Isolation of Dendritic Cells

This unit presents two methods for preparing dendritic cells (DCs), a highly specialized type of antigen-presenting cell (APC; see Background Information). The first method (see Basic Protocol 1) involves the isolation of DCs from mouse spleen, resulting in a cell population that is highly enriched in accessory cell and APC function. Collagenase digestion of splenocyte suspensions (see Support Protocol) can be used to increase the yield of dendritic cells. If it is only necessary to enrich APC function in general, protocols in *UNITS 3.12 & 3.15* can be utilized. The second method involves generating large numbers of DCs from mouse bone marrow progenitor cells (see Basic Protocol 2). In that technique, bone marrow cells are cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) to yield 5–10 $\times$ 10^6 cells, 60% of which express DC surface markers (e.g., B-7-2/CD86). Additional techniques for isolating DCs from mouse spleens or other mouse tissues, as well as from human tissues, can be found in Table 3.7.1.

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

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless noted otherwise.

Table 3.7.1 Tissues from Which Dendritic Cells Have Been Isolated

Tissue	Source	Reference
Lymphoid organs		
Spleen	Mouse	Steinman et al., 1979; Macatonia et al., 1989; Vremec et al., 1992
Lymph node	Rat	Klinkert et al., 1982
Tonsil	Human	Hart and McKenzie, 1988
Peyer's patch	Mouse	Spalding et al., 1983
Thymus	Mouse	Kyewski et al., 1986; Crowley et al., 1989
	Rat	Wong et al., 1982
	Human	Landry et al., 1990
	Avian	Guillemot et al., 1984
Circulation		
Blood	Human	Freudenthal and Steinman, 1990
Afferent lymph	Rat	Pugh et al., 1983; Mayrhofer et al., 1986
	Rabbit	Kelly et al., 1978; Knight et al., 1982
	Sheep	Bujdoso et al., 1989
	Mouse	Rhodes and Agger, 1987
Nonlymphoid organs		
Skin	Mouse	Schuler and Steinman, 1985
	Human	Romani et al., 1989
Liver	Rat	Klinkert et al., 1982; Lautenschlager et al., 1988
Synovial exudate	Human	Zvaifler et al., 1985
Lung	Rat	Holt et al., 1987; Rochester et al., 1988
	Human	Nicod et al., 1987
	Mouse	Pollard and Lipscomb, 1990
Gut	Mouse	Pavli et al., 1990

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BASIC PROTOCOL 1

ENRICHMENT OF DENDRITIC CELLS BY PLASTIC ADHERENCE AND EA ROSETTING

This technique for isolating dendritic cells has been used with only minor modifications for many years (Steinman et al., 1979). A collagenase-digested splenocyte suspension is centrifuged in dense bovine serum albumin to obtain a fraction with a low buoyant density. Plastic-adherent cells from this fraction are primarily dendritic (~80%) but other cell types such as macrophages and B cells are present. Dendritic cells become nonadherent after overnight culture and can be further purified (>95%) by depleting cells expressing Fc receptors in an approach known as EA rosetting, where erythrocytes coated with antibody bind to contaminating macrophages and B lymphocytes.

Materials

Collagenase-digested splenocyte suspension (see Support Protocol)
Dense bovine serum albumin (BSA) solution (see recipe)
RPMI 1640 medium (e.g., Life Technologies), 4° and 37°C
Complete RPMI-5 medium (see recipe), 4° and 37°C
Antibody-coated sheep erythrocytes (EA; see recipe)
Primary antibody for flow cytometry analysis (Table 3.7.2)
Secondary antibody for flow cytometry analysis (fluorescein-conjugated mouse anti-rat IgG and IgM; e.g., Boehringer Mannheim)

Beckman GH-3.7 and Sorvall HS-4 rotors (or equivalents)
15- and 50-ml conical polypropylene tubes
Autoclaved 9-in. (~23-cm) Pasteur pipets, plugged with cotton and unplugged
60-mm-diameter tissue culture dishes (e.g., Falcon)

Additional reagents and equipment for counting viable cells (*APPENDIX 3B*) and for flow cytometry (*UNITS 5.3 & 5.4*)

Prepare low-density splenocytes

1. Centrifuge collagenase-digested splenocyte suspension 10 min at 280 × *g* (1200 rpm in Beckman GH-3.7 rotor), 4°C, and aspirate the supernatant. Promptly resuspend the cell pellet in dense BSA, ~1 ml per spleen.

   Prompt resuspension reduces adherence and increases yield.

Table 3.7.2 Rat Monoclonal Antibodies Useful in Routine Flow Cytometry of Murine Splenic Dendritic Cells

Specificity[a]	Hybridoma	Antibody type	ATCC no.	Reference
Lymphoid DC	33D1	IgG2b	TIB 227	Nussenzweig et al., 1982
Thy-1.2	B5-5	IgG2b	—	Nussenzweig and Steinman, 1980
CD32 (FcγRII)	2.4G2	IgG2b	HB 197	Unkeless, 1979
CD45RA (B220)	RA3-3A1/6.1	IgG2a	TIB 146	Coffman and Weissman, 1981
Macrophage	SER-4	IgG2a	—	Crocker and Gordon, 1989
Macrophage	F4/80	IgG2b	HB 198	Austyn, 1981
MHC-I	M1/42	IgG2a	TIB 126	Springer, 1980
MHC-II (I-A/E)	M5/114	IgG2b	TIB 120	Bhattacharya et al., 1981
CD8	53-6.7	IgG2a	TIB 105	Ledbetter and Herzenberg, 1979
Interdig. cells	NLDC-145	IgG2a	—	Kraal et al., 1986

[a]Abbreviations: DC, dendritic cells; interdig., interdigitating.

