determined microscopically or by flow cytometry using vital stains or DNA-binding dyes. High throughput measurement of cell death is performed by a fluorescent or color marker.

Flow Cytometry

Flow cytometry allows the study of individual cells in a population with the detection stage requiring less than a minute. Specific cell components are stained by fluorescent antibodies or other reagents. Cells can be made more permeable to large proteins without changing overall cell shape. Simultaneously, cell viability, cell size, and internal structures (e.g. distinguishing lymphocytes from granulocytes with many vesicles) can be measured. After cells are stained, and fixed with glutaraldehyde if desired, the cell suspension is distributed into droplets containing one cell or no cell. Additionally, a mixture of cells can be analyzed by cell size.

Phase and Fluorescence Microscopy

Light microscopy shows the general state of cells, and combined with trypan blue exclusion, the percent of viable cells. Phase microscopy views cells in indirect light; the reflected light shows more detail, particularly intracellular structures. Fluorescence microscopy detects individual components in cells, after labeling with selective dyes or specific antibodies, and can distinguish cell surface from intracellular labeling. Microscopic observation of cell cultures is an integral tool for tissue culture, as it reveals the culture health during the maintenance, expansion and experimentation phases of the study.

Cell Signaling

Cell signaling for a number of activities is measured by a variety of techniques, such as calcium flux, change in intracellular pH, metabolic assays, proliferation, and gene expression.

Immunocytochemistry

The presence and the cellular localization of macromolecules can be determined by immunocytochemistry, in which cells are fixed on a microscope slide, and a molecule is stained by a specific labeling reagent and detected by fluorescence microscopy.

Reporter Gene Assays

Cells can be transfected with reporter genes that are activated when certain pathways are triggered. Pathway induction is quantitated by the reporter gene, such as the appearance of fluorescence or of an enzyme activity. These surrogate methods may be much more sensitive and rapid than detection of the primary gene response.

Tissue Culture

Cell Lines, Primary Cultures

Cell lines maintain growth and specialized properties during prolonged or indefinite culture in the laboratory. Primary cell isolates are derived fresh from tissues and will grow and maintain specialized properties for a limited time, about 10 passages. We have experience with explants of liver, breast, ovary, lung, skin, spleen, lymph node and brain. Both cell lines and primary cultures can be stored frozen in liquid nitrogen and then put back into culture. These methods facilitate biological studies that are convenient, reproducible, and cost effective. Cell lines allow studies that may be difficult with whole organs or *in vivo*, such as mechanism of action, radioactive experiments, or system manipulation. Cell lines with desired properties are obtained from repositories such as the American Type Culture Collection, or derived by Murigenics through selection techniques and/or DNA transfection.

Cell Culture, Medium Optimization, Serum-Free Adaptation

Each cell line must be matched to a particular growth medium. Small scale growth and maintenance in culture (1 mL to 100 mL) is carried out in tissue culture grade plastics, while scale-up utilizes roller bottles, porous bead supports or a hollow fiber bioreactor, or stir cells. Nonadherent cells are also economically grown up to 40 L in stir cell suspension culture. Some adherent cell types can be adapted to nonadherent growth resulting in a more simple production method. Adaptation to serum-free conditions allows convenient purification of a secreted protein product.

Cell Cloning

Cloning is used to obtain a stable culture of homogeneous cells. Over periods of many months in tissue culture, cells can change properties due to somatic gene mutation, and overgrowth of mutated cells. It may be desired to select a rare cell type or the few stably transfected cells in a transfection pool. Cloning is achieved by diluting a culture so that ½ the wells in a 96 well plate contain one cell and ½ contain no cells. At this low cell concentration, conditioned medium (medium from the same cell type harvested at high concentrations) is added to enhance growth. When the clone reaches suitable numbers, aliquots are frozen in order to retrieve cells with the same properties at a future date.

DNA transfection

Genes can be introduced into cells by suitable molecular biology methods such as electroporation, cationic lipid reagents, or calcium phosphate. A gene can be transfected into cells simply with its regulatory elements or after making a construct to achieve high overexpression levels. Cells can also be transfected with conditionally expressed genes of interest. Transfections in mammalian cells can be transient or permanent. Transient expression lasts only a few days. Permanent expression requires cotransfection with a dominant selectable marker and several rounds of selection for the cell populations that stably integrate the transfected DNA into a cellular gene. This takes approximately three to four weeks.

