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T Cell — Primary Culture from Peripheral Blood

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Abstract

Peripheral blood mononuclear cells (PBMC) can be used to assess cell-mediated immunity in general or, via antigen-specific stimulation, to detect previous exposure to a variety of antigens/allergens and to monitor the response to immunotherapies. Peripheral blood is the most common source of mononuclear cells for in vitro cultures, although mononuclear cells can be obtained from other sources involved in the allergic reaction. PBMC from individuals previously exposed to an antigen proliferate in vitro when stimulated with the specific antigen. Proliferation is measured by the incorporation of (^{3}H)-thymidine into newly synthesized DNA. This parameter is often used as an end point of lymphocyte stimulation induced by antigen or antigen fragments (e.g., synthetic peptides), mitogens, or anti-CD3/anti-CD28 combinations.

The aim of this chapter is to describe the culture of T cells obtained from peripheral blood and the collection of cell supernatants for cytokine measurement.

Key Words: Peripheral blood mononuclear cells; T-cell culture; proliferation; cytokines.

1. Introduction

The investigation of the human immune system is a key element in understanding allergic reactions. Peripheral blood mononuclear cells (PBMC) can be used to assess cell-mediated immunity in general or, via antigen-specific stimulation, to detect previous exposure to a variety of antigens/allergens and to monitor the response to immunotherapies.

Peripheral blood is the most common source of mononuclear cells (lymphocytes and monocytes; PBMC) for in vitro cultures, although mononuclear cells can be obtained from other sources involved in the allergic reaction (lung, skin, etc.). PBMC from individuals previously exposed to an antigen proliferate in vitro when stimulated with the specific antigen. Proliferation is measured by the incorporation of (^{3}H)-thymidine into newly synthesized DNA. This parameter

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is often used as an end point of lymphocyte stimulation induced by antigen or antigen fragments (e.g., synthetic peptides), mitogens, or anti-CD3/anti-CD28 combinations.

Measurable amounts of cytokines can be detected in such cultures. In some cases, an enhanced expression of cytokine receptors and activation markers can be detected on lymphocyte surfaces by flow cytometry.

In addition to basic nutrients, in vitro cultures of PBMC require a serum supplement, usually of human or fetal calf origin.

It is frequently difficult to perform studies on PBMC the same day they are obtained. However, it is feasible to freeze and thaw the separated PBMC without significant function loss.

2. Materials

2.1. General Comments on Sterile Working and Biosafety Practice

All solutions and equipment coming into contact with cells must be sterile and a proper sterile technique should be used accordingly. Usually a lamina flow hood filtering the air to remove airborne bacteria and fungi is necessary. Immunological studies of the human immune system pose special safety problems associated with the risk of infection with human disease agents. Invariably, blood samples should be treated as though they are a potential source of hepatitis or HIV infection and appropriate personal protective equipment should be used, i.e., wearing laboratory suits, latex gloves, and eye protectors. Disinfection of all equipment after use is recommended (e.g., with alcohol or Barrycidal).

Work in the laboratory environment is closely regulated by legislation; usually reinforced and exemplified by institutional and local codes of practice. Many of the requirements for "good laboratory practice" rely on common sense or are based on a thorough knowledge of the likely risks and their avoidance (e.g., hazardous procedures or chemicals). Eating, drinking, smoking, or application of cosmetics in laboratory areas should be prohibited. A large number of commonly used laboratory chemicals are potentially carcinogenic or mutagenic, for example, phorbol esters (PMA) or propidium iodide, and handling them with care is necessary ***(1,2)***.

In addition, there are defined legal requirements for the handling of radioisotopes (*see* (^{3}H)-thymidine labeling). The work area should be especially designed for their use. The design of apparatus and experimental procedures should minimize the handling of radioactive material and avoid exposure to radiation. Appropriate shielding and distance reduces the radioactive dose received.

