IMMUNOLOGY

Immunological techniques and their applications

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Keywords

Antibody response; precipitation assay; double immunodiffusion; immunoelectrophoresis; immunoassays; ELISA; immonofluorescence; flow cytometry; monoclonal antibodies; polyclonal antibodies; hybridomas; cytokines.

Introduction

The immune system comprises of cells that have the ability to recognize, respond to and bring about inactivation of microbial infections, neutralization of pathogen-derived toxins and provide protection against diseased states such as cancers. These cells are located in primary and secondary lymphoid organs which include bone marrow, thymus, spleen and lymph nodes and are also found in circulation in blood. There are two components of the immune system, the humoral and the cellular. The former mediates its function through antibodies, soluble molecules that are produced by bone marrow derived cells called B lymphocytes. On the other hand the cellular component is largely defined by cells which are dependent on thymus for their development and maturation. These cells are called T lymphocytes (T for thymus derived). Even though these two arms of the immune system perform independent functions, their functioning is dependent on mutual collaboration commonly referred to as T-B collaboration.

B cells can produce antibodies against virtually any molecule. This ability to generate a large diversity of antibodies is achieved through a process of recombination that involves shuffling of immunoglobulin genes. B cells recognize an antigen in its native state through specific B cell receptors. Each B cell receptor has a recognition module made of immunoglobulin heavy and light chains which are non-covalently associated with molecules that enable it to transduce signals inside the cell. The help provided by T cells enables B cells proliferate and differentiate into antibody-secreting cells called plasma cells. During this process antibodies also undergo somatic hypermutation which increases their affinities for their cognate antigens. The signals provided by T cells also enable B lymphocytes to produce antibodies with different functional capabilities by a process called isotype switching.

Unlike B cells, T cells recognize an antigen only after one of its fragments is presented in association with molecules of the major histocompatibility complex (MHC). This job of antigen presentation is carried out by antigen presenting cells which include B cells or specialized immune cells that can take up microorganisms. The two main cell types in the latter category are macrophages and dendritic cells; these cells internalize microorganisms by phagocytosis. Like B cells, T cells also express specific receptors for their cognate ligands, the peptide-MHC complexes, and respond by secreting soluble mediators called cytokines. The latter can help an infected macrophage to inactivate a pathogenic microbe or help B cells to secrete antibodies. T cells can also directly kill infected cells such as those infected with a virus; therefore these cells have been classified into helper T cells and cytotoxic T cells (or killer T cells). Helper T cells recognize antigenic fragments in association with MHC class II while cytotoxic T cells recognize peptide-MHC class I complexes.

Upon encountering a pathogenic microorganism, the immune system first senses the presence of a pathogen. The pathogen is phagocytosed by macrophages or dendritic cells and its molecules are broken down into smaller fragments. This process of fragmentation called antigen processing is carried out in a compartment which is enriched in degradatory enzymes and is also identified by the presence of MHC class II molecules. Optimal sized peptides are loaded onto MHC class II molecules, transported to the membrane and presented to antigen-specific T cells. Upon activation, T cells increase in number and perform different functions. Infection of a macrophage or a dendritic cell with a pathogen or its activation with a pathogen-derived entity such as bacterial lipopolysaccharide also leads to induction and upregulation of molecules called co-

stimulatory molecules which provide a second signal for activation of antigen-specific T cells. As mentioned earlier, antigenic peptides derived from infectious agents can also be presented to cytotoxic T cells in association with MHC class I molecules. These T cells can be identified by the presence of cell surface associated co-receptor molecule, CD8 while helper T cells are identified by the CD4 molecule.

The immune response to an infection / foreign agent is therefore a complex process which can be investigated at multiple levels. This chapter will describe some of the most commonly used assays that can be employed to evaluate an immune response, and discuss their applications. In addition, methods to generate antibodies will also be described.

Analysis of Antibody Responses

The antibody response to an antigen is determined by mixing the antigen with the cognate antibody followed by detection of the antigen-antibody complex. The latter can be achieved either by a precipitation technique or by an immunoassay. The precipitation assays are based on the formation of a precipitin line when an antibody complexes with an antigen, whereas immunoassays use a labeled antigen or a labeled antibody to detect antigen-antibody complex.

Precipitation assays

Double immunodiffusion

This is one of the simplest assays that can be employed to analyze antibody responses to an antigen without a need for any sophisticated instrument. This assay was first reported by Swedish scientist Orjan Ouchterlony 30 years ago. In this technique, an antigen and an antibody are allowed to migrate towards each other in a gel and the presence of antigen-antibody complex is revealed by the appearance of a line of precipitation. This line is formed at the point of equivalence; the precipitate is soluble in excess antigen or excess antibody (Plate 1). The rate of formation and the intensity of the precipitin line depend on the concentration and the molecular size of the antigen. Moreover, multiple precipitation lines are produced if the antigen preparation contains a mixture of several molecular species.

Materials
Glass slides
Agar
Barbitone buffer (0.1M, pH 8.6)
Antigen and antibody solutions
Gel-punching device
Staining solution (1g Coomassie brilliant blue dissolved in 40 ml glacial acetic acid and 148 ml distilled water).

Method

- Prepare a 2% solution of agar in barbitone buffer either by allowing it to melt on a hot plate or in a microwave oven.
- Bring the solution to about 60°C; pour it onto a clean glass slide placed on a leveled surface (3-5 ml/slide) and allow the agar to set.

- *(Slides can be pre-coated with 0.1% gelatin or a thin layer of 0.5% agar to ensure good adhesion*).

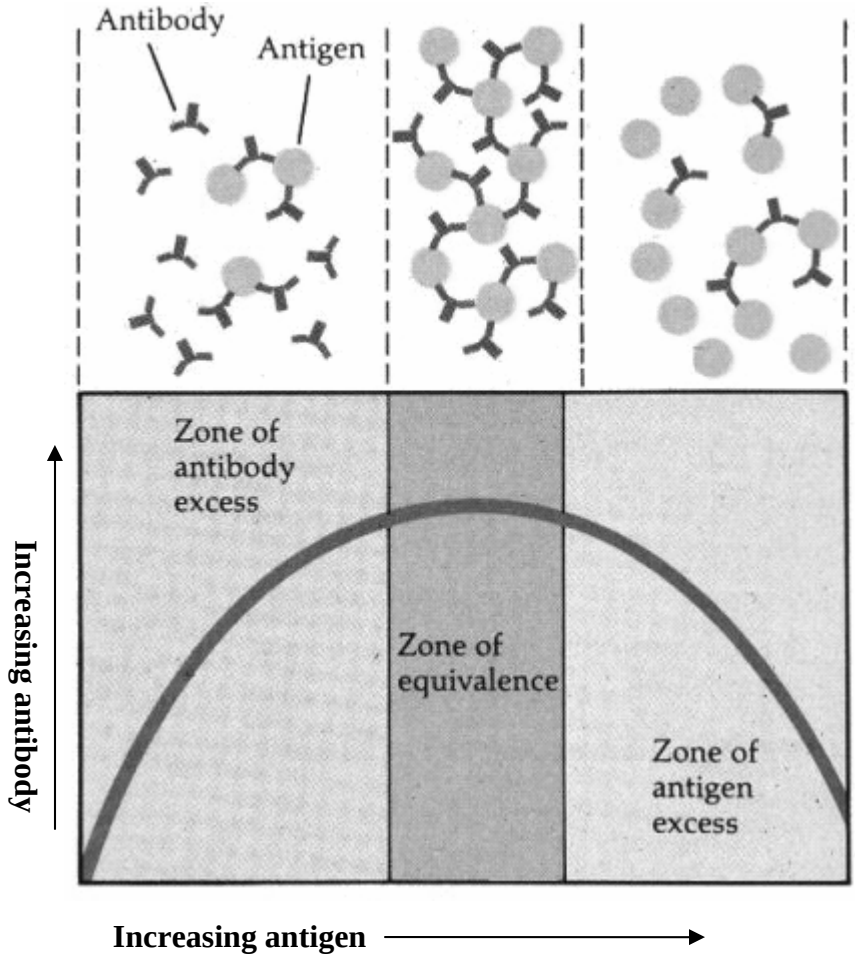


Fig. 1
(Source: eLabs Online Biomedical Sciences)

- Punch agar with a punching device (A typical punching pattern is shown in the figure below).