2. Prepare BSA columns by transferring 5 to 6 ml of cell suspension into a 15-ml conical tube and carefully overlaying with ~1.5 ml of 4°C RPMI-1640 medium, forming a sharp interface. Prepare four to five columns in this manner, until the full volume of cell suspension has been dispensed.

3. Centrifuge the BSA columns 15 min at 9500 × *g* (7000 rpm in Sorvall HS-4 rotor), 4°C, using slow acceleration and keeping the brakes turned off.

4. With a plugged Pasteur pipet, carefully collect the cells from the interface region, taking the full volume of RPMI-1640 and the top 1 ml of BSA. Pool the cells from BSA columns into a single fresh 50-ml conical tube (discard the BSA pellets). Fill the 50-ml tube with 4°C RPMI-1640 to dilute the BSA and mix by gentle inversion.

5. Centrifuge 10 min at 280 × *g*, 4°C, and aspirate the supernatants. Resuspend the cells in 5 to 10 ml complete RPMI-5 medium per interface and place them on ice.

6. Count viable cells by trypan blue exclusion (*APPENDIX 3B*).

   One spleen should produce ~10^7 low-density splenocytes.

Enrich dendritic cells by plastic adherence

7. Adjust the cell density to ~10^7 cells/ml using complete RPMI-5. Plate 4 ml of this suspension per 60-mm tissue culture dish until the entire volume has been plated. Incubate the dishes 90 min, allowing the dendritic cells to adhere.

   Plating more densely than ~4×10^7 cells per dish reduces adherence and yield.

8. With a plugged Pasteur pipet, remove and discard nonadherent cells by gently washing the surface of each dish with 37°C RPMI-1640 until all regions of its surface change from rough and turbid to smooth and nearly clear. Repeat this wash once.

9. Cover each dish with 4 ml fresh 37°C RPMI-1640 and incubate 30 to 60 min to release additional contaminating lymphocytes. Repeat the wash as in step 8.

10. View the dish by phase contrast under an inverted microscope. A majority of dark stellate dendritic cells, some bright flattened macrophages, and round cells that include both lymphocytes and monocytes should be seen. If dendritic cells are not predominant, repeat the wash and incubation in steps 8 and 9.

11. Remove the medium from each washed dish, replace it with 4 ml fresh complete RPMI-5, and incubate the dishes for 12 to 20 hr.

    During this incubation, initially adherent dendritic cells (and some contaminating macrophages) detach.

12. With a plugged Pasteur pipet, wash the surface of each dish with 37°C supplemented RPMI-5. Pool the eluted cells into 15-ml conical tubes (about three dishes per tube) on ice. Rinse each dish with ~2 ml complete RPMI-5 and pool the rinses. Repeat until all dishes have been harvested.

13. Centrifuge the collected cells 10 min at 280 × *g*, 4°C, and discard the supernatant. Resuspend the cell pellet in 1 ml fresh complete RPMI-5 per tube and place on ice.

14. Count viable cells by trypan blue exclusion.

    The usual yield is 0.5–1.0 × 10^6 cells per spleen. Dendritic cells should comprise ~80% of the population. While this purity may suffice for many kinds of experiments, dendritic cells may be further purified by EA rosetting, as described below.

Enrich dendritic cells by EA rosetting

15. Add 50 EA (antibody-coated SRBC) per leukocyte and mix well by inversion. Centrifuge 10 min at 70 × *g* (~600 rpm in GH-3.7 rotor), 4°C. Without aspirating the supernatant medium, place the tube containing the cell pellet on ice 30 min.

    During this period, B lymphocytes and macrophages bind the EA via their surface Fc receptors.

16. Remove all but 2 to 2.5 ml of the medium. Using a plugged Pasteur pipet, very gently pipet the cell suspension up and down 10 to 15 times to break up large aggregates without disrupting the rosettes.

    Check the appearance of the resuspended rosettes on a hemacytometer to confirm that rosette size is appropriate for a single white cell surrounded by red cells. If necessary, pipet the suspension again.