2.2. Culture Media, Supplements, Reagents, and Equipment

Many different culture media are available. RPMI 1640 was specifically designed for the culture of human cells. It is a double-buffering system containing

bicarbonate (24 mM) and HEPES (50 mM) (*N*-2-hydroxyethyl-piperazine *N*-2-ethanesulphonic acid) buffered by CO_2 in the air, as this approximates physiological conditions in closed culture conditions (5% CO_2 in air in a gassed incubator). Although 20 mM HEPES alone can control the pH of the culture medium within physiological limits, cells grow better in the presence of CO_2 and HCO_3^-. Oxygen tension is also important for the growth of cells. In static cultures, the depth of the medium should be greater than about 5 mM. Most cells grow well at pH 7.4. At this pH phenol red, the most commonly used indicator, is salmon. It is orange at pH 7.0, yellow at pH 6.5, and blue ("livid") under alkaline conditions.

For the prevention of bacterial contamination in cell cultures, an antibiotic cocktail of penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL) or gentamycin (30 µg/mL; more stable but more expensive) is recommended. Protection from yeast contamination is possible by adding fungicone (amphotericin B) up to a final concentration of 10 µg/mL.

The growth of cells in cultures requires a complex mixture of nutrients and other essential components. Theoretically, it should consist of a totally defined set of media additives that result in vigorous cell growth without causing extraneous cell stimulation. Such media additives have been devised but they are usually not as satisfactory as fetal calf serum (FCS), an easily obtained and relatively cheap material that normally contains all components necessary for the growth of human cells (heat inactivation for 30 min at 56°C reduces the number of viral and other adventitious contaminants and inactivate complements; filter-sterilized [0.45-µm filter]).

Alternatively, cell cultures can grow in serum-free medium (e.g., Ultraculture; Yssel's medium which is based on a modification of Iscove's modified Dulbeccos's medium) ***(3)***. In some cases, stimulation experiments will performed in serum-free ultra culture medium (Bioproducts, Heidelberg, Germanzy) supplemented with 2 mM GlutaMAX™ 1 (Life Style Technology), antibiotic/antimycotic solution (Sigma, Deisenhofen, Germany) and 20 mM β-mercaptoethanol (2-ME; Life Style Technology) ***(4)***.

In some experiments involving the culture of human lymphocytes, it is preferable to replace FCS with human AB serum because FCS can be mitogenic for human cells. Because the capacity of human AB serum to support lymphocyte cultures can vary, the screening of various batches is recommended. In addition, human AB serum should be heated inactivated for 30 min at 56°C.

Nonessential amino acids may be required for some applications. Glutamine is a nonessential amino acid that serves not only as a primary respiratory fuel, but also as a necessary substrate for nucleotide synthesis in rapidly proliferating cells such as lymphocytes. Glutamine can be produced in sufficient quantities under stable conditions, but it becomes a limiting substrate during metabolic

stress. Optimal lymphocyte functions have been demonstrated when the glutamine concentration is set around 0.6–2.0 mM ***(5,6)***.

Although the precise mode(s) of action of 2-ME has not been defined, it must be included in the media (final concentration 50 µM) used for procedures with primary cultures. 2-ME (50 mM in PBS diluted solution; stored at 4°C) must be added to media immediately before use because its activity rapidly declines if diluted.

A variety of ingredients and reagents are supplemented to the media shortly before use; they are typically made in large batches at 100× or 200× concentrations and stored in small aliquots of convenient size until use at –20°C and prewarmed at 37°C before use.

It is possible to purchase sterile media in solution. Alternatively, packaged, premixed powders can be purchased and reconstituted in 10 to 20-L batches, which must then be filter-sterilized (0.45-µM filter). The media can be stored at 4°C (*see* manufacturer's recommendations).