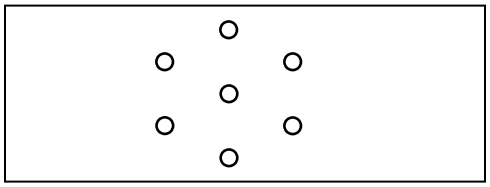
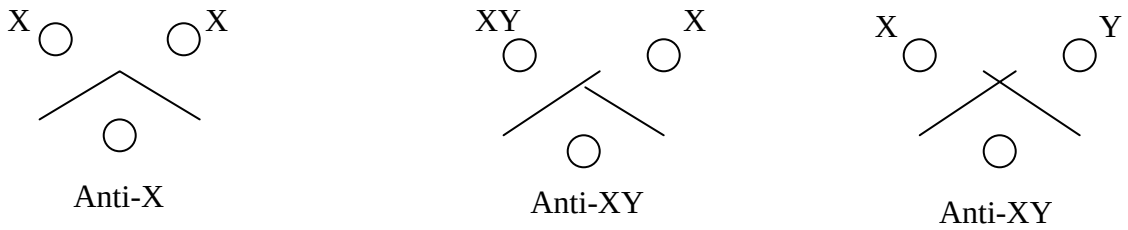


Fig. 2

- Remove agar from wells with a Pasteur pipette connected to a vacuum pump or with the help of an 18G needle.
- Dispense the antigen in the outer wells (in order to remember the identity of the material dispensed in each well, wells can be numbered on non-agar side or the identities can be recorded on a punching pattern drawn on a sheet of paper) and the antibody in the middle well (7-10µl/well) taking care that the solution does not overflow. Antigen and antibody concentrations should be titrated to obtain optimum results (The precise zone of equivalence can be obtained only after titrating various antigen and antibody concentrations).
- Place the slide in a humidified chamber and incubate overnight at room temperature (The rate of formation of a precipitin line depends on temperature; at 37°C this line can be seen within 4-5 h).
- Wash slides with several changes of saline for 24 h to remove unprecipitated proteins from the agar, dry with lint-free filter papers and stain for 10 minutes with a protein dye such as Coomassie blue. Destain with four successive 20 minutes washes of acetic acid-water (1:5). Rinse slides with tap water and air dry for storage.

Depending upon the nature of the antigen, three different precipitation patterns can be expected from the assay.

In case the antibodies recognize identical antigenic determinants, the lines of precipitation fuse to form one continuous arc (complete identity); in case the antigens share antigenic determinants - the presence of common as well as different determinants will give rise to the formation of a spur (partial identity) and in a situation where the two antigens do not share any determinants, two lines are formed independently without any interaction (non-identity).



Reaction of identity ***Reaction of partial identity*** ***Reaction of non-identity***

Fig. 3

A more sensitive version of double immunodiffusion in which antigen and antibody are driven towards each other with the use of electric current is called “Counter Immunoelectrophoresis”.

Immunoelectrophoresis

Immunoelectrophoresis combines electrophoresis with immunodiffusion. The combination of these two techniques increases resolution and enables to analyze interaction of antibodies with multiple antigens. A mixture of proteins is first allowed to separate into its components on the basis of charge by electrophoresis and following this, the separated components and the antibodies are made to interact with each other by allowing them to diffuse through the gel. The binding of an antigen with its complementary antibody results in the formation of a precipitin arc.

Materials and equipment
Electrophoresis tank and a power supply
2% agar solution in barbitone buffer
Barbitone buffer
Antigen mixture
Antiserum
Glass microscope slides

Method

- Prepare a 2% agar solution in barbitone buffer in a microwave. Bring the solution to about 60°C and pour 3-5ml onto a clean glass slide; allow it to set. Mark the end of the slide that will face the anode during electrophoresis.
- Make wells and a trough as shown in the figure (Fig. 3); wells can be made with a hypodermic needle that has been cut and sharpened or a Pasteur pipette, and the trough with a razor blade (well sizes in immunoelectrophoresis are smaller than those used in immunodiffusion).

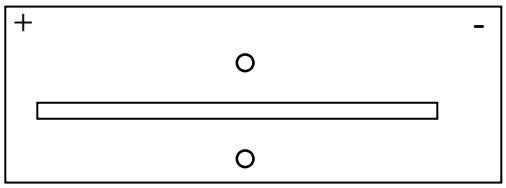


Fig. 4

- Take out agar plugs from the wells and fill them up with the mixture of proteins pre-incubated with a small amount of bromophenol blue.
- Fill the electrophoresis tank with barbitone buffer and place the slide in the tank. Connect the ends of the slide to the buffer chamber with Whatman paper wicks. Close the tank and connect it to a power supply.
- Apply a current of about 8-10 mA and run the gel for about an hour.
- Take the slide out of the tank, remove agar from the trough and fill it with the antiserum (standardize optimum dilution of the antiserum).
- Place the slide in a humid chamber and incubate overnight at room temperature.
- Examine precipitin arcs in the gel.

- Fix proteins by immersing the slide in 10% acetic acid. Dry the slide with a fine filter paper and stain it with Coomassie blue as described under Double immunodiffusion.

The sensitivity of immunoelectrophoresis can be increased by incorporating the antiserum in the gel. The formation of a precipitin line in this method is seen in the form of a rocket when an electric field is applied. This modified version is therefore named as “Rocket Immunoelectrophoresis”.

The precipitation assays while simple to perform are not very sensitive. Therefore, these assays have been replaced with more sensitive immunoassays.

Immunoassays

Immunoassays involve mixing of an antibody with an antigen followed by detection of the antigen-antibody complex with the help of a labeled antigen or a labeled antibody. These assays can be employed not only to analyze antibody responses during infection with a pathogen or upon vaccination with an antigen but also to detect antigens in biological fluids. Antibodies have over the years provided extremely powerful reagents in designing assays for laboratory diagnosis of infections and other diseased states. In 1960, Yalow and Berson first reported use of radiolabeled insulin for the detection of insulin in biological fluids. The presence of insulin in a biological sample was detected by allowing the sample to compete with radiolabeled insulin for binding to anti-insulin antibody. By using a set of standards, it was possible to determine the concentration of insulin in the unknown sample. The radioimmunoassay in effect laid the foundation for developing immunoassays. In recent years, this technique has been largely replaced by enzyme linked immunosorbent assay (ELISA) which uses an enzyme instead of a radioisotope. ELISAs combine the specificity of antibodies with the sensitivity of simple enzyme assays. Coupled with monoclonal antibodies, enzyme immunoassays have literally revolutionized laboratory diagnosis of infectious diseases. These assays can be classified as either competitive or non-competitive assays. In the competitive ELISA, unlabeled and labeled antigens compete for a limited number of binding sites on an antibody. In the non-competitive type of ELISA, the antigen (or antibody) to be measured is first allowed to react with antibody (or antigen) on a solid phase followed by measurement of the binding of enzyme-labeled antibody. One of the most commonly used non-competitive ELISA is the "sandwich ELISA." In this assay, immobilized antibody is incubated with either the standard or the test antigen. After washing, the immobilized antibody-antigen complex is incubated with an excess of enzyme-labeled antibody which binds to remaining antigenic sites. After washing away excess unbound labeled antibody, the bound labeled antibody is detected by an enzymatic reaction that converts a colorless substrate into a colored reaction product.

Indirect ELISA for the detection of specific antibody

This assay is extensively used to screen immune sera or B cell hybridomas for specific antibodies. Antigen is coated into the wells of a microtiter ELISA plate, followed by incubation with antibodies. The unbound antibody is washed and the bound antibody is detected by adding enzyme-labeled antibodies against the test antibody. This is followed by the determination of enzymatic activity.

Materials
Microtiter ELISA plates
Antigen and antibody solutions
Buffers for coating, blocking and washing
Enzyme substrate solutions
Stop solution
Microtiter Plate Reader

Procedure

- Dispense 50µl (or 100µl) antigen solution prepared in coating buffer into each well of a polystyrene (or polyvinyl) microtiter ELISA plate and incubate overnight at 4°C or at 37°C for 2-3 hrs (generally carbonate/bicarbonate buffer, 50mM, pH 9.5, is used for coating antigens but it is important to standardize coating conditions for each antigen. Similarly different antigen concentrations should be tested to standardize the optimum concentration of antigen for coating onto an ELISA plate).
- Aspirate and wash three times with the wash buffer. After the last wash, remove any residual buffer by inverting the plate onto a sheet of absorbent paper.
- Add 100µl or 200µl of blocking buffer to saturate residual binding sites; incubate plate for 1 h at room temperature or at 37°C.
- Wash the plate three times as in step 2.
- Add 50µl or 100µl of varying dilutions of antiserum or different concentrations of purified antibody diluted in blocking buffer into each of the coated wells and incubate for 1 h at 37°C or at room temperature. Use pre-immune serum or serum from a normal healthy subject as control.
- Aspirate and wash five times as in step 2.