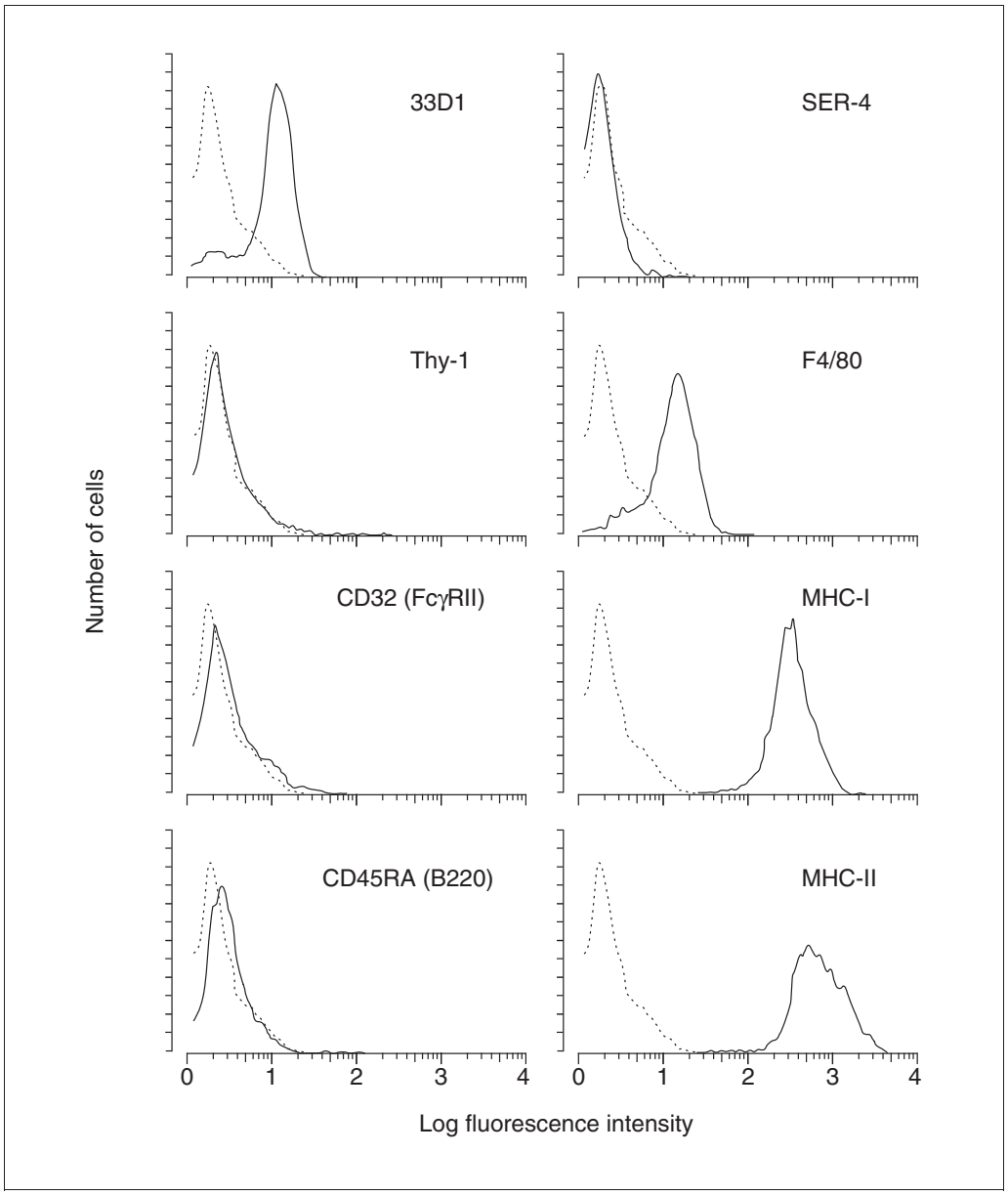

Figure 3.7.1 Flow cytometric analysis of murine spleen dendritic cells after purification by EA rosetting. Dashed lines: no primary antibody; solid lines: primary staining with the indicated hybridoma supernatant (see Table 3.7.2).

17. Prepare BSA columns by pipetting 5 ml of dense BSA into a 15-ml conical tube and carefully overlaying with ~2.5 ml of rosette suspension, forming a sharp interface.

18. Centrifuge the columns and collect interface cells as in steps 3 to 6 above.

    This suspension should contain >95% dendritic cells, with a yield of 2–7 × 10^5 cells per spleen.

19. Determine purity of dendritic cells by flow cytometry using a primary antibody chosen from Table 3.7.2. For the secondary antibody, use fluorescein-conjugated mouse anti-rat IgG and IgM.

    Table 3.7.2 identifies primary antibodies specific for the main features of the surface-antigen phenotype of dendritic cells, and the major contaminating cell populations in routine purifications.

    Results of a typical flow cytometric analysis of purified dendritic cells stained with a panel of rat MAbs and FITC-mouse anti-rat Ig are shown in Figure 3.7.1. More detailed analyses of dendritic cell-surface antigens are available elsewhere (Crowley et al., 1989; Metlay et al., 1990).

    While the purity of dendritic cells can be estimated using a variety of techniques—including hemacytometer counts and immunostaining after cytocentrifugation onto glass slides (Crowley et al., 1989)—flow cytometry (UNITS 5.3 & 5.4) offers superior quantitation and consistency (Crowley et al., 1989; Metlay et al., 1990).