1. Complete RPMI (*R*oswell *P*ark *M*emorial *I*nstitute) medium (500 mL) containing: 439.5 mL RPMI 1640 cell culture medium with GlutaMAX™ I-supplement and 25 mM HEPES (Gibco, BRL; Life Technologies GmbH Paisley; UK), 5 mL penicillin/streptomycin (10,000 U/mL penicillin and 10,000 µg/mL streptomycin; Gibco, BRL), 5 mL sodium pyruvate MEM (100 mM; Gibco, BRL), 0.5 mL 2-ME (1000×, Gibco, BRL), 50 mL FCS (Biochrom, Berlin), or human AB-serum (both heat-inactivated).
2. Hanks balanced salt solution (HBSS) without calcium and magnesium: D-glucose (1.0 g/L), KCl (0.4 g/L), KH_2PO_4 (0.06 g/L), NaCl (8.0 g/L), $NaHCO_3$ (0.35 g/L), Na_2HPO_4 (0.048 g/L).
3. PBS (phosphate-buffered saline; Dulbecco's) (10×): 80 g NaCl, 23 g $Na_2H\ PO_4 \cdot 2\ H_2O$, 2 g $KH_2\ PO_4$, 2 g KCl. Make up to 1000 mL with H_2O. For the ready-to-use PBS (1×), mix 100 mL PBS (10×) with 800 mL H_2O; adjust to desired pH (7.2–7.4) with NaOH. Make up to 1000 mL with H_2O.

For cell cultures and experiments, special equipment and utensils are necessary including CO_2-humidified incubator, biosafety cabinet (laminar flow hood; Kendro Laboratory Products), autoclave, centrifuges (e.g., Beckman Instruments, Munich), multiwell harvester or cell harvester (PHD, Cambridge Technologies or 1450 Microbeta Trilux, Perkin Elmer Wallac GmbH, Freiburg).

1. *Thymidine*: (^{3}H)-labeled thymidine-methyl is added to each cell-containing well for determination of the proliferation response. Purchased from Dupont, NEN Division, Dreieich, Germany.
2. *Ficoll-Paque* (or Ficoll-Paque plus; Amersham Biosciences Europe GmbH, Freiburg, Germany or Pharmacia LKB, Uppsala, Sweden) for in vitro isolation of lymphocytes; should be stored between 4 and 25°C and protected from direct light. Cold storage will increase the shelf life. Ficoll-Paque plus provides a sterile,

ready-to-use Ficoll-sodium diatrizoate solution (contains nonionic saccharose polymer, sodium-diatrizoate, and ethylenediamintetraacetylacid di-sodium) of the appropriate density, viscosity, and osmotic pressure for use in a simple and rapid lymphocyte isolation procedure.
3. *PBS, BSA, EDTA* buffers used for the pan T-cell isolation kit. PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA; should be degassed by applying vacuum or sonification.
4. *Eosin B* for microscopy (Merck Eurolab, Darmstadt, Germany). Prepare the 1% eosin stock solution by dissolving 100 mg eosin in 10 mL complete medium and sterile filtration; storage at +4°C for 1 mo is possible; dilute the stock solution 1:10 with complete medium for use.
5. *Freezing Medium*: A mixture prepared of 440 mL RPMI 1640 with Glutamax I, 5 mL penicillin/streptomycin, 5 mL sodium pyruvate, 100 mL FCS medium, and 50 mL dimethyl sulfoxide (DMSO). Aliquoted in 50 mL and stored at −20°C.
6. *PHA* (phytohemagglutinin; lectin from *Phaseolus vulgaris*; Sigma, Deisenhofen, Germany): 5 mg lyophylisate should be dissolved in 1 mL sterile HBSS (allowed to stand for 10 min for complete dissolution); carefully mixed, aliquoted in 10 µL portion (50 µg) and store at −20°C.
7. Hapten-antibody cocktail of antibodies for pan T-cell isolation kit: a cocktail of CD11b, CD16, CD19, CD36, and CD56 antibodies is used for the depletion of non-T cells. These antibodies are hapten-conjugated.
8. Reagents for polyclonal cell stimulation: use combinations of PMA (1 ng/mL) and calcium ionophore A23187 (500 ng/mL), both from "Sigma Chemicals"; PMA and soluble anti-CD3 mAb (1 µg/mL) (anti-CD3 were purchased from Ortho Pharmaceuticals); anti-CD28 mAb (1 µg/mL) and coated anti-CD3 mAb to plates.

3. Methods

3.1. Obtaining Peripheral Blood

Blood should be taken by trained staff in designated areas, and biosafety practices must be followed. Collect blood in heparinized tubes (20 mL heparinized blood for 2–3 $\times 10^7$ cells).