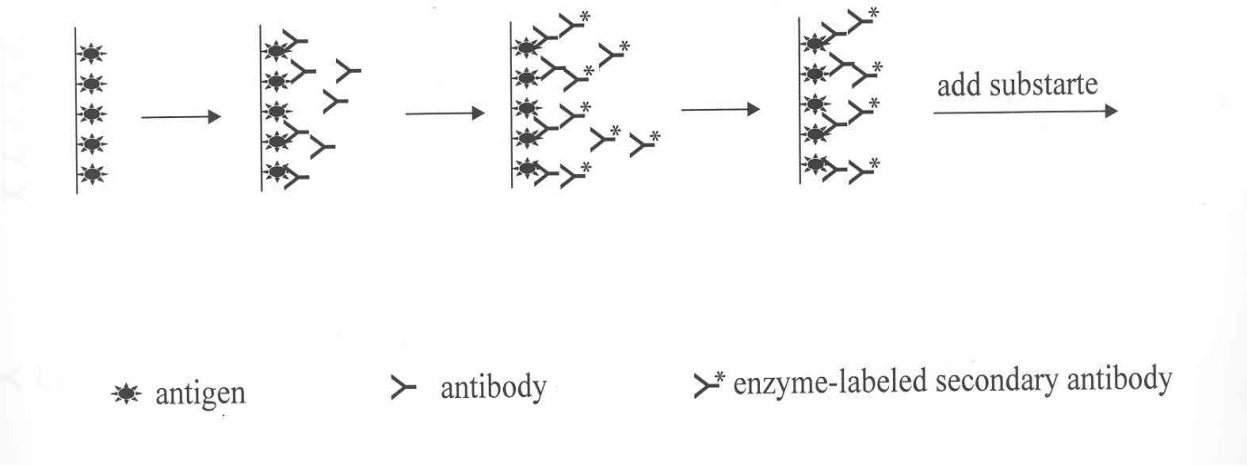


Fig. 5

- Dispense 50µl or 100µl of commercially available secondary antibody (such as HRP/AP-labeled anti-mouse immunoglobulin antibodies or HRP/AP-labeled anti-human immunoglobulin antibodies) and incubate for 1 hr at 37°C.
- Aspirate and wash the plate extensively. Add 100µl of substrate solution to each well and allow the color to develop (incubate up to 30 minutes in dark at room temperature).
- Stop the reaction by adding 50µl stop solution to each well. Read the plate within 30 minutes in a microtiter plate reader at an appropriate wavelength depending upon the enzyme/substrate/ chromogen mixture used in the assay.

Sandwich ELISA for the detection of an antigen

Materials

Same as mentioned under Indirect ELISA for the detection of specific antibody.

Procedure

- Dispense 50µl or 100µl antibody solution diluted in coating buffer into each well of an ELISA plate. Incubate the plate at 4°C overnight or at 37°C for a shorter duration (2-3 h).
- Aspirate and wash three times with the wash buffer. After the last wash, remove any residual buffer by inverting the plate onto an absorbent paper.
- Add 100µl or 200µl of blocking buffer to saturate residual binding sites; incubate plate for 1 h at room temperature or at 37°C.
- Wash the plate three times as in step 2.
- Add 100µl of varying concentrations of a standard or the test sample diluted in blocking buffer into each of the coated wells and incubate at room temperature or 37°C for 1 hr.
- Wash the plate five times with the wash buffer.
- Dispense 100µl of enzyme-conjugated detecting antibody (or biotin conjugated antibody + HRP-labeled avidin) diluted in blocking buffer to each well and incubate at room temperature or 37°C for 1 hr.

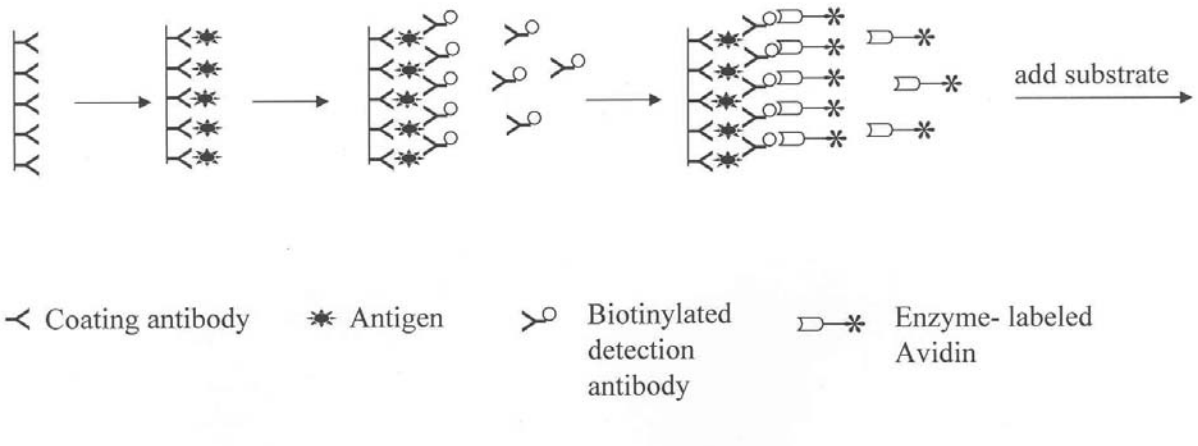


Fig. 6

- Wash the plate extensively, add 100µl of substrate solution to each well and allow the color to develop (incubation can be up to 30 minutes in dark at room temperature).
- Stop the reaction by adding 50µl stop solution to each well. Read the plate within 30 minutes in a microtiter plate reader at an appropriate wavelength depending upon the enzyme/substrate/ chromogen used in the assay.

The amount of color obtained after the addition of the substrate is directly proportional to the amount of antigen in the sample. The concentration of the antigen in the test sample can be calculated from a standard curve obtained after plotting absorbance values against known concentrations of the standard.

Common applications of ELISA

ELISA is routinely used for

(i) analyzing antibody responses during infection or after vaccination
(ii) laboratory diagnosis of different diseases, detection of infectious agents and pathogen-derived molecules in sera and other biological fluids.
(iii) for determining antibody production by B cell hybridomas and
(iv) for estimating cytokine levels during an immune response or during diseased conditions.

Immunofluorescence

Immunofluorescence is a technique that allows visualization of a cell or a tissue section with the help of a specific antibody. Visualization is achieved either by directly labeling the antibody with a fluorescent dye such as fluorescein isothiocyanate (direct immunofluorescence) or by using a secondary antibody that has been labeled with the dye (indirect immunofluorescence). Fluorescent samples are examined under a fluorescence microscope.

The most common fluorescent dyes are fluorescein, which emits green light, Texas Red, which emits red light, and rhodamine and phycoerythrin (PE) which emit orange/red light. By using selective filters, light coming from only the fluorochrome is detected in the fluorescence microscope.

Materials

Phosphate buffered saline (PBS)
Fixing solution (4% paraformaldehyde solution or 100% methanol)
Blocking buffer (5% normal serum depending upon the animal species in which the primary antibody has been produced)
Slides and cover slips
Assay diluent (PBS supplemented with 1% BSA)

Procedure

- Prepare a cell smear (or a tissue section) on a slide or a cover slip, dry it and fix with 4% paraformaldehyde for 10 minutes at room temperature or 100% methanol for 10 minutes at -20^{o}C.

- Rinse slides three times with PBS and incubate with blocking buffer for 1h at room temperature.
- Wash gently with PBS and incubate with an appropriate dilution of the primary antibody in PBS containing 1% BSA for 1 h (incubation time can vary depending upon the antibody) at 4^oC.
- Wash off unbound antibody with PBS and incubate with fluorochrome-tagged secondary antibody for 1h in dark at room temperature.
- Wash with PBS, add antifade reagent (available commercially) and observe the slide under microscope using appropriate filter.