PREPARATION OF COLLAGENASE-DIGESTED SPLENOCYTE SUSPENSION

SUPPORT PROTOCOL

Collagenase digestion of splenocyte suspensions increases the yield of dendritic cells 2-to 3-fold over disruption of spleens by nonenzymatic means (*UNIT 3.1*). This treatment also helps release dendritic cells that lie within periarterial sheaths (see commentary).

Materials

4000 U/ml collagenase D (see recipe), thawed and placed on ice
Hanks' balanced saline solution (HBSS), sterile, with Ca^{2+} and Mg^{2+} (*APPENDIX 2*; may also be purchased from Life Technologies)
Mouse spleens (*UNIT 1.9*)

Hypodermic needles, 22-G × 1½-in. (Becton Dickinson)
10- and 5-ml disposable syringes (e.g., Becton Dickinson)
100-mm-diameter petri dishes (e.g., Falcon)
Two serrated, semimicro dissecting forceps, autoclaved (e.g., Roboz)
Autoclaved stainless-steel screen: cut 5 × 5–cm squares from 40-mesh stainless steel wire cloth, fold cut edges underneath, then bend up all 4 sides to form a shallow rectangular bowl; wrap in foil before autoclaving

1. Dilute two 1-ml aliquots of 4000 U/ml collagenase D as follows: 1 ml into 9 ml of HBSS (to 400 U/ml, to be used in step 8), and 1 ml into 39 ml of HBSS (to 100 U/ml). Place on ice.

   The indicated volumes are sufficient for twenty spleens.

2. Attach a 22-G needle to a 10-ml syringe and fill the syringe with 100 U/ml collagenase. Place ~10 ml of the 100 U/ml solution into a 100-mm petri dish.

3. Perform work over a second, dry 100-mm petri dish. Hold the forceps in the nondominant hand and hold the syringe in the dominant hand, with the thumb on the plunger. Pick up a mouse spleen with the forceps and pierce the capsule at its

narrowest edge with the needle. Inject ~100 µl of 100 U/ml collagenase. Using the forceps to push the spleen onto the needle, advance the needle a few more millimeters. Repeat the collagenase injection, continuing until ~1 ml collagenase has been injected and the needle has tracked through the whole spleen, emerging at its opposite end.

The spleen will distend and will change from dark maroon to reddish-orange. In places, the capsule will be clear.

4. Tear open the spleen with the needle and transfer it into the petri dish containing the collagenase solution (step 2). Repeat until all spleens have been injected and torn.

5. Collect the cell suspension from the second dish and pool into a 50-ml conical tube on ice. Rinse the dish with ~3 ml of 100 U/ml collagenase and add to the 50-ml tube.

6. Place ~3 ml of 100 U/ml collagenase into the dish. One by one, transfer the torn spleens into the dish. Using two forceps, tease them into small fragments ~1 mm on a side, small enough to pass through the bore of a 5-ml serological pipet. When all torn spleens have been teased, transfer the collagenase solution from the dish in which the spleens were standing into the dish with the fragments. Pipet vigorously up and down several times with a 5-ml pipet.

7. Tilt the dish and, leaving the larger fragments behind, remove the fine cell suspension, pooling it into the iced 50-ml tube from step 5. Rinse both dishes with a few milliliters of 100 U/ml collagenase and add the rinses to the tube.

8. To the spleen fragments remaining in the dish, add the 10 ml of 400 U/ml collagenase. Pipet up and down several times, then place the dish in the incubator for 30 to 90 min.

9. At the end of the incubation, pipet the fragments vigorously up and down, then pipet them onto a sterile screen in a fresh 100-mm petri dish.

10. Hold one edge of the screen with a forceps. Rinse the fragments with a few milliliters of 100 U/ml collagenase, then press the fragments into the screen using the sterile plunger from a 5-ml syringe. Mash and smear until all red color has been removed from the tissue in the screen. Rinse the screen with a few milliliters of 100 U/ml collagenase.

11. Remove the screen from the dish and discard the colorless adherent capsule fragments. Vigorously pipet the fine suspension up and down, then transfer it into the 50-ml tube from step 7. Rinse the dish with ~5 ml of 100 U/ml collagenase and add to the tube.

Dendritic cells should comprise only ~0.5% or less of the viable leukocytes in this suspension, which is now ready for centrifugation in dense BSA as described in Basic Protocol 1.

GENERATION OF DENDRITIC CELLS FROM PROLIFERATING MOUSE BONE MARROW PROGENITORS

This protocol (Inaba et al., 1992) overcomes a major obstacle to prior work with DCs—i.e., the limited numbers that are available as "endogenous APCs" in all tissues that have been examined. Bone marrow progenitor cells are cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) to stimulate proliferation and maturation of DCs. Once generated, the large numbers of DCs can be used for cell biology studies, genetic modification, and in vivo immunization. This method has also been important for studying the biology of DC development.