3.2. Preparation of Human Mononuclear Cell Population (PBMC)

The use of human peripheral blood as a source of lymphoid cells is facilitated by Ficoll-Hypaque density gradient centrifugation *(7)*. This simple and rapid method of purifying PBMC takes advantage of the density differences between mononuclear cells and other blood materials. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density: in contrast, erythrocytes and granulocytes (neutrophils and eosinophils) have a higher density than Ficoll-Hypaque and collect at the bottom of the gradient. Platelets are separated from the mononuclear cells by subsequent washing.

Removal of immature cells (contaminants of cord blood and peripheral blood from infants) requires additional steps. All procedure steps have to be done under sterile conditions using a laminar flow cabinet.

1. Transfer the fresh heparinized blood into a 15- or 50-mL conical centrifuge tube, add an equal volume of HBSS (room temperature) or PBS and mix it well. (Using blood from a leukophoresis donor, blood dilution with buffer of 1:3 or 1:4 [blood:buffer] is recommended.) (Alternatively, instead of 50-mL conical centrifuge tubes use LeucoSep tubes [Greiner, Frickenhausen, Germany] which have a special permeable disk membrane in the lower part of the tube).
2. Place Ficoll-Paque Plus solution (Pharmacia LKB, Uppsala, Sweden; density 1.077 g/L) in a 15- or 50-mL conical centrifuge tube. Use 3 mL Ficoll-Paque solution per 10 mL blood/buffer mixture.
3. Slowly layer the blood/buffer mixture over the Ficoll-Paque solution by placing the tip of the pipette containing the blood/buffer mixture at the surface of the Ficoll-Paque solution. (Alternatively, slowly layer the Ficoll-Paque solution underneath the blood/buffer mixture by placing the tip of the pipette containing the Ficoll-Paque solution at the bottom of the tube).
4. Centrifuge the capped tube 30 min at 900*g* (room temperature) using no brake.
5. Use a sterile pipette and remove the upper layer containing the plasma and most of the platelets. Change the pipette and transfer the next layer containing the mononuclear cells (lymphocytes, monocytes, and basophils) to another centrifuge tube.
6. Wash the PBMC by adding excess HBSS (more than three times the volume of the cell layer) and centrifuge them for 10 min at 400*g* at room temperature. Discard the supernatant into an appropriate disinfectant solution, resuspend the cell pellet carefully in HBSS (step by step; first in a small volume, mixing gently, adding HBSS to a final volume of 10 mL into the tube), and repeat the washing procedure once again.
7. Resuspend the PBMC in complete RPMI medium. Count cells and determine viability (*see* **Section 3.4.**).

3.3. Isolation of T Cells (Immunomagnetic Purification of T Cells)

For some research applications, isolation of purified T cells is necessary (e.g., studies on signal transduction, regulation of T-cell cytokine expression, induction of T-cell anergy). We used the magnetic activated cell sorter (MACS®) T-cell isolation kit for indirect isolation of untouched $CD3^+$ T cells from human peripheral blood mononuclear cells (MACS®, Miltenyi Biotech, Bergisch-Gladbach, Germany). This T-cell isolation kit is based on an indirect magnetic labeling system with depletion of B cells, monocytes, NK cells, dendritic cells, erythroid cells, platelets, and basophils. For this purpose, a cocktail of CD11b, CD16, CD19, CD36, and CD56 antibodies is useful for the depletion of non-T cells. The T cells are isolated following the manufacturer's instructions.

In brief, a MACS® column type LS^+/VS^+ is used. It is a prerequisite to isolate PBMC cells without any clumps as the cells pass through a 30-mm nylon mesh or filter.

