Immunomagnetic Purification of T Cell Subpopulations

This unit describes a general method for physical separation of cell subpopulations from a heterogeneous mixture of cell types. The technique relies on having an appropriate monoclonal antibody or mix of monoclonal antibodies that distinguish between the cell types being separated. Its advantages over other antibody-mediated selection techniques are purity of resulting cell preparation, reproducibility of separation, and ease of handling small to large numbers of cells (10^6 to 10^{10} cells). The basic protocol outlines preparation of purified human T lymphocytes. The method gives good yields of pure T cells prepared by negative selection (i.e., not labeled with antibody). The alternate protocol applies similar principles for purifying $CD4^+$ T cells, and presents refinements of the basic protocol that minimize cost and improve convenience.

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.

IMMUNOMAGNETIC NEGATIVE SELECTION OF T CELLS FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

BASIC PROTOCOL

In this protocol, T cells are isolated by depletion of all non–T cells (B cells, macrophages, and natural killer cells). Peripheral blood mononuclear cells (PBMC) are incubated with a mixture of monoclonal antibodies to coat unwanted cells. Suspensions are then incubated with magnetic beads coated with goat anti-mouse (IgG), which bind the antibody-coated cells. Subsequent exposure to a strong magnetic field removes the unwanted bead-coated cells, leaving behind the desired cell population. Cells from spleen, lymph node, tumor, and peritoneal fluid can also be used.

Materials

Appropriate monoclonal antibodies (10× MAb mix including CD14, CD16, CD20 and anti-glycophorin; Table 7.4.1 and reagents and solutions)
Peripheral blood mononuclear cells after Ficoll-Hypaque separation (PBMC; *UNIT 7.1*)
Coating medium, room temperature and 4°C, freshly prepared
Goat anti-mouse IgG–coated magnetic beads (Dynabeads M-450, Dynal #11005 and #11006; see critical parameters)

15-ml polypropylene tube (e.g., Falcon #2059)
Sorvall RT 6000 centrifuge with H-1000B rotor (or equivalent)
Magnetic separation apparatus (Dynal MPCI #12001 or Advanced Magnetic #41025) *or* rare earth cobalt magnets (Edmund Scientific #A30964)

Additional reagents and equipment for preparation and titration of monoclonal antibodies (*UNIT 2.5*), counting cells (*APPENDIX 3*), flow cytometry (*UNITS 5.4 &.7.9*), and cryopreservation of cells (*APPENDIX 3*)

NOTE: Perform all steps at 4°C on ice or in a cold room under sterile conditions. All resuspensions of cells and washing of magnetic beads are done using coating medium.

1. Prior to initiation of the separation procedure itself, choose the appropriate combination of antibodies for selection of T cells and determine the concentrations to be used. A guide to antibody selection is given in Table 7.4.1.

   For preparing total T cells, include antibodies specific for CD14 (on monocytes),

Table 7.4.1 Examples of Antibodies Used to Label Cells to be Removed

Contaminating cell types	Receptor[a]	Monoclonal antibody and source[b]
Monocytes	CD14	MMA (ATCC) My4 (Coulter) Leu-M3 (Becton Dickinson) 63D3 (ATCC)
	CD11b[c]	LM2/1 (ATCC) M1/70 (ATCC)
Natural killer	CD16	Leu-11 (Becton Dickinson)
RBC	Anti-glycophorin	10F7 (ATCC)
B cells	CD20	B1 (Coulter) Leu-16 (Becton Dickinson)
CD8	CD8	OKT8 (Ortho) Leu-2 (Becton Dickinson)

[a]See also Table A.4A.1 (*APPENDIX 4*) for complete listing of CD molecules.

[b]Addresses and phone numbers of suppliers are provided in *APPENDIX 5*.

[c]Optional, depending on precise cell population desired.

CD16 (on natural killer cells) and CD20 (on B cells). Anti-glycophorin MAb may be included to remove contaminating RBC; this depends on the degree of contaminating erythrocytes in the starting cell population and the need for RBC exclusion.