Materials

Mice, 6 to 7 weeks old (preferably male)
70% ethanol
RPMI-1640 medium (e.g., Life Technologies), ice-cold and room temperature
Ammonium chloride solution (*UNIT 2.5*)
Antibodies (hybridoma supernatants) for lysing lymphocytes (optional)—e.g: hybridoma RA3-3A1 (for MAb to B220; ATCC #TIB 146), GK1.5, (for MAb to CD4; ATCC #TIB 207), 3.155 (for MAb to CD8; ATCC #TIB 211), and M5/114 (for MAb to MHC II; ATCC #TIB 120)—also see *UNIT 2.5*
Rabbit complement (Pel-Freez)
Complete RPMI-5 medium (see recipe)
Mouse GM-CSF (mGM-CSF): either purified recombinant proteins or conditioned medium from cell lines transduced with the mouse GM-CSF gene (gift of Dr. A. Lanzavecchia, Basel, Switzerland)
Antibodies for flow cytometric detection of DCs: e.g., hybridoma supernatant for MHC class II (ATCC #T1B120), hybridoma supernatant for B7-2/CD86 (Pharmingen), and MAb NLDC-145 for DEC-205 (ATCC #HB 290)

Dissecting equipment
Sterile gauze pads
100-mm petri dishes (e.g., Falcon)
3 ml syringes with 25-G, 5/8-in. (1.58-cm) needles
9-in. (~23-cm) Pasteur pipets, plugged with cotton and autoclaved
Nytex filters (Mesh 3-40/26; Tetko)
15- and 50-ml conical polypropylene tubes
24-well tissue culture plates (e.g., Corning)

Additional reagents and equipment for preparation of mouse bone marrow cells (*UNIT 6.4*, Support Protocol 1), counting viable cells (*APPENDIX 3B*) and flow cytometry (*UNITS 5.3 & 5.4*)

Prepare bone marrow suspension

1. Remove femurs and tibias from mice and keep in RPMI-1640 medium on ice until all the mice have been prepared. Remove the muscles from the bones (usually on a sterile gauze pad) and place the clean bones in a new petri dish. Finally immerse the bones for 2 min in a petri dish containing 70% ethanol, then wash twice with ice-cold RPMI-1640 (also see *UNIT 6.4*, Support Protocol 1).

   Male mice are preferred because they have bigger bones and therefore yield larger numbers of progenitor cells.

2. Cut off both ends (epiphyses) of each bone with a scissors and transfer to a separate dish. Obtain the marrow by flushing out each of the shafts with 2 ml RPMI-1640 using a syringe. Mince the epiphyses in a separate dish. Resuspend the minced epiphyses together with the marrow plugs from the bone shafts, breaking up the

clumps with Pasteur pipets. Pass the suspension through Nytex mesh (to remove particles) into a 15- or 50-ml collection tube (also see *UNIT 6.4*, Support Protocol 1).

The epiphyses contain a good deal of marrow.

3. Lyse red blood cells by adding 3 to 10 ml ammonium chloride solution. Let stand 3 min at room temperature, then centrifuge the cell suspension 10 min at 280 × *g* (1200 rpm in Beckman GH-3.7 rotor), room temperature, and remove the supernatant.

4. *Deplete lymphocytes (optional):* Treat cells at a concentration of 1×10^7 cells/ml final with appropriate concentrations of hybridoma supernatants (usually 1:20 final) and rabbit complement (usually 1:17-1:20 final). Incubate 1 hr in a 37°C water bath with swirling every 20 min.

It is not essential to deplete lymphocytes to generate large numbers of proliferating DCs. However this step removes contaminating B and T cells from the final population of mature DCs. The authors use antibodies to B220 (hybridoma RA3-3A1), CD4 (hybridoma GK1.5), CD8 (hybridoma 3.155), and MHC II (hybridoma M5/114).

Set up cultures of proliferating immature dendritic cells

5. Wash the marrow cells twice with RPMI-1640, each time by centrifuging 10 min at 280 × *g* (1200 rpm in a Beckman GH-3.7 rotor), room temperature. Count viable cells (*APPENDIX 3B*) and adjust cell concentration to 1×10^6 cells/ml in complete RPMI-5 medium.

6. Add murine recombinant GM-CSF at 700 to 1000 U/ml or ~20 ng/ml. Plate cell suspension at 1 ml/well in a 24-well plate.

The authors have used either pure recombinant GM-CSF or conditioned medium (e.g., 3% v/v) from a cell line transduced with the mGM-CSF gene.

Generally, the authors recover 4–5 × 10^7 marrow cells from one mouse (if the culture was not treated with antibodies and complement), and therefore establish 40 to 50 cultures.

7. Wash and feed cells every two days, each time by removing the old medium, then inclining the 24-well plate and gently washing the well along the walls with RPMI-1640 and aspirating the wash, and finally replacing the old medium with 1 ml fresh complete RPMI-5 medium containing 700 to 1000 U/ml (~20 ng/ml) mGM-CSF (see step 6).

Early in the course of the culture, on days 2 to 4, one is rinsing off granulocytes, which are developing often as nonadherent balls of very round cells. Residual lymphocytes are also removed by washing—e.g.,B cells if the culture was not treated initially with antibodies and complement in step 4. By day 4, one begins to see aggregates of growing DCs attached to the adherent stroma. The aggregates rise above the monolayer, in contrast to the flattened dispersed macrophage colonies. Spiky processes (dendrites or veils) can also be recognized, extending from the periphery of the aggregates, giving them a "hairy" or "thistle" appearance. By day 6, the wells are usually covered with many aggregates or balls of proliferating immature DCs.

