Purification of T Cell Subpopulations

This unit describes a procedure for isolating T cell populations or subpopulations using the method of indirect panning (basic protocol). In this method, cells are selected by their capacity to bind to antibody-coated plates on the basis of particular cell-surface markers. It is superior to the antibody/complement lysis method (alternate protocol) because the nonselected cell population can be retrieved. For example, to obtain subpopulations of purified T cells, T cells are incubated with a mouse anti-human monoclonal antibody against a surface antigen present on a T cell subpopulation, then poured onto petri dishes precoated with anti-mouse immunoglobulin. The subpopulation that binds the mouse anti-human antibody will adhere to the antibody-coated petri dish, whereas the subpopulation (which does not bind the mouse anti-human antibody) will not. The nonadherent and adherent subpopulations can then be separated physically.

Antibody/complement–mediated cytotoxicity is commonly used to deplete a heterogeneous cell population of a specific subpopulation recognized by a cell-surface antibody. This procedure can be used as the primary negative-selection method for isolating any subpopulation of cells not interacting with a particular cell-surface antibody. In addition, it can be used to eliminate contaminating cells from a partially purified cell population obtained by other methods, e.g., removing residual T cells from a non–T cell population after rosetting with sheep red blood cells (UNIT 7.2). Unfortunately, cell populations interacting with the antibody are irretrievably lost in this procedure owing to lysis.

CAUTION: When working with human blood, cells, or infectious agents, biosafety practices must be followed (see Chapter 7 introduction).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique must be used accordingly.

BASIC PROTOCOL

ISOLATION OF T CELL POPULATIONS BY INDIRECT PANNING

In the following protocol, isolated T cells (E-rosette-positive cells; UNIT 7.2) are separated into CD4+ and CD8+ T cells using an anti-CD8 antibody.

Materials

- Affinity-purified and human-Ig-absorbed goat anti-mouse Ig (Tago #4150)
- 0.05 M Tris \( \cdot \) Cl, pH 9.5
- Specific mouse antibodies (polyclonal or monoclonal antibody; e.g., Coulter, or Becton Dickinson)
- Phosphate-buffered saline (PBS; APPENDIX 2)
- T cell population (UNIT 7.2)
- 5% and 1% heat-inactivated (1 hr, 56°C) FCS in PBS
- Complete RPMI medium (serum-free and filter sterilized; APPENDIX 2)
- 15 × 100-mm plastic petri dish, bacteriological grade (not treated for tissue culture)
- 15-ml centrifuge tube
- Beckman GPR centrifuge with GH-3.7 horizontal rotor (or equivalent temperature-controlled centrifuge)
- Flat surface at 4°C
- Inverted microscope

Additional reagents and equipment for immunofluorescent staining (UNIT 5.3) and flow cytometry (UNITS 5.4 & 7.9)
1. Dilute the goat anti-mouse Ig to 10 µg/ml in 0.05 M Tris-Cl, pH 9.5. Add 10 ml to a petri dish and incubate 40 min at room temperature, or 24 hr at 4°C.

   *The dish needs to be swirled to make certain the entire bottom is coated. The coated petri dish can be stored for 1 to 2 weeks at 4°C.*

2. Determine appropriate concentration of specific mouse antibody (in this case, anti-CD8 antibody) to stain mononuclear cells for flow cytometry. Dilute antibody to this concentration with PBS.

3. Place 2-3 × 10^7 T cells in a 15-ml centrifuge tube and spin 10 min in a GH-3.7 rotor at 1300 rpm (400 × g), 4°C to 10°C. Decant supernatant and resuspend cell pellet in appropriate amount of antibody as determined in step 2. Incubate cell suspension 30 min on ice.

4. While T cells are incubating, remove unbound goat anti-mouse Ig from the dish using a sterile pipet. Wash by adding 5 to 7 ml of 5% FCS in PBS to the dish. Gently swirl and remove the FCS. Do this three times.

   *The unbound goat anti-mouse Ig can be reused. Transfer the unbound Ig (the fluid in the initial pipet) immediately to another petri dish and repeat coating and incubation of step 1.*

5. Add 5 to 10 ml of 5% FCS to the T cells and centrifuge 10 min at 1300 rpm (400 × g), 4°C. Remove supernatant and resuspend pellet in 5% FCS in PBS. Repeat the wash.

6. Resuspend cell pellet in 3 ml of 5% FCS in PBS. Remove the 5% FCS from the coated petri dish with a sterile pipet and immediately pour cells onto the dish.