1. Count the isolated PBMC cells (*see* **Section 3.4.**) and wash them with PBS with 2 mM EDTA and 0.5% BSA (*see* **Note 1**) by centrifugation of the cell suspension for 10 min at 400*g* at room temperature.
2. Remove supernatant completely and resuspend cell pellet in PBS with 2 mM EDTA and 0.5% BSA to a total volume of 80 µL/1×10^7 total cells.
3. Add 20 µL haptene antibody cocktail to 1×10^7 total cells, mix well, and incubate for 10 min at 6–12°C.
4. Wash the cells carefully with PBS with 2 mM EDTA and 0.5% BSA by adding 10–20 times the labeling volume.
5. Centrifuge for 10 min at 400*g* at room temperature and remove the supernatant.
6. Repeat the washing step. Resuspend the cells carefully in 80 µL of PBS with 2 mM EDTA and 0.5% BSA for 1×10^7 total cells.
7. Add 20 µL MACS® anti-haptene microbeads to 1×10^7 total cells, to label the cells magnetically.
8. Mix the cell suspension well and incubate them for 15 min at 6–12°C.
9. Wash the cells carefully with PBS with 2 mM EDTA and 0.5% BSA by adding 10–20 times volume (approx. 15 mL) and centrifuging at 400*g* for 10 min.
10. Place the column in the magnetic field of an appropriate MACS® separator.
11. Prepare the LS^+/VS^+ column by washing with 3 mL PBS with 2 mM EDTA and 0.5% BSA.
12. Apply cell suspension onto the column; the unlabeled cells will not be retained on the column and will come out in the effluent.
13. Collect the effluent as negative fraction, representing the enriched T-cell fraction.
14. Rinse with 3–4 mL of PBS with 2 mM EDTA and 0.5% BSA and collect the effluent (enriched T-cell fraction).
15. Remove the column from the separator and elute outside of the magnetic field all positive non-T cells.
16. Check the purity of the untouched T cells by staining an aliquot of the T-cell fraction with the fluorochrome conjugate antibody against T cells (e.g., CD3-PE) and analyze by flow cytometry. To determine if any remaining non-T cells in the T-cell fraction are leukocytes or rather erythroid cells, counterstain an aliquot of the cell fractions with an antibody against CD45 coupled to another fluorochrome (e.g., CD45-FITC). In this case, T cells are double positive staining.

3.4. Determination of Cell Count and Cell Viability

Cells can be counted using Coulter counter or a hemocytometer. A Coulter counter is an electronic particle counter. It is based on the principle that particles (cells suspended in an electrolyte) passing through an aperture in which an electronic current is flowing will alter the electrical resistance of the electrolyte and give rise to changes in the current flow and voltage. A hemocytometer

(improved by Neubauer ruling; Brand, Wertheim, Germany) is a special type of microscope slide (cell counting chamber), which is divided into squares of a defined area over which a defined volume of cell suspension is distributed. Counting the number of cells in a defined volume can give us the number of cells per milliliter in a cell suspension. The hemocytometer can be used to count all cells or determine the number of viable cells in a culture. Viability is determined by either trypan blue or eosin dye exclusion test (*see* **Note 2**). Determination of cell viability is recommended when using thawed PBMC.

The following procedure can be used for the determination of viable cells with eosin dye exclusion test.

1. Centrifuge the cell suspension (10 min at 400*g* at room temperature), remove the supernatant completely, and resuspend in a defined volume (e.g., 1 mL) in complete medium RPMI. The cells need to be well mixed and uniformly suspended in the suspension.
2. Ensuring that both are clean and free from any grease, place coverslip on hemocytometer.
3. Gently mix 50 µL cell suspension with 450 µL eosin ready-to-use solution in a small plastic tube.
4. Dip a 0.1- or 1-mL pipette into the cell–eosin suspension to form a small drop on the end of the pipette and transfer gently to the surface of the slide at periphery of the coverslip.
5. Place hemocytometer on the stage of the microscope and focus on the cells.
6. Count the number of viable cells (unstained, in contrast to the red dead cells) at each of the four corner squares (the total volume of each corner square is 0.1 mm^3) (*see* **Note 3**). Cells falling across the top and left border lines of a square are considered to be in that square, whereas those that lie on the bottom and right borders are excluded. Count any clumps of cells as one cell.
7. Calculate the viable number of cells per milliliter of cell suspension as follows:viable cell number/mL = (total number of cells in four corner squares/4) $\times 10^5$—the factor 10^5 includes the cell dilution of 1:10 in eosin.