The features of the aggregates can be verified by pulse labeling with [^{3}H]thymidine, where 10% to 20% of the cells are in S-phase, and by flow cytometry (UNITS 5.3 & 5.4), where the cells have intermediate levels of surface MHC II but undetectable B7-2/CD86 costimulator. MHC II is found in intracellular compartments (MHC II compartments or MIICs) of immature DCs.

Subculture to promote maturation of dendritic cells

8. Between day 5 and day 8 (when aggregates are present in sufficient numbers), dislodge aggregates by gently pipetting RPMI-1640 or RPMI-5 over the adherent stroma. Pool dislodged cells, centrifuge 10 min at 280 × *g*, room temperature, and

remove the supernatant. Resuspend the pellet in RPMI-5 at a maximum 1×10^7 cells/ml, then place in 100-mm petri dishes at a maximum of 1×10^7 cells/dish in 10 ml medium per dish.

The original 24-well plates will often keep generating aggregates for at least several days.

9. During the 24 to 48 hr following transfer, collect the nonadherent, nonproliferating, maturing DCs as they are released, by gently swirling the dish at intervals of 24 hr and transferring the resuspended cells to a collection tube for use in further studies.

10. Count viable cells (*APPENDIX 3B*). Assess the yield of DCs by flow cytometry (*UNITS 5.3 & 5.4*) or by simply counting large, irregularly shaped cells in a hemacytometer (*APPENDIX 3B*).

The yield is usually 5–10 $\times 10^6$ cells per animal, of which ≥60% have the surface markers of mature DCs (i.e., positive for CD86, MHC II, NLDC-145, and DEC-205).

Mature DCs have very high levels of MHC class II (usually 1 to 2 log units higher than typical B cells) and high levels of B7-2/CD86. Mature DCs also have surface DEC-205, a multilectin receptor that is detected with MAb NLDC-145 (Jiang et al., 1995). Lymphocytes are usually not detectable if they have been removed initially with antibodies and complement (step 4), but some can be found nevertheless, especially B220-positive B cells. Macrophages remain firmly adherent on the culture vessels. The main contaminants are less mature DCs that have lower levels of surface MHC II and lack surface CD86 and DEC-205. However, the immature DCs at all times may have high amounts of MHC II in intracellular vacuoles (MIICs).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 5****.*

Antibody-coated sheep erythrocytes (EA)

Wash 2.5 ml of packed sheep erythrocytes (obtained in Alsever's solution; Cocalico) three times with PBS (*APPENDIX 2*), centrifuging each time for 5 min at 350 × *g* (2000 rpm in Beckman GH-3.7 rotor), 4°C. Dilute to a 5% cell suspension with fresh PBS (~10^9 erythrocytes/ml). Dilute rabbit anti-erythrocyte hyperimmune serum (homemade) 1:50 in the 5% erythrocyte suspension (this should be a subagglutinating dose of antibody). Incubate 2 hr at room temperature with occasional gentle inversion. Wash the resulting EA three times with RPMI-1640 (Life Technologies) and dilute to a 5% suspension with RPMI.

The EA are now ready for use. Treated cells can be stored up to two weeks at 4°C, but should be washed once with RPMI immediately before use.

Collagenase D, 4000 Mandl U/ml

The specific activity listed by the manufacturer is in Wünsch units (1 Wünsch unit = 750 Mandl units) and varies from lot to lot. Dissolve the entire vial of lyophilized collagenase D (Boehringer Mannheim) to 4000 Mandl U/ml in HBSS (containing Ca^{2+} and Mg^{2+}; *APPENDIX 2*) to a final volume given by:

$$\text{ml/vial} = \frac{(x\ \text{Wünsch U/mg}) \times (750\ \text{Mandl U/Wünsch U}) \times 1000\ \text{mg/vial}}{4000\ \text{Mandl U/ml}}$$

where *x* is the specific activity listed in the package insert.

Filter-sterilize the clear, light-brown solution in a 115-ml filtration unit, divide into 2.2-ml aliquots, and freeze at −20°C. One aliquot is sufficient for ~20 spleens.

continued

Collagenase D is a Ca^{2+}-dependent enzyme; therefore, calcium-containing HBSS is essential to preserve activity. It is unnecessary to add DNase to the collagenase D solution because the technique used (see Support Protocol) is sufficiently gentle that very few cells lyse, resulting in low amounts of DNA.

Complete RPMI-5 medium

RPMI-1640 medium (e.g., Life Technologies) containing:
5% heat-inactivated fetal bovine serum
10 mM HEPES
20 µg/ml gentamicin sulfate (Life Technologies)
50 µM 2-ME
Filter sterilize

Dense BSA solution

1. In a 1-liter beaker, combine 186 ml PBS (*APPENDIX 2*), 29 ml of 1 N NaOH, and 65 ml water (avoid splashing the solution onto the inner walls of the beaker). Without stirring, layer 106 g BSA (Cohn fraction V; Intergen) onto the surface of the solution. Cover the beaker with foil and refrigerate overnight, allowing the albumin to dissolve slowly.