Flow Cytometry

Flow cytometry is a technique for detecting, counting and sorting light signals generated by microscopic particles as they flow in a fluid stream past a beam of laser light. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of single cells flowing through an optical/electronic detection apparatus.

Cytometers use lasers as the source of illumination for their ability to provide intense focused illumination. Numerous detectors are aimed at the point where the stream of cells passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. Each particle passing through the beam scatters the light in some way, and fluorescent chemicals in the particle may be excited into emitting light at a lower frequency than the light source. This combination of scatters and fluorescent light is detected and fluctuations in brightness at each detector are analyzed. This gives information about the physical and chemical structure of the particle. For example FSC correlates with cell volume and SSC with inner complexity of the particle (like granularity or membrane roughness).

Major applications of flow cytometry include analysis of specific cell populations in a mixture, determination of expression of cell surface or intracellular molecules, analysis of cell cycle and cell death. Flow cytometry is also used for cell sorting.

Materials
Phosphate buffered Saline (PBS)
Assay buffer – PBS containing 1% BSA

Procedure
- Wash cells with PBS and dispense into wells of a round bottom 96 well plate. Centrifuge cells at 300 x g, add primary antibody (culture supernatant derived from a hybridoma or purified antibody diluted in the assay buffer), mix well and incubate for 1h at 4^oC.
- Wash cells two times with the assay buffer.
- Resuspend cells in fluorochrome-labeled secondary antibody diluted in the assay buffer and incubate for 1hr at 4^oC.
- Wash cells with the assay buffer, resuspend in PBS and analyze in a flow cytometer.

Evaluation of cellular responses

Isolation of immune cells from human peripheral blood

Peripheral blood is a convenient source of immune cells. Blood is readily obtainable and, in healthy donors, even small volumes can provide sufficient number of cells for most experimental purposes. In small animals such as mice, immunological investigations are carried out mostly with cells obtained from lymhpoid organs such as spleen, lymph node, thymus and bone marrow. To study immune cells from blood, it is important to prevent blood clotting. This is achieved by collecting blood in the presence of anti-coagulants such as sodium heparin, EDTA and acid citrate dextrose. Blood contains a high proportion of red blood cells (RBCs) and it is desirable to separate these cells from the cells of interest (mononuclear cells). Isolation of cells should be carried out immediately after blood collection or else blood should be stored overnight at 4^{o}C. RBCs are separated from mononuclear cells by selective osmotic lysis of erythrocytes or by density gradient centrifugation.

Osmotic lysis of RBCs

This is an inexpensive and quick method, and gives high yields of leucocytes. The most commonly used reagent for osmotic lysis is ammonium chloride solution. The protocol for osmotic lysis is described below.

- Take 5ml whole blood collected in the presence of an anti-coagulant in a sterile conical centrifuge tube.
- Centrifuge at 300 x g for 5 minutes at room temperature.
- Add 3ml of freshly prepared ammonium chloride solution.
- Incubate for 10 minutes at room temperature.
- Centrifuge at 300 x g for 5 minutes at room temperature.
- Wash the pellet (containing peripheral blood mononuclear cells; PBMCs) two times with saline or tissue culture medium.

Density gradient separation

The most commonly used reagent for density gradient separation of mononuclear cells is Ficoll-Hypaque (available commercially). This method yields a highly purified mononuclear cell population and also has the advantage of removing dead cells from the sample.

Materials

Blood collected in the presence of an anti-coagulant
Ficoll-Hypaque
Conical centrifuge tubes
Phosphate-buffered saline (PBS)
Tissue culture medium

Procedure

- Using a sterile pipette, place the desired volume of Ficoll-Hypaque in a conical centrifuge tube.
- Mix the desired volume of blood with an equal volume of PBS and slowly overlay the diluted blood over Ficoll-Hypaque solution and centrifuge at 400 x g for 25-30 minutes at

room temperature. The mononuclear cells are located at the interface between the plasma (top) and the Ficoll layer (bottom). Remove this layer very carefully by aspirating with a pasteur pipette. Transfer the mononuclear cells to a fresh tube and wash with 10ml of PBS or tissue culture medium, by centrfuging at 300 x g for 5minutes.

- Remove supernatant and repeat the washing step.

Mononuclear cells obtained by any of the methods given above can be further separated into individual cell types by different methods. Cells can be separated into monocyte, eosinophil and lymphocyte populations by using the Percoll density gradient centrifugation method. Cells can also be separated on the basis of cell surface markers by using the Magnetic Cell Sorting (MACS) or by Fluorescence Activated Cell Sorting (FACS) methods.

Isolation of mouse immune cells

In experimental animals like mice, lymphocytes are isolated from lymphoid organs, such as spleen, thymus, bone marrow, lymph nodes, or mucosal-associated lymphoid tissues.

Materials

Sterile instrument box
Sterile petri dishes (60 x 15mm)
Syringes and 19G needle
Sterile 200μm mesh nylon screen
Erythrocyte lysis buffer containing 0.15M NaCl

Procedure

- Place freshly removed organ (spleen/lymph node/thymus) in a 60 × 15 mm petri dish, containing 3-5ml complete medium RPMI-1640 or DMEM. Cut the organ into several pieces with the help of scissors.
- Using circular motion, press tissue pieces against bottom of the petri dish with the plunger of a 10ml syringe until mostly fibrous tissue remains.
- Disperse cell clumps by drawing up and expelling the suspension several times through a 6ml syringe fitted with a 21G needle.
- Pass the cell suspension through a 200μm mesh nylon screen and collect single cells in a 15ml centrifuge tube.
- Centrifuge at 300 x g for 5minutes, resuspend the pellet in medium and centrifuge again.
- Resuspend cells in tissue culture medium and count cells in a hemocytometer. Lymph node and thymic cells can be used without further processing. Erythrocytes need to be removed from spleen cells.
- Resuspend cell pellet in 3-5ml of erythrocyte lysis buffer. Incubate at room temperature for 5 minutes. Wash with medium three times by centrifuging cells at 200 x g for 5 minutes. After the final wash, resuspend cells in appropriate medium.

Magnetic cell Sorting

Immuno-magnetic cell sorting can be used to isolate any specific cell lineage like B cells or T cells according to cell surface antigens. This can be achieved by either positive or negative selection. Cells from spleen, lymph node, tumor or peritoneal fluid are specifically labeled with a mixture of monoclonal antibodies followed by binding to magnetic beads. After magnetic labeling, cells are passed through a separation column which is placed in a strong magnet. The magnetically labeled cells are retained in the column and separated from unlabeled cells, which pass through. After removing the column from the magnetic field, the retained fraction can be eluted. Magnetic fraction contains positively selected cells whereas non-magnetic fraction contains negatively selected cells.

Materials

- Appropriate monoclonal antibody
- Magnetic beads
- 1x PBS
- 2mM EDTA + 0.5% BSA
- Magnetic separation apparatus

Procedure

- Incubate $20x10^7$ cells with an appropriate monoclonal antibody at $4^{\circ}C$ for 30minutes.
- Centrifuge at 200 x g for 5 minutes at $4^{\circ}C$ to remove unbound antibody.
- Incubate antibody-labeled cells with magnetically labeled anti-antibody beads (or avidin - labeled beads in case the first antibody is biotinylated) at $4^{\circ}C$ for 1h.
- Wash unbound beads.
- Separate antibody-labeled cells using the magnetic apparatus by allowing the cell suspension to run through magnetic column and collecting the effluent. This would be the negatively selected fraction.
- Wash column with 3 x 500µl buffer.
- Remove the column from separator and place it in a new collection tube.
- Apply 1ml of buffer to the column and firmly flush out cells using the plunger supplied with the column. This would be the positively selected fraction.
- Analyze the cell population by flow cytometry to assess purity.
- Use cells immediately for phenotypic or functional analysis.

Assays for lymphocyte functions

Lymphocytes play a critical role in the regulation of immune responses and are responsible for mediating many of the effector functions of the immune system. Assays to measure accurately the activity of lymphocyte populations in both animal and human systems are thus of vital importance, not only to monitor the immune response, but also to understand the immune processes that are involved. Lymphocyte functions are studied in vitro by analysing the ability of cells to proliferate in response to activation with specific antigens, secrete cytokines following this recognition and in the case of cytotoxic T cells by their ability to kill target cells.