7. Incubate dish on a flat surface 30 min at 4°C. Remove dish and gently swirl for 30 sec. Incubate another 30 min at 4°C.

8. Collect and save nonadherent cells by gently pipetting the fluid from the dish. Wash dish gently with 5 to 7 ml of 1% FCS in PBS by placing the pipet tip against the side of the dish and slowly adding the FCS. Pipet off the fluid and repeat 2 to 3 times. Add these washes to the nonadherent cells to isolate the negatively selected population.

   *For maximum purity of the nonadherent population, use only the initial fluid removed from the petri dish.*

9. Examine the dish with an inverted microscope and determine the number of nonadherent cells. Continue washing until all nonadherent cells are removed.

10. Remove adherent population from the dish by adding 5 to 7 ml of 1% FCS to the dish and gently scraping with a sterile scraper. Repeat 2 to 3 times.

   *Alternatively, add 15 ml of 1% FCS in PBS to the dish and pipet vigorously to remove the bound cells. Examine the dish for any remaining adherent cells as in step 9 and remove them.*

11. Centrifuge adherent and nonadherent populations (separately) 10 min at 1300 rpm (400 × g), 4°C to 10°C. Resuspend cells in complete serum-free RPMI medium. Remove an aliquot of the cell suspension to determine the purity by flow cytometry.

   *The purity of the populations can be increased by panning twice (repeating steps 6 to 11).*
ISOLATION OF T CELL POPULATIONS BY ANTIBODY/COMPLEMENT–MEDIATED CYTOTOXICITY

In this procedure, a T cell preparation is depleted of a T cell population by lysing the latter with antibody and complement. In the example illustrated, CD4+ T cells are depleted by incubation with anti-CD4 antibody, and then with rabbit complement. Cells bearing CD4 that have bound the anti-CD4 are lysed (killed), leaving a cell population containing mainly CD8− T cells, as well as a small number of CD4−, CD8− T cells (γδ T cells).

Additional Materials

- Baby rabbit complement (Cedarlane Laboratories)
- Complete RPMI-1 medium (APPENDIX 2)
- Additional reagents and equipment for cell counting and viability staining (APPENDIX 3) and Ficoll-Hypaque cell separation (UNIT 7.1)

1. Screen baby rabbit complement for the presence of nonspecific toxicity against human mononuclear cells. Determine the concentration of complement that gives maximum antibody-specific cytotoxicity.

   *Freezing and thawing complement destroys its activity.*

   *Lysis of cells by complement in the absence of a specific antibody is known as nonspecific cytotoxicity. This is determined by following the procedure without adding the specific antibody (steps 3 and 4).*

   *The concentration of complement that gives maximum antibody-specific cytotoxicity is generally 20% to 50% (depending upon the source). This can be determined by simultaneously performing the procedure with different dilutions of the complement. The concentration of complement that gives minimal nonspecific cytotoxicity and maximum antibody-specific cytotoxicity is the appropriate concentration to use.*

2. Determine the amount of antibody needed to stain mononuclear cells for flow cytometry.

   *Alternatively, measure the titer of the antibody and make 2-fold serial dilutions. Determine the concentration necessary to kill the cell population in the presence of complement.*

3. Pipet 10^6 to 10^7 T cells into a centrifuge tube. Centrifuge 10 min at 1300 rpm (400 × g), 18° to 20°C. Pipet off supernatant and resuspend cells in appropriate dilution of antibody (as determined in step 2).

   *Controls include cells incubated with the antibody but without complement, cells without antibody but with complement, and cells resuspended in complete RPMI-1 without antibody or complement.*

4. Incubate cells 30 min on ice. Centrifuge 10 min at 1300 rpm (400 × g), 4° to 10°C. Pipet off supernatant.

5. Dilute baby rabbit complement with complete RPMI-1 to the appropriate concentration as determined in step 1. Immediately resuspend cells in this suspension to a concentration of 1 × 10^7 cells/ml. Incubate 1 hr in a 37°C water bath.