It is advisable to obtain BSA only from the manufacturer listed above (see discussion). However, other laboratories have used a prepared 35% BSA solution from Sigma.

2. The next day, gently swirl the clear, honey-brown solution, and remove a small sample. Measure the refractive index of the sample to the fourth decimal place—the correct density of BSA, 1.080 g/ml, corresponds to a refractive index between 1.384 and 1.385 at 25°C.

If necessary, correct the concentration of BSA as follows: add 1.5 g BSA to increase its refractive index by ~0.0005 or add 5 ml PBS to reduce its refractive index by ~0.0005. When the refractive index is correct, check the pH with an indicator strip. It should be between 7.0 and 7.4 and does not usually require adjustment.

3. Filter sterilize the solution as follows. Place a 47-mm prefilter (Millipore type) on top of the membrane of a 500-ml sterile filtration unit (Nalgene #156-4045). Moisten the prefilter with a few milliliters of the BSA solution. Apply vacuum for a few seconds, just until the BSA begins to pass through the membrane. Release the vacuum and carefully pour the rest of the BSA solution into the upper reservoir (avoid foaming). Apply vacuum to the unit and filter the remainder. Store ≤3 months at 4°C.

This viscous solution usually requires ≥10 min to filter.

COMMENTARY

Background Information

One of the earliest models for studying immune responses in tissue culture was the primary antibody response to sheep red blood cells by mouse splenocytes (*UNIT 3.8*). Antibody formation in this system required both radiosensitive lymphocytes and radioresistent nonlymphocytes. These two populations could be separated as plastic-nonadherent and -adherent, respectively. Because of their adherence to glass or plastic, the nonlymphocytes were presumed to be macrophages; however, adherence is not a cell-specific criterion (in fresh spleen and other lymphoid suspensions, even lymphocytes adhere).

It became apparent early on that the adherent population of splenocytes was a particularly good source of a novel cell type termed the dendritic cell. Dendritic cells lack all the fundamental features of macrophages including Fc and complement receptors, phagocytic activity, and prolonged adherence to glass or plastic. What emerged from subsequent studies in spleen and many other tissues was the concept of a dendritic cell system specialized for initiating immune responses from dendritic resting lymphocytes. Whereas most studies of antigen

presentation utilize freshly primed T cells or chronically stimulated T cell clones and hybridomas, dendritic cells prove to be potent accessory cells for several kinds of primary responses, both in vitro and in situ.

Dendritic cells are a trace but highly specialized subset of antigen-presenting cells (APC). Because no dendritic cell line exists, these cells must be freshly isolated from tissue before each experiment. In every tissue and species where they have been studied (Table 3.7.1), dendritic cells exhibit a set of common features: an irregular shape, a distinct cell-surface phenotype including very high levels of MHC class II proteins, active motility, and potent stimulatory activity for T cell–dependent responses. In most assays of antigen presentation, dendritic cells are 30 to 100 times more active than unfractionated spleen cells. Dendritic cells activate unprimed T cells in vitro and in vivo.

The method described here for isolating dendritic cells from spleen has been optimized over the years. The fact that many dendritic cells are plastic-adherent initially allowed for removal of the vast majority of lymphocytes in a splenocyte suspension. Further enrichment of mouse spleen dendritic cells is made possible by several other traits: low buoyant density (Steinman and Cohn, 1974; Steinman et al., 1979), lack of Fc receptors (Steinman and Cohn, 1974; Steinman et al., 1979), loss of plastic adherence (Steinman et al., 1979; Nussenzweig and Steinman, 1980), and improved viability in granulocyte/macrophage colony stimulating factor (GM-CSF; Naito et al., 1989; Crowley et al., 1990).

An alternative procedure is to float spleen cells on metrizamide columns (Macatonia et al., 1989; Vremec et al., 1992). These metrizamide methods have the advantage of including those dendritic cells that are nonadherent, and the disadvantage that very high levels of purity are more difficult to achieve.

Other methods for enriching dendritic cells, particularly nonadherent dendritic cells, emphasize negative selection with monoclonal antibodies (Vremec et al., 1992). A positive-selection approach to isolating mouse spleen dendritic cells utilizes the N418 MAb to CD11c, the alpha chain of the p150/90 member of the β2 integrin family (Crowley et al., 1990). While CD11c is expressed primarily on dendritic cells in the mouse in the steady state, it is known that CD11c in man is expressed on monocytes and stimulated B cells. Therefore there is a possibility that sorting with CD11c includes a few contaminants.

Many approaches to enriching dendritic cells from other tissues and species have been described (Table 3.7.1). In all cases, useful features are low buoyant density, nonadherence to plastic, and a lack of markers that are found on other cell types. The enrichment procedures are monitored by identifying cells with the peculiar shape and motility of dendritic cells, and very high levels of MHC class II proteins.