Lymphocyte proliferation assays

The proliferation of lymphocytes following stimulation with specific antigens, mitogens or cross linking with surrogate antigens, the anti-antigen receptor antibodies, can be investigated either by measuring incorporation of radioactive thymidine into DNA, or by non-radioactive enzymatic assays.

Lymphoproliferation assay using 3H-Thymidine

In this assay, incorporation of radiolabeled tritiated thymidine (3H-Thy) into the DNA of dividing cells is measured. Lymphocytes are incubated with a specific antigen or a mitogen [a T cell mitogen activates T cells independent of their antigen specificity] for 3–5 days before adding 3[H]-Thymidine for 6–18 hrs. The total amount of the radiolabel that is incorporated into cells provides a measure of the rate of synthesis of DNA by the entire population of cells.

Materials

Tritiated thymidine; 3[H]-Thymidine
Cell harvester
Scintillation counter

Procedure

- Dispense 1x105 – 3x105 lymphocytes into each well of a 96-well tissue culture plate and stimulate with an appropriate antigen, mitogen or an antibody. Each stimulation is set up in triplicate.

  For stimulation with antigens, T cells need to be activated in the presence of antigen presenting cells such as dendritic cells, macrophages or B cells. Unfractionated cells (these will contain lymphocytes as well as antigen presenting cells) isolated from a lymph node or spleen of an immunised mouse are therefore used for analysing antigen-specific activation of T cells; cells are incubated with varying concentrations of an antigen. Purified T cells (T cell clones or T cell hybridomas) can also be activated with anti-TCR antibodies or anti-CD3 antibodies coated into the wells of a 96-well tissue culture plate.
- Incubate cells for 3 – 5 days at 37oC (for T cell hybridomas stimulation with antigens or anti-TCR antibodies for 24 hrs is sufficient to study specific activation).
- Pulse each well with 1(Ci 3[H]-Thymidine (10(l/well) and incubate for 6 – 18 hrs at 37oC.
- Harvest cells on glass fiber filters using a cell harvester. If harvesting cannot be performed immediately, plate may be frozen and stored at -20 oC until harvesting, but not more than a few days.
- Dry filters at room temperature, punch filters into scintillation vials and add 3-5ml scintillation fluid into each well. Alternatively, process filter sheets for counting in a betaplate counter.
- Count vials/ filters in a (-scintillation counter to determine counts per minute (cpm).

MTT assay

The reduction of tetrazolium salts by metabolically active cells is an easy and reliable way to measure cell proliferation. The yellow tetrazolium MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5 tetrazolium bromide) is reduced by dehydrogenase enzymes produced by metabolically active cells to generate an intracellular purple formazan, which can be solubilized and quantified by spectroscopy.

Procedure

- Dispense 1x105 – 3x105 lymphocytes into each well of a 96-well tissue culture plate and incubate at 37 oC with an appropriate stimulus for optimum time duration.
- Add 10(l of MTT reagent to each well (make a stock solution of 5mg/ml MTT in PBS and filter sterilize) and incubate for 2- 4 hrs at 37 oC until purple precipitate is visible. Add 100µl of a detergent solution or DMSO to each well and swirl gently. Leave in dark at room temperature for 2 - 4 hrs and read absorbance in a spectrophotometer at 570nm.

The activation state of T lymphocytes can also be studied by analyzing expression of activation markers on the surface of lymphocytes using flow cytometry. Cell-surface markers that can be used for this purpose include CD25, CD69; these are upregulated following lymphocyte activation. In human T cells, MHC class II expression is also upregulated following activation.

Determination of cytokines

T lymphocytes produce a variety of cytokines following activation with specific antigens or polyclonal stimulators such as mitogens or antibodies. The amount of cytokines and the kind of cytokine that a T cell produces depends upon the nature of the stimulus that the cell receives. This ability to secrete different kinds of cytokines empowers T cells to perform different effector functions. The cytokine levels can be measured in culture supernatants derived from activated cells by commercially available enzyme immunoassays which employ cytokine specific antibodies (see Sandwich ELISA for detection of antigen for details of the assay). These assays can also be used to determine cytokine levels directly in body fluids. Alternatively, cytokine levels can be determined by intracellular single cell staining using flow cytometry.

Intracellular staining for cytokines in activated T cells

Materials

Brefeldin A (20µg/ml culture medium)
Anti-cytokine antibodies
PMA and ionomycin (50ng/ml PMA and 500ng/ml ionomycin)
Saponin (0.05%)
Paraformaldehyde solution (4%)

Procedure

- Incubate immune T cells (these cells could be PBMCs derived from subjects previously immunized with a vaccine or individuals recently infected with a pathogen, or these could be cells isolated from the lymph node/spleen of an immunized mouse) with a specific protein or a peptide for 60 h at 37°C. As a positive control, cells can be stimulated with PMA and ionophore which activates many cytokines from T cells.
- 48 h post stimulation, add brefeldin A to accumulate cytokines intracellularly (Brefeldin A blocks intracellular transport processes and results in the accumulation of most cytokine proteins in the endoplasmic reticulum)
- After washing, permeabilise cells by treating with saponin for 5 minutes followed by washing with PBS.
- Incubate with an appropriate anti-cytokine antibody for 45-60 minutes at 4°C followed after washing with fluorophore-labeled secondary antibody.
- Wash and analyse cells on a flow cytometer.

Intracellular staining for cytokines can be combined with MHC tetramer (fluorophore or biotin-labeled MHC tetramers pre-loaded with specific peptides which can bind cognate T cell receptors) staining to analyze individual antigen-specific responses.

Note
Critical parameters for cytokine staining include the cell type and the activation protocol, the time of cell harvest following activation, the inclusion of a protein transport inhibitor during cell activation and the choice of anti-cytokine antibodies. Some antibodies which recognize native cell surface markers may not bind fixed/ denatured antigen. For this reason, it is recommended that the staining of cell surface antigens be done with live, unfixed cells prior to fixation / permeabilization and staining for intracellular cytokines.

CTL assays

T cells perform a variety of effector functions including activating macrophages/dendritic cells and helping B cells to proliferate, differentiate and secrete antibodies. In addition, a subset of T cells has the ability to kill infected cells or tumor cells. These cells, referred to as cytotoxic T cells are particularly important during viral infections. Cytotoxic T cells recognize antigens in association with MHC class I expressed on target cells and signal these cells to undergo apoptosis. The most widely used assay to determine the cytotoxic ability of $CD8^+$ T cells is the radioactive chromium release assay in which target cells are labeled with ^{51}Cr and then incubated with cytotoxic cells. As target cells die, they release ^{51}Cr into the medium which gives a measure of the killing efficiency of cytotoxic cells.

^{51}Cr release assay

- Prepare a single cell suspesion of target cells (these could be ex-vivo cells [lymphoblasts prepared by stimulating splenocytes with concanavalin-A – 5µg/ $5x10^6$/ ml cells or with LPS – 5-10µg/$5x10^6$/ ml] or cell lines expressing appropriate MHC molecules – tumor cell lines that are often used for this purpose include P815 mastocytoma cell line, EL4

thymoma cell line) in complete medium. Make sure that there are no cell clumps in the cell suspension.

- Load these cells with the relevant antigen (this could be done by incubating cells with a peptide, a protein or infecting them with a virus).
- Centrifuge and incubate antigen-loaded cells with ^{51}Cr. Use about 200µCi for $50x10^6$ cells in a total volume of approximately 300µl. Incubate cells with the radiolabel for 45 minutes (for lymphocytes) to 1 to 2 hrs for tumor cells at 37°C. It is important to start with target cells which have high viability because cells with poor viability generally leak ^{51}Cr. (The half life of ^{51}Cr is 28 days).
- Prepare $CD8^+$ effector T cells which have been activated *in vivo* (either by immunization or infection with a virus; it is not necessary to purify CD8 T cells but it is helpful to remove monocyte-macrophages) while targets are getting labeled with ^{51}Cr.
- Wash target cells 3-4 times with complete medium and mix them with effector cells as soon as possible after the last wash. Use different effector to target ratios (starting from 100 and go down to 3) to establish an optimum dose response [E:T ratio refers to the total number of effector cells not just $CD8^+$ T cells]. Effector cell not stimulated with an antigen or stimulated with an irrelevant antigen should be used as a control. Set up the assay in triplicate.
- Centrifuge cells at 200 x g for 5 minutes so as to promote contact between target and effector cells and incubate at 37 ^oC for 3 to 6 h. Incubation time varies depending on the activity of the target cells and degree of spontaneous ^{51}Cr release from the target cells.
- Take out 100µl supernatant from each well and determine the amount of radioactivity released in the medium by counting in a β-scintillation counter. Determine maximum releasable radioactivity by lysing just the target cells with 0.1ml of 2% Triton X-100.