Drug Action

Functional Bioassays

Drug action can be followed in a bioassay by measuring the drug binding to a cell surface receptor or other initial protein interaction. Bioassays may be developed to detect the drug being transported into intracellular compartments or may measure intracellular signaling events such as receptor phosphorylation, calcium flux, or gene activation (Reporter Assays, PCR). Bioassays could measure metabolic effects and other biological events on cell growth or death. Additionally, bioassays could detect induction of new protein synthesis and protein secretion and cell component rearrangements.

Drug And DNA Delivery Drug Metabolism

Drugs may be presented to biological systems by themselves, as prodrugs that have to be metabolized to a form more readily taken up or in liposomes that facilitate transport across lipid membranes. Drugs may also be formulated in degradable polymers or other slow-release systems, or attached to carriers to facilitate transport, to decrease clearance or to maintain circulating concentrations. Drug capture by a cell surface receptor is measured by localization (subcellular fractionation), or by its action of triggering a biological reaction (cell signaling, reporter gene assays, proliferation, cell death, metabolic assays). The concentration of drug in its initial formulation, and of free drug, internalized drug, and subsequently metabolized or degraded drug can be measured.

IMMUNOLOGY

IMMUNOLOGY OVERVIEW

Our scientists are experienced in producing monoclonal antibodies as well as developing and running ELISA assays and other immunological assays for research and development. Our expertise in cell biology enables us to develop, validate and perform cell-based assays from primary tissue, such as bone marrow, spleen, liver, blood, as well as with tissue culture cell lines.

CELL-BASED IMMUNOASSAY

Many of our clients develop pharmaceuticals that require cell-based immunoassays to measure an immune response. We can test sera not only for IgG or other antibody isotype responses but can determined whether cell activation is involved, by using cell-based immunoassays such as the mixed

lymphocyte reaction (MLR) or natural killer cell activation. We can determine for toxicity studies whether cytotoxic T lymphocytes have been sensitized to your drug or whether the immune system cells have been activated by the detection of released cytokines or the presence of a specific cellular receptor

MONOCLONAL ANTIBODIES

Often cell-based immunoassays require the pairing of two antibodies, one for capture and one for detection (see ELISA assay below). Since polyclonal antibody affinities change over time as the T cells mature, an assay employing monoclonal antibodies may result in a more reproducible assay. Additionally, affinity purification using a monoclonal antibody will also result in reproducible yields. Our scientists will consult with you about producing hybridomas that generate monoclonals with the desired characteristics. We will conjugate your hapten, or if your antigen is a protein, we will suggest an immunization protocol. We will develop your screening assay or implement the assay you've developed and screen your initial cell cultures after fusion as well as your clones. We will deliver to you conditioned media for further screens or after consultation with you, purify the antibody and deliver the purified monoclonal antibodies as well as the hybridomas. If you need further characterization of the monoclonal antibodies, our scientists will discuss with you your needs and perform the needed analyses.

ELISA ASSAY

ELISA assay development and validation is frequently needed for drug purification, stability or lot release. We can develop your ELISA assay using polyclonal or monoclonal antibodies supplied by you or use the antibodies we develop here at MuriGenics. Our scientists will consult with you about your final use for the ELISA assay and discuss various formats that can be designed into you assay development. Should you have an ELISA assay already developed and wish to have our scientists use it to test drug metabolism, drug stability or lot release we will employ your protocols.

Cellular Assays

Isolation and Characterization of Cell Populations

Cells of the immune system are commonly purified from blood, spleen or lymph nodes. Separate cell populations (lymphocytes, granulocytes and monocyte / macrophages, erythrocytes, and cancer cells) are usually prepared by density gradient centrifugation through Ficoll-Hypaque or Percoll solutions. Separation is based on the buoyant density of each cell subpopulation at the given osmolality of the solution. Monocytes and neutrophils are also purified by selective adherence.