Evidence for two populations of splenic dendritic cells has become apparent in recent work (Crowley et al., 1989; Metlay et al., 1990; Vremec et al., 1992). First it was noted that only a subpopulation of splenic dendritic cells expressed CD8 and NLDC-145 (Crowley et al., 1989), the latter being a marker found on the central or periarterial "interdigitating cells." Second, the dendritic cell– restricted N418 anti-CD11c MAb stained not only the traditional interdigitating cells in the periarterial sheaths, but also very large nests of cells at the periphery of the T area (Metlay et al., 1990). These peripheral dendritic cells are literally positioned as "doors," where the T cells enter the white pulp, breaking through the marginal zone in regions where typical phagocytic macrophages are absent (Metlay et al., 1990; Agger et al., 1990). Third, peripheral dendritic cells are readily released when spleens are teased apart manually, while the central periarterial, or interdigitating, $CD8^+$ dendritic cells are associated with the stroma and require collagenase for their release (Crowley et al., 1989; Vremec et al., 1992).

A system for generating DCs from human progenitors, analogous to that for mouse cells, has been described (Caux et al., 1992; Szabolcs et al., 1995). This system begins with $CD34^+$ cells that are enriched in progenitors and therefore lacking many of the stromal elements that are part of the marrow suspensions described in Basic Protocol 2. Both GM-CSF and TNFα must be added to the $CD34^+$ cells, which then form similar proliferating aggregates of DCs as described. In mice, the authors have found that TNFα is being released into the culture medium, possibly from stromal cells, and that TNF does mediate the terminal maturation of DCs.

In humans, an additional system for generating large numbers of DCs from nonproliferating progenitors in the monocyte fraction has been described (Romani et al., 1996; Bender et al., 1996). This system has two components: an initial (priming) culture in GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Romani et al., 1994; IL-13 will substitute for IL-4 but TNFα is without effect here) and a second

(differentiation) culture in which a monocyte-conditioned medium provides a variety of cytokines including TNFα, which, by itself, is insufficient. Some laboratories are adding IL-4 to mouse bone marrow cultures. It is possible that in the authors' method there is some endogenous source of IL-4, IL-13, or some comparable factor. It is also possible that there is an endogenous source of the monocyte-conditioned medium, since macrophages are abundant in the GM-CSF treated cultures.

The culture method described in Basic Protocol 2 for generating DCs from mouse bone marrow progenitors reproducibly provides typical DCs in large numbers—i.e., the cells are nonadherent, large, irregularly shaped, and motile and have abundant surface MHC II and accessory molecules and potent T cell stimulatory capacity. In terms of subsequent function, for example, in human cells, it is clear that triggering DCs via CD40 sustains viability, maturity, and function (Caux et al., 1994).

Critical Parameters

Bovine serum albumin from only a single supplier (i.e., Intergen) has been used successfully to prepare mouse spleen dendritic cells. Mice should not be stressed prior to sacrifice because corticosteroids reduce yields. Dehydration also reduces yields, so mice should be allowed to rest ≥5 days after shipment.

The single most technique-sensitive step is washing tissue culture dishes free of contaminants after plastic adherence as described in Basic Protocol 1. Care must be taken to clear the entire surface of nonadherent cells, pipetting with sufficient force to dislodge them but avoiding excessive force, which can strip off adherent cells. It is essential to use warm medium in this step, because cold medium detaches dendritic cells from plastic.

While in principle dendritic cells could be enriched from total spleen adherent cells instead of the low-density fraction, the latter is a time saver and makes it easier to obtain high purity. The difficulty with total adherent cells is that the percentage of dendritic cells is very low. To prepare adequate numbers requires washing many plates free of nonadherent cells, and there is a larger proportion of contaminants, especially B cells. By preparing a low-density population prior to adherence, the dendritic cells from a large number of spleens are concentrated into a small volume, reducing the number of dishes and increasing the cell density of the adherent monolayers. There are then many fewer plates to wash, it is easier to insure that lymphocytes have been dislodged, and the distinct dendritic cell cytology is more readily observed.

Depleting contaminating cells with EA rosetting provides a highly enriched dendritic cell population (Fig. 3.7.1). Obviously, it is simpler to omit the EA rosetting step, and we know of no differences in function between dendritic cells that are partially versus highly enriched. The reason is that low density spleen adherent cells from specific pathogen free mice are typically 60% to 80% dendritic cells. The difference between 60% to 80% and 95% to 100% purity is not of major consequence in most assays. On the other hand, the EA rosetting step provides a safeguard for a poor washing technique. If most lymphocytes were not dislodged during washing, there is a second chance for purification when EA rosetting and resedimentation of the suspension on dense BSA is carried out.

Anticipated Results

The final yield of viable, purified dendritic cells ranges from 2-7 × 10^5 per spleen. Purity is monitored by cytologic and flow cytometry criteria. The simplest test of functional capacities is to use the dendritic cells as APC for allogeneic T cells in a mixed leukocyte reaction (*UNIT 3.12*). Dendritic cells are 30 to 100 times more potent than bulk spleen cells.

Time Considerations

In Basic Protocol 1, 5 to 6 hr of work are required on the first day of the purification, from removal of spleens to the overnight incubation on plastic. On the second day, harvesting detached dendritic cells and EA rosetting requires 2 to 3 hr.

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Cytologic study of dendritic cells in different organs and species.

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