3.5. Cryopreservation of Cells (Freezing and Thawing of Cells)

Freshly isolated PBMC or cell lines should be used for culturing and stimulation experiments. However, they can be stored in culture medium at 4°C for at least 2 d prior to use or frozen as soon as possible. Cell concentrations up to 10^7 cells/mL can be successfully frozen in special cryovial tubes (sterile; volume 1.8 mL; Nunc, Roskilde, Denmark). A stepwise freezing process is recommended (1–2°C/min for the first 30°C). DMSO (or alternatively, glycerol) as an additive in the culture medium (cryoprotection solution) avoids the development of ice crystals during the freezing process. DMSO must be handled with care (avoid skin contact), and avoid the accumulation of toxic peroxide in the DMSO solution by using a fresh bottle every 3 mo.

1. Prepare freezing medium with DMSO (RPMI with GlutaMAX™ with 20% FCS, 10% DMSO, 1% penicillin/streptomycin, 1% sodium pyruvate) and store at −20°C (add DMSO to medium and not vice versa).
2. Centrifuge the PBMC at 500*g* (room temperature) using a 50-mL conical centrifuge tube, discard the supernatant, and save the cell pellet. Place the cells in the tube in an ice bath.
3. Add the precooled freezing medium (+4°C) to the cell pellet to achieve a final cell concentration of 2×10^6 cells/mL and resuspend cells gently (avoid cell sedimentation).
4. Transfer 1 mL of the resuspended cells to each labeled cryovial (use an indelible marker). Cap the vial and immediately place it for 1 h in a freezer storage box in −20°C freezer. Then, place the cryovials overnight into a −70°C freezer. For long-term storage (several years for human freshly isolated PBMC), transfer cells to a liquid nitrogen container (−196°C).
5. Thawing the frozen PBMC begins with precooling the cells (+4°C). Retrieve the cryovial containing the cells from the "liquid nitrogen freezing container".
6. Take the cryovial to the laminar flow and disinfect the outside of the vial with alcohol (70%).
7. Swirl cryovial gently in glove-protected and disinfected hands until PBMC are thawed or alternatively thaw vial quickly in 37°C water bath.
8. Open the cryovial gently and transfer the cells into a sterile 15-mL Falcon tube containing 12 mL of the precooled media. (Alternatively, resuspend the cells gently using a sterile 1-mL pipette, transfer them into a sterile 15-mL Falcon tube, and add dropwise 9 mL complete RPMI media with gentle shaking over a time period of about 2–3 min).
9. Cap the tube, centrifuge for 5 min at 500*g* at room temperature, and discard the supernatant (under sterile conditions in the laminar flow), resuspend the cells in complete RPMI, and repeat the washing procedure as above twice. Examine the cell viability and determine cell count. The cells are ready for use at this stage and should be transferred to tissue culture plates or flasks.
10. In some cases, it is necessary to remove the dead cells. For this purpose, centrifuge the cells (10 min at 400*g* at room temperature), resuspend in 2 mL sterile PBS, and by using a 2-mL pipette, place 2 mL of FCS under the cell suspension and centrifuge the suspension for 5 min at 500*g* and remove the supernatant. Wash the cells once with complete RPMI and determine the number of viable cells.

3.6. Culturing and Stimulation of PBMC

A number of agents can specifically or nonspecifically induce T-cell activation resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Some agents are capable of nonspecific activation of unprimed T cells in culture. Calcium ionophore and phorbol ester stimulation can directly crosslink the T-cell receptor (TCR) on a large percentage of responder cells. Lectins, such as PHA, indirectly crosslink the TCR; accessory cells are necessary with concentrations of 1–5 μg/mL.

The use of anti-CD3 or anti-TCR antibodies in soluble forms (rather than plate bound) also requires accessory cells. When using super antigens, such as staphylococcus enterotoxin, accessory cells must express the appropriate MHC class II molecules.