Determine CTL activity as follows

$$\% \text{ lysis} = \frac{\text{test } ^{51}Cr \text{ released} - \text{control } ^{51}Cr \text{ released}}{\text{maximum } ^{51}Cr \text{ released} - \text{control } ^{51}Cr \text{ released}}$$

where test refers to effector cells having cytotoxic activity and control refers to non-cytotoxic cells.

Production of antibodies

Polyclonal antibodies

Antibodies can be produced by injecting experimental animals with an antigen of interest. The most widely used animal for generating polyclonal antibodies in a laboratory set-up is the rabbit. Large amounts of antibodies can be produced by immunizing sheep, goats or donkeys. For monoclonal antibodies, mice are the most extensively used animals. Small molecules (haptens) that can't generate antibodies on their own need to be covalently linked to a carrier protein that provides T cell help. The ability of an antigen to produce an antibody response is considerably increased when it is injected in an insoluble/particulate form. This is achieved by mixing it with

immune potentiators/ immunostimulators called adjuvants; particulate antigens such as bacteria can be injected without an adjuvant.

Materials

Antigen
Animals
Adjuvant (Complete Freund's adjuvant, Alum, Titermax, Montanide, Ribi etc)
Two 2ml glass syringes, a 3 way stopcock (*or a small tubing that can be used to connect the two syringes*) and a needle.

Procedure

- Prepare a solution of the antigen in normal saline (Haptens and small peptides can be conjugated to a carrier protein by a number of methods. For details see Refs. 1 - 3).
  The amount of antigen to be used for immunization can vary from one antigen to another and would also depend upon the kind of animal to be used for generating antibodies. However, for a rabbit one could start with 100µg of antigen.
- Mix the antigen solution (100µg in 500µl saline) with equal volume of Complete Freund's Adjuvant (CFA) with the help of two glass syringes connected through a 3 way stopcock. Continue mixing till a stable water-in-oil emulsion is obtained. This can be checked by allowing a drop or two to fall on water; an optimum emulsion does not disperse but floats as an intact, cohesive drop.
  Complete Freund's adjuvant is a mixture of oil and heat killed Mycobacterium tuberculosis.
  Alternatively, an antigen can be adsorbed onto alum, an adjuvant that is permissible for use in humans. Alum (aluminium hydroxide) is commercially available as a gel (alhydrogel). The antigen is absorbed onto the gel for about 1-6 hrs at 4°C (50-200µg protein/mg alhydrogel).
- Shave fur from a small region on the back of a rabbit. Inject antigen at multiple sites with 50-100µl of the emulsion. Before immunization, bleed animals through the ear vein so as to prepare pre-immune serum.

Routes of Immunization

Intraperitoneal (i. p.)

In intraperitoneal immunizations, antigen is administered at the lower part of the abdomen in the peritoneal cavity taking care not to rupture any internal organ like intestine.

Subcutaneous and intramuscular (s.c and i.m)

Subcutaneous injection is given at the hind limb flap or around the neck. Intramuscular injection is given in the muscles around thighs directly between muscle and fibers.

Intravenous (i.v)

During intravenous injections, antigen is injected in the caudal vein using a 30G needle; caudal vein can be dilated with xylene.

- 3-4 weeks later, prepare an emulsion of the antigen with Incomplete Freund's Adjuvant (IFA) (*this is prepared the same way as CFA*) / or adsorb it onto alum and inject intramuscularly into the thigh muscle.
- 7-10 days after the booster, bleed the animal through the ear vein (or through retro-orbital plexus in the case of small animals such as mice).
- Prepare serum and check for antigen-specific antibodies with an appropriate assay such as ELISA (discussed earlier).
- The animal can be boosted again after 4 weeks and high titre antisera can be generated.

Monoclonal antibodies

Polyclonal antibodies can be produced in large amounts with much ease and without a need for any specialized experimental facility. However, lack of reproducible characteristics and ill defined specificity are major problems with polyclonal anti-sera. These problems were overcome in 1975 when George Kohler and Cesar Milstein reported that antigen-specific B cells could be fused with cancerous myeloma cell lines that would lead to generation of immortal cell lines with an ability to secrete antibodies indefinitely. This technique now routinely called hybridoma technique, for which Kohler and Milstein were awarded Nobel Prize in Physiology and Medicine in 1984, has made a revolutionary impact not only in the field of immunology but in biomedical research in general. This technique has been extensively used to produce mouse monoclonal antibodies but it is also possible to make rat, hamster, human and rabbit monoclonal antibodies as well.

Before outlining the procedure of this technique, a brief account of the principle of the technique is described. Spleen cells from an immunized mouse are fused with a mouse myeloma cell line which is a cancerous cell line of B cell origin. This cell line does not produce any antibody on its own. Another characteristic feature of this cell line is that it lacks the enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT) and therefore cannot use salvage pathway of DNA synthesis. If the *de novo* pathway of DNA synthesis is inhibited in these cells, these cells die. It is this property which has been exploited to generate hybridomas. After fusion, three different types of hybrid cells are generated: spleen x spleen, spleen x myeloma and myeloma x myeloma. Fused cells are grown in a medium which inhibits *de novo* protein synthesis but can support salvage pathway. Spleen x spleen hybrids die because these cells have a short half life, myeloma x myeloma die in HAT (Hypoxanthine Aminopterin Thymidine) selection medium because these are HGPRT negative and aminopterin blocks their *de novo* pathway of DNA synthesis but spleen x myeloma hybrids survive because these have HGPRT and can therefore use the salvage pathway for growth and survival.

Materials

Mice
Mouse myeloma cell line, Sp2/0 (can be obtained from American Type Cell Culture, USA)
Cell culture medium; RPMI-1640 or DMEM
(Medium is prepared according to the manufacturer's instructions and supplemented with 10% FCS)
Fetal Calf Serum (FCS)

Polyethylene glycol
(PEG of mol.wt. 1500 is most frequently used for mouse fusions; 1g PEG is autoclaved in a screw cap glass tube and 1ml medium is added to it to make a 50% solution)
HAT (hypoxanthine, aminopterin and thymidine) selection medium
Dimethyl sulfoxide (DMSO; for freezing cells)

Procedure

- Immunize mice with the antigen of interest as described under "Polyclonal antibodies".
- Boost immunized mice intravenously or intraperitoneally with the antigen 3-4 days before fusion.