   *An aliquot of the cells can be removed to determine the efficiency of cytotoxicity. A single treatment with complement may be insufficient to eliminate all of the positive cells; repeat incubation with antibody and complement if necessary.*

6. Add 10 ml complete RPMI-1 to the cells. Centrifuge 10 min at 1300 rpm (400 × g), 18° to 20°C. Remove supernatant and resuspend pellet in complete RPMI-1. Repeat two times.
7. Remove supernatant and resuspend cells in complete RPMI-1. Count cells and determine viability and percent cytotoxicity, where

\[ \text{% cytotoxicity} = \left( \frac{\text{number of dead cells}}{\text{number of live + dead cells}} \right) \times 100. \]

8. Remove the dead cells by passage on a Ficoll-Hypaque gradient (UNIT 7.1).

Dead cells will pellet at the bottom of the tube; live cells will be the top layer (above the Ficoll-Hypaque layer).

**COMMENTARY**

**Background Information**
Separation of cells based on the differential expression of cell-surface antigens is an important technique that has facilitated the study of specific subpopulations of lymphocytes. Cell separation can be a negative selection process, in which the isolated subpopulation lacks the selected cell-surface antigen, or it can involve positive selection, in which the isolated population has the selected antigen.

The traditional method of negative selection is based on antibody/complement-mediated lysis, which allows the recovery of a population of cells in which a specific subpopulation has been removed (Bianco et al., 1970). The advantage of this method is that the isolated population has not had a cell-surface antigen cross-linked during the procedure. The disadvantage of this method is the loss of the subpopulation undergoing lysis. The method requires that complement-fixing antibody must be used and that the complement be screened for effectiveness and for low background (nonspecific) lysis. The latter may be reduced with the use of baby rabbit complement rather than rabbit serum.

The panning technique can be used to both negatively and positively select a specific subpopulation of cells. It is superior to the antibody/complement lysis technique in that it does not result in the loss of a cell population. In addition, it is used instead of cell sorting of fluorescence-labeled cells when large numbers of cells are desired.

The panning technique was initially described to separate mouse B cell from T cells (Wysocki and Sato, 1978; Mage et al., 1977). In this initial description, polystyrene surfaces coated with anti-mouse Ig were used to separate B cells (which bound to the polystyrene) from T cells (which did not). This method, known as direct panning, required high amounts of affinity-purified antibody or high-titered specific antiserum.

Indirect panning as described here is a sandwich technique that requires lower concentrations of the specific antiserum. This modification makes panning for human T cell subpopulations feasible (Engleman et al., 1982; Payne et al., 1981), but its application is limited to cell suspensions in which the nonselected cells do not spontaneously adhere to plastic. Good separation depends partly on the antibodies used. Not all antibodies successfully separate cells by panning because of differences in affinity and in binding to plastic. The yield and the purity of the subpopulation is compatible with results obtained when cell populations are selected by cell sorting of fluorescence-labeled cells. The purity of the selected populations can be increased by repeated panning.

Recently, magnetic monosized polystyrene microspheres coated with antibodies have been used to remove specific populations from heterogeneous populations. This method is described in UNIT 7.4 and by Padmanabhan et al. (1980).

**Critical Parameters**
For antibody- and complement-mediated cytolysis, it is critical to screen both the antibody and source of complement to ensure depletion of the selected population. The antibody must be a complement-fixing IgG2a or IgM. Freezing and thawing the rabbit serum or rabbit complement destroys the complement.

The purity of the nonadherent and adherent populations obtained by panning is dependent on the thoroughness of the washing regime used to remove the subpopulations from the plate. In general, both populations will be >90% pure, but the nonadherent population will contain more contaminating cells than the adherent population. The purity of the population can be increased by repeating the panning procedure. The purity of the nonadherent population can be increased by employing only the cells initially removed from the panning dish.
Also, if the antibody is complement-fixing, purity of the nonadherent cell population can be increased by eliminating contaminating cells by an incubation with the complement.

The positively selected population is isolated by cross-linking an antibody to a cell-surface antigen. It is important to realize that positive selection may alter the function of the positively selected cell subpopulation. When possible, the function of a specific subpopulation should be compared using both negative and positive panning techniques.

**Anticipated Results**

When using appropriate antibodies, either technique should yield a population of >90% purity and viability. Similar results are obtained when using either commercially available monoclonal antibodies or supernatant containing these antibodies. As determined by flow cytometry (UNITS 5.4 & 7.9), the adherent cells are 97% to 98% pure and the nonadherent cells are 90% to 95% pure.

**Time Considerations**

Antibody- and complement-mediated cytolysis takes ~2 hr. If the complement incubation must be repeated in order to obtain maximum purity, another hour is required. Indirect panning requires 2 to 3 hr.

**Literature Cited**


**Key Reference**


Reports in detail the procedure for both direct and indirect panning.

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