For antigen-specific stimulation of PBMC, peripheral blood cells from donors previously exposed to antigen can be used successfully. In the case of tetanus toxoid, donors should ideally have received a booster injection about 1 mo to 1 yr prior to the use of their PBMCs. To study PBMC reaction induced by allergens, peripheral blood from atopic donors can be stimulated specifically with allergens, which induced the IgE response in this subject (for example, house dust mite allergen [Der p 1] or grass pollen [Phl p 1–5], latex allergen [e.g., Hev b 1–10] and so on). Patients should be tested for allergen-specific serum IgE levels. Although there is not always a positive correlation between RAST score and success rate in the induction of allergen-specific PBMC response, in some cases (e.g., for the generation of Th2 clones) donors with a RAST class > 3 should be chosen preferentially.

The antigen concentration used for T-cell proliferation assay needs to be optimized. It is common for Th2 responses to be induced preferentially with low allergen concentrations, whereas Th1 responses are more frequently induced by higher allergen concentration. The sensitization is not important for stimulation of PBMCs, as cells from nonallergic but exposed subjects also demonstrate proliferation response in the presence of allergen ***(8)***. This is not surprising, as cells from nonallergic but exposed subjects recognize the antigen and show strong IgG binding to the antigen, and immunocompetent cells are stimulated in this polyclonal system. The observation that nonallergic subjects show a proliferative response to allergen is in accordance with the observation of other authors, although there is some controversy regarding whether the proliferation of PBMC from allergic and nonallergic subjects is similar or lower for the latter ***(3,8)***.

3.7. Measurement of Proliferative Responses of Cultured Lymphocytes (Mitogen- and Antigen-Induced)

Although proliferation is not a specific effector function of T lymphocytes, in contrast to helper function for B lymphocytes or cytotoxicity, proliferation assays are reliable, simple and easy to perform, and have been widely used to assess the overall immunocompetence of the T cells.

1. For the proliferation assay, PBMC were "seeded" at a concentration of 2×10^4 cells/well in a 96-well culture plate (U-bottom; total volume 200 µL) in triplicate or increased number of replicates (hexaplicates) and stimulated with the antigen (*see* **Note 3**). Appropriate dilutions of various antigens (10–0.01 µg/mL) in complete

medium were then added in 0.1-mL aliquots. As a positive control, stimulation with the mitogen PHA (5 µg/mL) is used. Cells incubated with no antigen provide a medium control, to give a baseline level of T-cell proliferation.
In the case of mitogen-specific stimulation, an incubation time of 3 d is performed. In the case of allergens, 5 or 6 d is a good time, although this needs to be optimized for each allergen. For (^{3}H)-thymidine incorporation with allergens we performed a 5-d incubation in a humidified atmosphere at 37°C and 5% CO_2 (*see* **Notes 4–8**).
2. For the final 12 h of incubation, 37.4 kBq (1 µCi) of (^{3}H)-thymidine-methyl (Dupont, NEN Division, Dreieich, Germany) was added to each well (20 µL) and the incorporated radioactivity was assessed.
3. A semiautomated cell harvesting apparatus is used to lyse the cells with water and precipitate the tritium-labeled DNA onto glass fiber filters. The dried filter pads were counted by liquid scintillation spectroscopy.
4. Results were calculated as stimulation indices (SI-value). The SI-value is the ratio of the mean counts per minute obtained in three (or more) similar cultures with allergen or antigen to that obtained in the antigen-free culture (the medium control).
5. In the case of allergen-specific stimulation, it is important to set a cutoff point for determination of a positive response. An SI-value of 1.0 indicates that there is no antigen-specific response. An SI-value of 2 or 3 is often considered as a positive stimulation index.

3.8. Measurement of Receptors and Activation Markers on Lymphocyte Surfaces by Flow Cytometry

T-cell activation can be determined by measuring activation markers expressed on the cell surface of stimulated cells. For example, markers like CD25 (IL-2 receptor) and/or CD71 (transferrin receptor) are expressed on proliferated cells. The expression of these special markers is time-dependent, e.g., CD69 is an early proliferation marker. In addition, expression of TCRs or intracellular cytokines for further characterization of T cells can also be determined in PBMC cultures by flow cytometry.