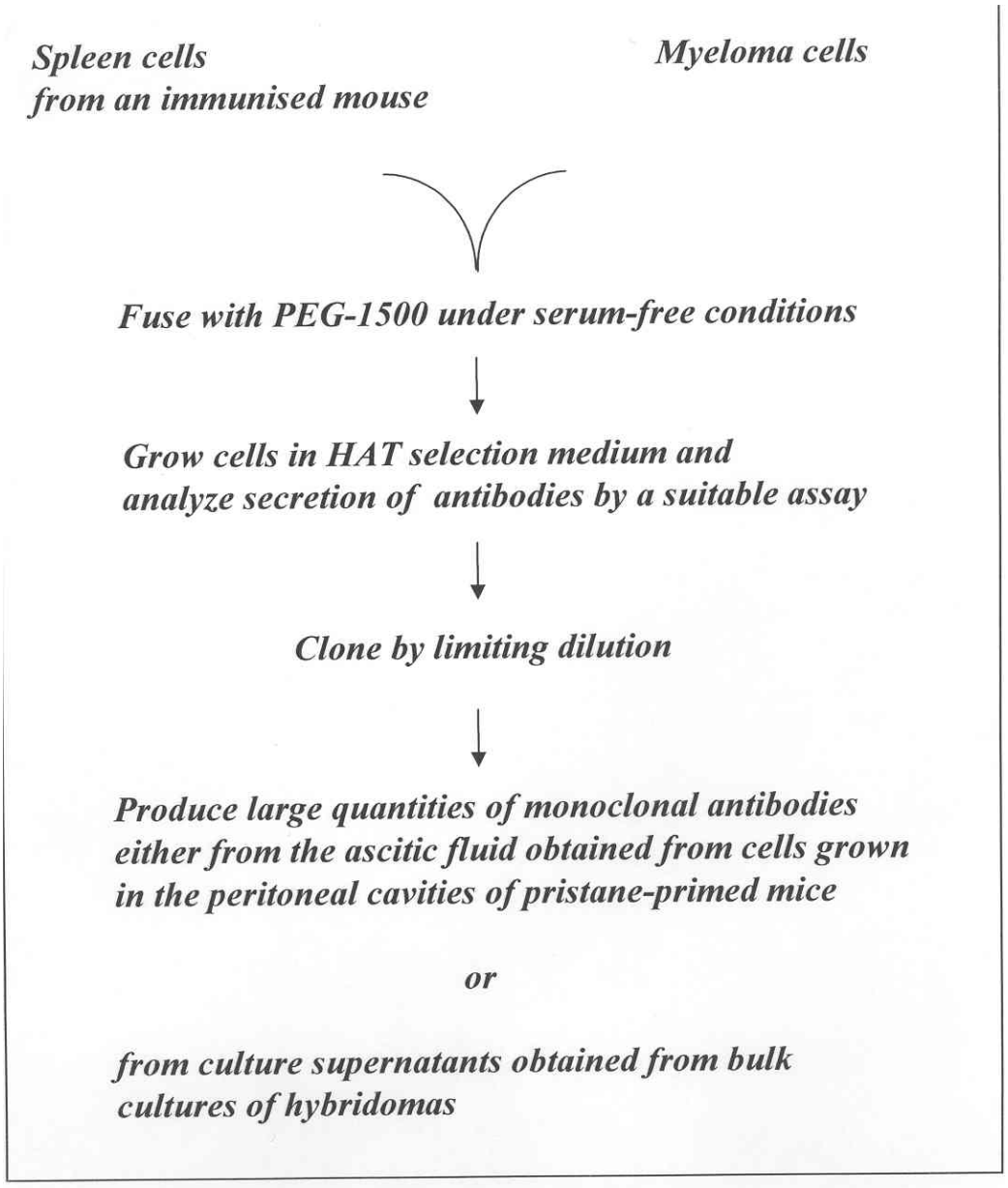


Fig. 7

- Subculture myeloma cells a day before fusion to a cell density of 3-5 x 105 cells/ml.
- Take out spleen from the immunized mouse under aseptic conditions and make a single cell suspension in serum-free medium. Wash twice with serum-free medium and resuspend in serum-free medium.
- Harvest Sp2/0 myeloma, wash twice with serum-free medium and resuspend in serum - free medium. Myeloma cells should be used for fusion only if the cell viability is greater than 95%. Viability can be determined by trypan blue exclusion).
- Count cells using a hemocytometer.
- Mix spleen cells and myeloma cells in a ratio of 1:1, 2:1 or 4:1 and pellet them down. (Keep aside some myeloma cells and some spleen cells to be used as control). Break the cell pellet by gently tapping the tube against the palm of your hand. Add 1ml of 50% PEG solution dropwise over a period of 60-90 seconds with gentle shaking of the tube. Add 10ml serum free medium over a period of 5-10 minutes with gentle tapping and swirling of the tube. Start with smaller volume and increase it with time.
- Incubate cell suspension for 10 minutes at 37(C.
- Centrifuge at 200 x g and resuspend cells in HAT selection medium.
- Dispense cells in a 24 well plate (0.5 -1 x 106 cell/ml) or in a 96 well plate (3-4 x 105 cells/well). Keep the plate in a humidified 5% CO2 incubator at 37^0C.
- Replace HAT medium every 3-4 days.
- About 10-14 days after the fusion, when hybrids are macroscopic and clearly visible, test culture supernatants for antibodies by an appropriate assay (ELISA etc).

Screening of hybridomas

This is a crucial step in the generation of hybridomas. It is important to have a well standardized and sensitive assay for determining antibodies before embarking on making hybridomas. Care should be taken not to take supernatants very early because you might miss low secretors. It is also important not to allow the colonies to overgrow.

Procedure

- Remove supernatants from wells containing hybrids and determine antibody activity.
- Replenish cultures with fresh medium and re-test the supernatants 48-72 hrs later. Re-screening also helps to establish that the antibody reactivity was not due to surviving spleen cells.

Cloning by limiting dilution

Cloning of positive hybridomas is done to establish monoclonality. This should be done immediately after the first screening in order to reduce chances of overgrowth by non-secreting hybridomas. It can be done either by limiting dilution or by using semisolid agar. Cloning by limiting dilution is described here.

- Plate 2-3 x 105 spleen cells or 104 peritoneal exudates cells per well of a 96 well plate. (Isolation of spleen cells has been described above. Peritoneal exudate cells can be obtained by injecting cold medium into the peritoneal cavity of a mouse).

- Count cells from a positive hybridoma in a hemocytometer. Resuspend 50-100 cells in 10ml cell culture medium supplemented with 20% FCS and HAT. Dispense cells in a 96 well tissue culture plate such that each well does not get more than 1 cell.
- Examine plates daily and look for growing hybrids. Normally small colonies start appearing in the wells 7-10 days after cloning. When colonies become macroscopic, screen them for their ability to secrete antibodies. Choose positive hybrids which have only one colony (*determined by microscopy*). Repeat cloning twice till all the growing subclones are positive for the antibodies.

Cryopreservation and thawing

Once a B cell hybridoma has been established, it is important to preserve these cells under appropriate conditions. These hybridomas can be stored in liquid nitrogen and revived whenever needed.

- Harvest healthy cells (grown at a density of approximately 0.5 x 10^6 cells/ml), centrifuge at 200 x g and resuspend in freezing medium (*consisting of 10% DMSO and 90% FCS*) at a cell density of 2 – 5 x 10^6 cells/ml. Dispense 1ml in a sterile 2ml cryovial, cap tightly, wrap the vials in cotton and store overnight at -70°C in a deep freezer. Transfer vials to liquid nitrogen the next day.
- To revive cells, clean the frozen vial with 70% alcohol and thaw cells by adding medium prewarmed to 37°C. Transfer cells into a 10-15ml tube containing growth medium.
- Centrifuge at 200 x g, resuspend the pellet in growth medium and culture cells in a 25cm^2 flask in a humidified 5% CO_2 incubator at 37°C.

Ascites

One of the convenient methods of producing large quantities of antibodies is by growing hybridomas as ascites in the mouse peritoneal cavity.

Procedure

- Inject 0.5ml pristane (2,6,10,14 tetramethyl pentadecane) intraperitoneally into mice. One week later, inject 2-5 x 10^6 hybridoma cells suspended in 1ml serum-free medium into the peritoneal cavity.
- Growth of cells in the peritoneal cavity results in bulging of the cavity; this usually takes 7-10 days.
- Drain ascitic fluid with an 18G needle. Centrifuge the fluid at 200 x g and analyze antibodies in the supernatant. Resuspend the pellet in fresh medium and examine cells under the microscope. These cells can be re-injected in pristane-primed mice.

Aliquot ascitic fluid and store at -20°C.

One limitation of this procedure is that unless one uses an antigen-specific affinity chromatography to purify these antibodies, the monoclonal antibodies in the acsitic fluid will be contaminated with small amounts of endogenous mouse immunoglobulins. Alternatively, one can also grow hybridomas in fermenters for large scale production of monoclonal antibodies. Since many monoclonal antibodies are in use for therapeutic purposes in humans, cell culture media which do not use animal sera (FCS) have been devised for culturing hybridomas.

Furthermore, it is possible to clone and express antibodies in high yield in mammalian expression systems. It is not within the scope of this chapter to discuss those procedures in detail.

Isotyping of monoclonal antibodies

The effector functions of an antibody depend on the isotype of the antibody. The most commonly used assay for determining the isotype of an antibody is ELISA.

Procedure

- Coat an ELISA plate with the antigen of interest as described under "Indirect ELISA for the detection of specific antibody".
- After blocking it with the blocking buffer and subsequent washing, dispense culture supernatant containing the monoclonal antibody and incubate for 1 h at 37°C.
- Wash the plate, dispense HRP-labeled isotype-specific secondary antibodies available commercially and incubate for 1 hr at 37°C. After subsequent washing, determine the enzyme activity as described under "Immunoassays".

The isotype of an antibody can also be determined by double immunodiffusion.

Production of human monoclonal antibodies

Human hybridomas can be generated by infecting B cells with Epstein Barr Virus (EBV) followed by fusion of immortalized B cell lines with a suitable myeloma line. EBV infects and immortalizes human B lymphocytes which become a good source of antigen-specific lymphocytes suitable for producing human hybridomas.