2. Following selection of monoclonal antibodies, determine the saturating concentration of each in preliminary flow cytometry studies of the relevant MAb on PBMC. With this information in hand, prepare a 10× MAb mix.

   Generally 1 μg/ml per 1×10^6 cells is a saturating concentration; however, pre-titration may allow use of a 10-fold lower concentration.

3. In a 15-ml polypropylene tube, resuspend PBMC in coating medium at 2×10^7 cells/ml (e.g., 2×10^8 cells in 10 ml coating buffer).

   Perform all incubations with cells at this concentration.

4. Add 1 ml of 10× MAb mix (from step 1) to 10 ml cell suspension. Incubate on a rotator with end-over-end rotation, 30 min at 6 to 10 rpm, 4°C.

   If the monoclonal antibodies have not been premixed, add them individually at saturating concentrations.

   Rotation keeps the cells from forming a pellet during incubation; however, some workers have obtained satisfactory results in its absence.

5. Wash cells to remove any unbound MAb. Centrifuge 10 min in Sorvall H-1000B rotor at 1000 rpm ($150 \times g$), 4°C. Resuspend in cold, freshly prepared coating medium, filling tube. Repeat wash a second time, resuspend to 10 ml, and transfer to a new tube.

6. Wash 1 ml of anti-IgG-coated magnetic beads as follows. Vigorously resuspend the bead suspension in the stock vial, then transfer 1 ml to a 15-ml polypropylene tube. Add coating medium, agitate, then pull beads to the side of tube with magnetic apparatus. After beads have accumulated adjacent to magnet (allow ~5 min), aspirate the medium with an aspirator tip placed at the bottom of the tube. Repeat medium addition, agitation, and removal. Resuspend the beads in 1 ml coating medium.

   The tube can be placed in a magnetic stand, or a strong rare earth magnet can be held in apposition to the tube by an elastic band.

7. Add the 1-ml suspension of washed beads to cell suspension from step 5 and rotate 1 hr at 6 to 10 rpm, 4°C.

   Rotation must be vigorous enough to maintain cells and beads in suspension, and gentle enough not to damage cells. It is helpful to examine an aliquot of the suspension microscopically to observe the beads rosetting around some cells but not others.

8. Separate the cells labeled with MAb and coated with beads using the magnetic apparatus or strong magnet as in step 6. After 5 min, transfer the unbound cell suspension to a fresh tube and perform a second magnetic separation. Count the cells microscopically and resuspend in coating medium at 2×10^7 cells/ml.

   It is not usually necessary to readjust the cell concentration after the first bead separation, as only a small fraction of the cells are generally removed.

9. Repeat steps 6 to 8 if complete depletion of monocytes is necessary.

   The need for a second round of bead depletion is determined by the purity required at the end of the preparation. For many studies of T cell function, as few as 1 monocyte/10,000 T cells represents a significant contamination. It is not usually necessary to repeat the MAb incubation because antibodies of reasonable affinity will remain bound.

10. Analyze the cell population by flow cytometry to assess purity.

    Suboptimal purity may result from inadequate coating of the cells with MAb, or inadequate removal of MAb-coated cells. When checking for inadequate removal of MAb-coated cells, be sure to include controls (see critical parameters).

11. Cryopreserve cells or use immediately for phenotypic or functional analysis.

    Storing cells 12 to 24 hr at 4°C does not seem to impair cellular functional response.

IMMUNOMAGNETIC NEGATIVE SELECTION OF $CD4^+$ T CELLS

ALTERNATE PROTOCOL

In this protocol, the $CD4^+$ T cell subpopulation of PBMC is selected. The procedure includes an overnight incubation that will allow a shorter working day, and uses less expensive magnetic particles than the basic protocol for the initial round of negative selection. A more complex mixture of selecting monoclonal antibodies is also employed.