If known subpopulations are to be isolated, for example CD4+ or CD8+ T cells, fluorescence activated cell sorting (FACS) will be employed or magnetic beads coated with specific anti-CD4 or anti-CD8 monoclonal antibody are used. The beads are mixed with peripheral blood leukocytes and only CD4+ or CD8+ cells will bind to the beads, which are then separated out from the non-specific cells with a magnet. Another method depends on killing the undesired populations with specific antibodies and complement. In some cases, a noncytotoxic antibody or other inhibitor can block the activity of a cell subtype.

Characterization of cell types and subpopulations can be performed using markers such as specific enzymes, cell surface proteins detected by antibody binding, cell size or morphological identification.

Cell Activation

Purified or unseparated lymphocytes can be activated for proliferation and DNA synthesis is measured by ^{3}H-thymidine incorporation. Other measures of activation such as cytokine production, expression of activation antigens, or increase in cell size are utilized. Activation is accomplished by incubating cells with nonspecific activators such as Concanavalin A, phytohemagglutinin (PHA), phorbol myristic acetate (PMA), an ionophore, an antibody to T cell receptors, or stimulation with specific antigen to which the cells are sensitized.

Mixed Lymphocyte Reaction (MLR)

A common method to assess the cellular immune response is to co-culture lymphocyte populations from two individuals, (e.g. two humans or two mice of different strains) where the T cells recognize the foreign cells from the other strain, activate and proliferate. One population of cells can be restricted to being stimulator cells (e.g. a preparation of tumor cells), by pretreating them with a mitotic poison (mytomycin B), thereby turning the assay into "one-way" MLR.

Cytotoxic T Lymphocyte (CTL) Assay

A key activity of cellular immunity in rejection of transplants, reactions to pathogens such as viruses and tumors, is the development of T lymphocytes that specifically kill target cells. These activated cells develop during *in vivo* exposure or by in vitro sensitization. The CTL assay consists of increasing number of sensitized lymphocytes cultured with a fixed number of tumor or other target cells that have been prelabeled with ^{51}Cr. To prelabel the target cells, the cells are incubated with the radiolabel. The ^{51}Cr is taken up and reversibly binds to cytosolic proteins. When these target cells are incubated with sensitized lymphocytes, the target cells are killed and the ^{51}Cr is released.

Natural Killer (NK) Activity

Natural killer (NK) cells are an essential defense in the early stage of the immune response to pathogens. NK cells are active in naïve individuals and their numbers can be enhanced in certain circumstances. The NK assay typically uses a ^{51}Cr-labeled tumor target and is similar to the CTL assay described above.

Cytokine, Activation Antigens and Receptor Analysis

Specifically activated lymphocytes synthesize and secrete a number of distinctive cytokines. These are quantitated by various ELISA methods. Alternatively, induced cytokines are detected by fluorescence activated flow cytometry (FACS) using fluorescent antibodies that enter permeabilized cells. Activated cells also express new cell surface antigens where the number of cells is quantitated by immunofluorescent microscopy, flow cytometry, or ELISA. Unique cell surface receptors that distinguish cell populations are detected by similar immunochemical methods or by the binding of their specific labeled ligand.

Immunoassays

Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA assays are very sensitive, precise and quantitative. Some assays such as fluorescence and time-resolve fluoresence are as sensitive as radiometric assays. They can be used to measure an antigen or an antibody, or generally, any macromolecule that binds another molecule or cell. A typical format is to coat a protein on the bottom of a plastic 96 well plate, block remaining potential protein binding sites by incubation with bovine albumin, casein, or other blocking agents, add the test sample which can be in a crude mixture such as serum, wash out any material that does not bind to the first protein, and detect the bound molecule by an enzyme-conjugated specific antibody. Detection is amplified many-fold using an enzyme-linked detection system, because the substrate is continuously turned over and the product is measured. Murigenics uses colorimetric, fluorescent, time-resolved, and homogenous (requiring no washing step) ELISA formats.

Luminex

Luminex color-codes tiny beads, called microsphere are separated into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, xMAP technology allows multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely. Pro and anti-inflammatory cytokines, Th1/Th2 cytokines and chemokines as well as markers of cardiovascular disease are just a few examples of the analytes available.