Materials

Heparinized peripheral blood
B95-8 marmoset cell line
Heteromyeloma fusion partner K_6H_6/B_5
RPMI-1640 medium
Fetal Calf serum
Phosphate buffered saline
Ficoll-Hypaque
Polyethylene glycol (PEG)
HAT medium

Preparation of EBV

- Grow B95-8 marmoset cell line, which secretes EBV, at a density of $1x10^6$cells/ml RPMI-10 for 3 days in a humidified 37°C CO_2 incubator.
- Harvest culture supernatant (cells should be > 90% viable) and clarify it by centrifuging at 400 x g for 15 minutes at 20°C.
- Filter the supernatant through a 0.45μ filter.

- Make 1ml aliquots and store at -130°C. Culture supernatant prepared this way usually contains $>10^2$ to 10^3 transforming units/ml of EBV.

Caution

- Prevent repeated freezing and thawing of the culture supernatant stored at -130°C.
- Inactivate all the waste material by autoclaving at 121°C for 30 minutes or by treating with hypochlorite solutions such as Chloros (final conc. 10% v/v) for 18 h.
- Handle EBV carefully since it may cause disease in non-immune individuals.

Preparation of lymphocytes for EBV transformation

- Dilute 15ml heparinized peripheral blood 1:3 in PBS.
- Take a 50 ml conical tube, add 10 ml of Ficoll-hypaque and overlay it with 40ml of diluted blood taking care not to mix these two solutions.
- Centrifuge tubes at 400 x g for 25-30 minutes at room temperature.
- Isolate peripheral cells using Ficoll-hypaque as described under "Density gradient separation".
- Thaw an aliquot of B95-8 culture supernatant
- Suspend 5 x 10^6 lymphocytes in 1ml B95-8 culture supernatant and incubate for 2 h at 37°C in a CO_2 incubator with intermittent shaking of tubes to keep cells in suspension.
- Dilute cell suspension with 14 ml RPMI-1640 and centrifuge at 200 x g for 10 minutes.
- Wash cells once with RPMI-1640 supplemented with 5% FCS.
- Resuspend cells in RPMI-10 and plate cells at 10^6/ 2 ml/ well in a 24 well flat bottom tissue culture plate.
- Replace half of the culture supernatant with fresh culture medium at weekly intervals taking care not to disturb cells.

Note: *Transformed B-cells are microscopically visible as small clumps of cells usually 1-2 weeks after infection with EBV.*

- After 3 weeks collect culture supernatant from each well and assay for antibody by ELISA.
- Re-assay the culture supernatant after 5 days to confirm antibody secretion.
- Expand antibody-secreting cultures by transferring them from a 24 well plate to a 6 well plate and replenish fresh culture medium. Freeze antibody-secreting B cell lines and store in liquid nitrogen.

Generation of human hybridomas

Preparation of feeder cells

Inject 10ml cold RPMI-1640 into the mouse peritoneum.
Tap the abdominal cavity gently and withdraw peritoneal exudates using an 18G needle. Plate 50 µl of this exudate into each well of a 96-well tissue culture plate.

Note: *Feeder cells can be prepared on the day of fusion or one day before fusion. Mouse spleen cells can also be used as feeder cells.*

Fusion

- Wash 10^7 K6H6/B5 cells and 10^7 EBV-transformed cells with RPMI-1640.
- Mix cells and centrifuge at 200 x g for 10 minutes.
- Decant the supernatant carefully and completely and tap the base of the centrifuge tube to dislodge the cells in the pellet.
- Add 0.5-1 ml of warm PEG-1500 solution over a period of 1 minute and stir gently.
- Add 10ml of warm RPMI medium very slowly with gentle mixing over a period of 6-10 minutes and incubate at 37°C incubator for 10 minutes.
- Centrifuge the suspension at 200 x g for 10 minutes and resuspend in RPMI medium supplemented with 20% FCS, HAT (hypoxanthine, thymidine and aminopterin) and ouabain (0.001mM). Plate 10^5 cells per well of a 96-well plate containing feeder cells..

Note: *Keep controls for each cell type (K6H6/B5, EBV-transformed cells and feeder cells).*
Screeing of hybridomas, cloning by limiting dilution and expansion of stable hybridomas has been described under "Mouse monoclonal antibodies".

Purification of antibodies

Monoclonal antibodies can be purified from tissue culture fluids or ascitic fluids by a number of methods including salt fractionation, ion exchange chromatography, gel filtration or affinity chromatography. However, affinity chromatography using Protein A- Sepharose or Protein G-Sepharose offers the most convenient method and is widely used for purifying IgG class of antibodies. Protein A and Protein G bind to the Fc region of the antibody. These proteins have different affinities for antibodies from different animal species as well as for different isotypes from the same animal species.

Materials

Protein A / Protein G - Sepharose beads (available commercially)
Ascitic fluid/ tissue culture supernatant
Phosphate buffer (0.5M, pH 8; 0.1M, pH 8)
Phosphate-buffered saline (0.1M phosphate, 0.15M sodium chloride, pH 8)
0.1M acetic acid solution prepared in 0.15M NaCl

Procedure

- Pack Protein A/G – Sepharose in a syringe and wash with 0.1M phosphate buffer, pH 8. (*Alternatively, swell Protein A/G – Sepharose beads in 0.1M phosphate buffer, pH 8 (1.5g dry gel gives 5 ml swollen gel)).*
- Dilute 1ml ascitic fluid with 2 volumes of 0.5M phosphate buffer, pH 8, filter through a 0.45µ membrane and allow to bind to the beads for 30-45 minutes at room temperature.
- Wash the beads with PBS until no proteins are detectable in the affluent (A280 < 0.05).

- Elute the antibody from the beads with 0.1M acetic acid solution containing 0.15M NaCl. Neutralize it immediately and dialyze against PBS (50mM phosphate, 0.15M NaCl, pH 7.4).
- Wash the beads with PBS and store in PBS containing thiomersol (0.1%) as the preservative.

Applications of monoclonal antibodies

Monoclonal antibodies have not only contributed immensely to biological research but have also revolutionized the field of laboratory diagnosis. Some of their important applications are in

i) the measurement of proteins, hormones and drugs in biological fluids.
ii) tissue and blood typing.
iii) the identification of infectious agents.
iv) the analysis of cell surface antigens on leukemias, lymphomas and other kinds of cancers.
v) imaging tumors.
vi) in immunotherapy of cancers.

Buffers and other reagents

Phosphate Buffered Saline (PBS; 50mM phosphate, 150mM NaCl, pH 7.4)

Na_2HPO_4	41mM
$NaH_2PO_4.2H_2O$	9.5mM
NaCl	150mM

Coating buffer for ELISA (carbonate buffer, pH 9.5)

Na_2CO_3	16mM
$NaHCO_3$	37mM

Blocking buffer

1% milk protein or 1% BSA in PBS

Wash buffer for ELISA (PBS containing 0.05% Tween-20)

PBS	1 litre
Tween-20	500µl

Citrate-phosphate buffer, pH 5.6

Citric acid	22.2mM
Na_2HPO_4	51.3mM

Erythrocyte lysis buffer

NH_4Cl	0.15M
$KHCO_3$	10mM
Na_2EDTA	0.1mM

Adjust pH to 7.2-7.4 with 1N HCl. Filter sterilize through a 0.22µ filter.

HAT solution

100X stock solution – 13.6 mg/ml hypoxanthine, 0.019 mg/ml aminopterin and 0.388 mg/ml thymidine. Store in aliquots at -20°C.

HRP Substrate

200µl of TMB/TBA-BH solution [41mM 3, 3', 5, 5' – Tetramethyl-benzidine (TMB) and 8.2mM Tetrabutylammonium-borohydride (TBA-BH) in N, N'- dimethyl acetamide (DMA)] and 3µl H_2O_2 to 8 ml citrate phosphate buffer, pH 5.6.

Stop solution

2N H_2SO_4

Suggested Readings

1. Antibodies – A Laboratory Manual. Edited by Ed Harlow and David Lane. Cold Spring Harbor Laboratory, USA.
2. Practical Immunology. Edited by Leslie Hudson and Frank C. Hay. Blackwell Scientific Publications, Oxford, London.
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6. Kohler G and Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-7.
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