Additional Materials

10× MAb mix including receptors for CD14, CD16, CD20, anti-glycophorin, CD8, and CD11b (Table 7.4.1 and reagents and solutions)
Goat anti-IgG coated magnetic particles (Advanced Magnetics #4340)

1. Choose appropriate antibodies for $CD4^+$ T cell selection.

   Include all the antibodies used in basic protocol and add MAb specific for CD8 (on $CD8^+$ cells). A CD11b MAb can be added that binds to a subset of $CD8^+$ cells as well as monocytes and NK cells, to further guarantee satisfactory depletion of those contaminating cells (see also APPENDIX 4).

2. Determine optimal MAb concentrations as in the basic protocol, step 2, and prepare 10× MAb mix.

3. Coat unwanted cells in the cell preparation with the MAb mix as in the basic protocol, steps 3 to 5.

4. Wash 6 ml of anti-IgG Advanced Magnetic particles as described in the basic protocol (step 6) for Dynal beads, except allow ~15 min for them to migrate to the magnet. After washing, reconstitute in 1/6 vol (1.0 ml) to maintain the same efficacy per volume as the Dynal bead suspension.

5. Add the washed particles to the cell suspension (from step 5 of basic protocol) and rotate overnight at 6 to 10 rpm, 4°C.

   Overnight incubation is convenient because steps 1 to 5 may be performed one day and subsequent steps the next morning. This convenience compensates for the longer time required for Advanced Magnetic particles to bind to the MAb-coated cells.

6. Magnetically remove the cells labeled with MAb and coated with particles as in step 8 of the basic protocol—allowing 20 min, instead of 5, for migration of the cells toward the magnet.

7. Centrifuge the cells 10 min at 1000 rpm (150 × *g*), 4°C. Discard the supernatant. Resuspend the pellet in 2 ml coating medium and count the viable cells microscopically. Add coating medium to bring to 2×10^7 cells/ml (~1 ml).

   Because a greater number of cells are depleted, the cell concentration will be considerably lower in this preparation than in the basic protocol. Expected yield is 6-7 × 10^8 cells (20% to 35% of the starting cell number).

8. Prepare for the second round of magnetic depletion by washing coated Dynal magnetic beads as in the basic protocol, step 6. Approximately 50 µl of suspended Dynal beads are required for every milliliter of cell suspension.

   Few remaining PBMC are target cells, as the majority will have been removed by the first step. Empirically, one Dynal bead per remaining cell is required. This is also true for the basic protocol, but for simplicity this refinement has not been employed.

9. Add washed and suspended beads to cell suspension and rotate 1 hr at 6 to 10 rpm, 4°C.

10. Magnetically remove the cells labeled with MAb and coated with beads as in step 8 of the basic protocol.

11. Analyze the cell population by flow cytometry to assess purity.

REAGENTS AND SOLUTIONS

Coating medium

Hanks balanced salt solution (HBSS; APPENDIX 2) without Ca^{++}, Mg^{++}, or phenol red, containing:

10% fetal calf serum (Biofluids #200; heat-inactivated 1 hr, 56°C)
20 mM HEPES (GIBCO/BRL #380-5630)
Store at 4°C

Monoclonal antibodies

Ascites fluid, purified protein, or culture supernatant may be used as the MAb (see Table 7.4.1 for recommended monoclonal antibodies and sources). Good ascites preparations are diluted 3000-fold before use and preservatives (such as azide) are removed by dialysis (*APPENDIX 3*). Premix the appropriate antibodies (Table 7.4.1) to ten times the final concentration—e.g., prepare a mixture containing 300-fold dilutions of ascites fluids containing antibodies specific for CD14, CD16, CD20, and glycophorin. Many antibodies are damaged by repeated freezing and thawing; therefore, it is best to maintain the mixture at 4°C under sterile conditions. Most monoclonal antibodies are stable for several months at 4°C.