High Throughput Screening Assays (HTS)

To screen a library of thousands of compounds, a high throughput screening assay will facilitate the number of "hits" in less time than a typical assay. Antibody based immunoassays are often used as the first screen before using cell based high throughput screening assays. A high throughput assay typically is not complicated, must be very reproducible and have a high signal-to-noise ratio in order to minimize false positives. The follow-up research on the "hits" is time intensive, thus the number of "hits" should enable identification of only active compounds. Additionally, stabilizing the assay components so that production of the completed assay for use in the screens is an objective when developing the initial configuration of the assay.

Western Blot and Dot Blot

The Western blot identifies specific protein antigens and their approximate size. Proteins are separated by polyacrylamide gel electrophoresis (PAGE) after denaturation with sodium dodecyl sulfate (SDS) which makes proteins more linear and migrate in inverse proportion to their molecular weight. A unique protein band is detected by "blotting" or electrophoretically transferring the protein onto nylon or nitrocellulose support. Detection of the protein band results when the support is incubated with a specific antibody that is conjugated with a radiolabel, enzyme or other method.

The dot blot is a simpler method involving binding a small amount (10 microliters) of a protein mixture onto a solid support and detecting it as described above. Both methods can be quantitative by use of concurrently run standards.

Monoclonal Antibody Production

Hybridoma Production of Monoclonal Antibodies

Immunization of the appropriate specie with antigen emulsified with adjuvant occurs on days 1, 14, and 28. Sera are tested for antibody titer in an ELISA assay (or with the investigator's screening method) and if titers are high, hyperimmunization is performed and spleen cells are isolated and fused to myeloma cells with polyethylene glycol (PEG). Hybridoma cells are selected with HAT medium that kills non-fused myeloma and spleen cells.

Hybridoma Antibody Screening

Hybridoma cells are subcultured in microwells and supernatants are screened for IgG secretion in the investigator's antigen specific system. Cells from positive wells are cloned. A solid phase ELISA assay or FACS screening can be used to identify positive wells.

Clone Selection

The cells from the best five cell cultures producing antibody, will be cloned by limited dilution in order to isolate a monoclonal line. The clones will be tested and the best clones selected for expansion. Hybridomas continue to "throw-out" genes as they are kept in culture (immunoglobulin genes are lost early since they are not needed for survival) and may need to be recloned to find the best producer after 4-6 months in continuous culture.

Antibody Characterization

Immunoglobulin Class

The IgM, IgG subsets or other classes of antibody are determined by immunoassay methods. The kappa or lambda light chain class is similarly determined.

Binding Affinity Determination

The strength of antibody binding to its ligand is assessed by, ELISA, or binding in a column support format. Dissociation is performed by increasing denaturating conditions, or by competition with a related ligand. The dissociation constant, K_d, is determined by a Scatchard plot.

IN VITRO TOXICOLOGY OVERVIEW

Murigenics performs a variety of *in vitro* toxicity tests and assays. Performing *in vitro* cytotoxicity assays prior to animal testing may be a cheaper alternative for initial drug screens. Since many of the

assays require cell-based assays, our experience in cell biology, enables us to analyze cells morphologically as well as using molecular and biochemical techniques.

APOPTOSIS

You may want to know whether your drug candidates demonstrate cytotoxicity through apoptosis or necrosis. We will measure different parameters for apoptosis compared to necrosis. Apoptosis is characterized by early events such as expression of phosphotidylserine on the cell surface and fragmentation of the DNA, followed by loss of membrane integrity and mitochondrial function. We will discuss with you parameters such as drug concentration, time of exposure, measurement of DNA fragmentation in order to customize your in vitro cytotoxicity assay. Necrosis occurs through the action of toxic factors that act within the cell, such as irreversible inhibitors of protein, RNA or DNA synthesis, or mitotic poisons. We would measure protein or nucleic acid synthesis rates to determine your drug toxicity.

CELL GROWTH

Another measure of cytotoxicity is the inhibition of cell growth or proliferation. No one mechanism may be responsible for this observation, however, proliferation rates can be measured as a response to drug concentration. Some drugs may have a threshold level rather than a linear response for inhibition of cell growth. We will discuss parameters such as optimal target cell line, drug concentration, drug exposure time and drug delivery to customize your in vitro cytotoxicity assay.

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