COMMENTARY

Background Information

Isolation of lymphocyte subpopulations is a prerequisite to studying them phenotypically and functionally. There are a variety of available techniques, many of which are based on antibody-mediated selection—e.g., immunomagnetic selection, panning on immobilized monoclonal antibody (*UNIT 7.3*), antibody/complement-mediated lysis (*UNIT 7.3*), and cell sorting of fluorescence-labeled cells (*UNITS 5.4 & 7.9*). The most refined method is the cell sorting; however, the low yield of cells resulting from this technique is severely limiting. Complement-mediated lysis has the disadvantage of requiring a good complement-fixing monoclonal antibody as well as complement. Immunomagnetic selection and panning are very similar in concept, differing only in the means of antibody immobilization (plastic surface vs. magnetic beads, respectively). The immunomagnetic approach is preferred for negative selection, as it offers increased ease of handling of large numbers of cells as well as purity of the populations recovered. Greater purity may result from the uniformly low shear conditions under which the negatively selected cells are recovered using magnetic beads.

The procedures described in this unit are for negative selection, although the approach has also been used to positively select for lymphocyte subsets (Gaudernack et al., 1986). Two disadvantages to positive selection are that beads cannot be removed easily and must be carried along with the cells, and that antibody binding to the positively selected cells may affect their subsequent functional responses.

Immunomagnetic selection can be adapted to isolate any desired lymphocyte subpopulation or, indeed, other cell types (e.g., hematopoietic cells, Langerhans' cells, and transfected cells). The only limitation in this regard is the availability of appropriate antibody mixtures that will effectively remove all possible contaminating cells (Hansel et al., 1988; Horgan et al., 1990). An example of the "creative" use of the immunomagnetic protocol is the addition of an anti–major histocompatibility complex (MHC) class II monoclonal antibody to the mixture to assure that the isolated subpopulation consists of resting T cells (the anti–MHC class II monoclonal antibody will eliminate T cells that express this marker of T cell activation).

Other kinds of bead preparations and protocols are possible, but they are not well suited to the separations outlined here. For example, it is possible to link the antibody directly to the beads, rather than linking via anti-Ig, or to precoat the beads rather than the cells with antibody. These are poor strategies when negatively selecting subsets from complex cell mixes in which the combination of determinants on particular unwanted cells may vary.

Critical Parameters and Troubleshooting

Throughout these procedures, manipulations should be performed at 4°C to minimize capping and shedding of monoclonal antibody–bound molecules.

Appropriate IgG antibodies must be added in saturating concentrations in the monoclonal antibody mixture, so all unwanted cells are labeled by antibody and will be coated by the beads and removed. Ideally, the monoclonal antibody should bind to the target cells to be removed and not bind to the desired cell population. Immunoglobulin G–monoclonal antibodies are preferable because goat anti-serum with which the beads are coated is raised against mouse IgG. Therefore the resulting antiserum (and bead) reactivity with IgM has been found to vary between preparations (C. June, pers. comm.). The antibody on the beads may also react with κ light chains, making κ-type IgG–monoclonal antibody preferred reagents.

Complete removal of unbound antibody is also necessary. Following the antibody incubation, it is vital to wash cells at least twice to remove unbound antibody so that it does not occupy the anti-IgG binding sites on the beads.

Approximately seven Dynal beads per target cell are needed for negative selection when two sequential depletions are performed. Because ~30% of the PBMC are target cells (i.e., non–T cells coated with monoclonal antibody which will be bound by the beads and removed) two beads are needed for every cell in the sample. Thus, for 2×10^8 starting cells, 4×10^8 beads are needed. This is equivalent to 1 ml of bead suspension (as prepared in step 7).

Two sequential bead depletions at modest bead/target ratios (e.g., 7:1 using Dynal beads) are cheaper and at least as effective as single depletions at much higher bead/target ratios (e.g., 40:1 as recommended by Dynal; Hansel et al., 1989, and Funderud et al., 1987). This is because during the first depletion, the beads tend to be taken up by cells with the highest density of bound antibody, resulting in neglect of the cells with less bound antibody; adding

additional beads at this step results in further overloading of the high-density coated cells. By removing these high-density coated cells in the first step, only the low-density coated cells remain to be efficiently removed in the subsequent step.

Advanced Magnetic particles are designed for applications in ligand-binding assays and are similar to Dynal magnetic beads except that they are about 1/10 the diameter (0.5-µm versus 4.5-µm average diameter, respectively) and are irregular in shape rather than spherical. The Advanced Magnetic particles are less expensive than the Dynal beads, but their size dictates that procedures be done more slowly. In addition, they seem less able to remove cell populations that bind antibody weakly. Therefore, in the alternate procedure, the bulk of unwanted cells are removed in a first depletion step using Advanced Magnetics particles, while any residual unwanted cells are removed in a second step with Dynal beads. This regimen reduces the cost of bead separation by 75%, compared with the basic protocol in which only Dynal beads are used.

In the alternate protocol, about 60% of the PBMC are target cells (i.e., non–T cells and $CD8^+$ T cells coated with monoclonal antibody, which will be bound by the particles and removed). Every target cell requires seventy Advanced Magnetics particles for the first round of negative selection; therefore, ~40 Advanced Magnetic particles are needed for each cell in the preparation. For 2×10^8 starting cells, 8×10^9 particles are required, which translates to 6 ml of particle suspension.

Adequate time must be allowed for magnetic removal of the beads. Advanced Magnetic particles require ≥20 min versus 5 min with the Dynal beads. Adequate bead removal may be assessed microscopically or visually (the pellet will appear gray).

When checking for inadequate removal of monoclonal antibody–coated cells by flow cytometry, include controls without antibody, plus and minus FITC-conjugated anti-IgG. If the FITC-conjugated anti-IgG control stains brighter than the control without it, the preparation still contains coated cells (those detected by the conjugation reagent). In this case, depletion steps 7 to 9 were incomplete.

To assess overall antibody coating, stain the cell suspension with CD3 monoclonal antibody (*UNIT 5.3*). If CD3-negative cells are present, check for contaminating cell types by staining for specific markers on B cells, natural killer cells, and monocytes. (For example, use CD20, CD16, and CD14 monoclonal antibodies individually.) If cells are detected by staining with these markers, but not with FITC-conjugated anti-Ig alone, the antibody coating of that cell type was unsatisfactory. In this case, repeat coating steps 5 and 6.

For the alternate protocol, include additional antibodies to assess the purity of the $CD4^+$ cells and the exclusion of any $CD8^+$ cells.

Anticipated Results

The isolated cells are >98% pure with regard to T cell content and do not respond to lectin in the absence of additional monocytes. The yield is consistently 20% to 35% of the starting number for $CD4^+$ T cells and 30% to 50% for pure T cells.

Time Considerations

The basic protocol from initial PBMC suspension to cells ready for flow cytometry requires ~6 to 8 hr. The alternate protocol (with overnight incubation of the first batch of beads) requires 2 to 3 hr the first day and 3 to 4 hr the next day.

Literature Cited

Funderud, S., Nustad, K., Lea, T., Vartdal, F., Guadernack, G., Stensted, P., and Ugelstad, J. 1987. Fractionation of lymphocytes by immunomagnetic beads. *In* Lymphocytes: A Practical Approach (G.G.B. Klaus, ed.) pp. 55-61. Oxford University Press, New York.

Gaudernack, G., Leivestad, T., Ugelstad, J., and Thorsby, E. 1986. Isolation of pure functionally active $CD8^+$ T cells. Positive selection with monoclonal antibodies directly conjugated to monosized magnetic microspheres. *J. Immunol. Methods* 90:179-187.

Hansel, T.T., Pound, J.D., Pilling, D., Kitas, G.D., Salmon, M., Gentle, T.A., Lee, S.S. and Thompson R.A. 1989. Purification of human blood eosinophils by negative selection using immunomagnetic beads. *J. Immunol. Methods* 122:235-241.

Horgan, K.I., Van Seventer, G.A., Shimizu, Y., and Shaw, S. 1990. Hyporesponsiveness of "naive" ($CD45RA^+$) human T cells to multiple receptor-mediated stimuli but augmentation of responses by co-stimuli. *Eur. J. Immunol.* 20:1111-1118.

Key Reference

Funderud, et al., 1987. See above.

Comprehensive description of magnetic bead technique.

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