1. Using sterile technique, 2×10^6 PBMC/mL are incubated in a 24-well culture plate in the presence or absence of antigen/allergen at different concentrations and for different times at 37°C in 5% CO_2.
2. After incubation, the cells are harvested and transferred to tubes and centrifuged for 10 min at 500*g*. Discard the supernatant.
3. Resuspend the cells in medium and count. Adjust cell count to 2×10^6/mL.
4. Pipette 50 µL PBMC cell suspension in a polypropylene tube and incubate with 8–10 µL of the chosen fluorochrome-labeled antibody to determine its expression.
5. Incubate in the dark for 30 min at 4°C.
6. Add 2 mL PBS and centrifuge the labeled cell suspension for 5 min at 500*g*. Discard the supernatant.

7. Repeat the washing step. Add 250 µL PBS with 0.2% paraformaldehyde to fix the cells.
8. Measure activation and cell surface marker expression by flow cytometry.

3.9. Collecting Supernatants for Further Cytokine Determination

The concentrations of soluble cytokines (like IL-4, IL-5, IL-10, IL-13, and interferon-gamma [IFNγ]) in culture supernatants could be measured with commercially available sandwich ELISAs (technique using combinations of unlabeled and biotin-coupled mAb of different epitopes of each cytokine).

1. Incubate 10^6 PBMCs/mL in the presence or absence of antigen/allergen in either 24 (final volume 1 mL), 48 (500 µL), or 96-well (200 µL) plate.
2. For polyclonal stimulation, use combinations of PMA (1 ng/mL) and calcium ionophore A23187 (500 ng/mL), both from "Sigma Chemicals"; PMA and soluble anti-CD3 mAb (1 µg/mL); anti-CD28 mAb (1 µg/mL and coated anti-CD3 mAb) to coat plates with anti-CD3 mAb, incubate a 96-well flat bottom plate with 10 µg/mL anti-CD3 mAb diluted in PBS and washed twice with 100 µL of RPMI.
3. Incubate the cells at 37°C, 5% CO_2 for 16 h/24 h (for IL-4 and IL-2) and 48 h or longer for IFNγ, IL-5, IL-13, etc., and harvest the supernatant which can be frozen (−20°C or better −70°C) prior to cytokine analysis.
4. Determine cytokine production levels by specific cytokine ELISAs. Be sure that the supernatant is cell-free (supernatant should be used after centrifugation).

4. Notes

1. Buffer is degassed by vacuum pump or sonification. Excess gas in buffer can form bubbles in the matrix of the column during separation, which may lead to clogging of the column and decrease the quality of separation.
2. Dye exclusion test is based on the principle that nonelectrolyte dyes penetrate into dead cells, whereas live cells with intact plasma membranes do not allow the entry of such substances. Exclusion dyes will eventually penetrate live cells, so quantification should be performed within 30 min of adding dye to cell suspension. Antigen preparations can lead to nonspecific activation of T cells, e.g., casein which if contaminated with lipopolysaccharide (LPS) can result in unspecific T-cell stimulation ***(9)***. LPS can be removed from the protein fractions by polymyxin B coupled to a solid phase as described ***(10)***.
3. As an alternative to (^{3}H)-thymidine incorporation, cell growing (proliferation) can be monitored using nonradioactive methods. Proliferation can be determined by photometric method using MTT (3-[4,5 dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or else by flow cytometry using a combination of ethidium bromide and brom desoxyuridin (BrdU).
4. The level of (^{3}H)-thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA, and "cold" thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be

accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of non-activated cells will also interfere with the accuracy of this last parameter.

5. The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in (^{3}H)-thymidine incorporation. When values obtained in triplicate cultures correspond poorly (for example, >5% differences in CPM values > 1000), technical problems such as cell clumping, dilution, and pipeting should be considered.
6. Excessively high values may be obtained from contaminated wells, as thymidine will be incorporated into replicating bacteria; therefore, it is a good practice to check the wells from microtiter plates under inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes. It is also useful to check the blast formation by microscopic examination of the cultures. Activating lymphocytes will tend to enlarge, and detection of blast will give a general indication of successful activation ***(2)***.
7. The main problem that may occur with proliferative response assays is high levels of background (^{3}H)-thymidine incorporation in control cultures without antigen. This problem is frequently because of the FCS used to supplement the cultures which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weekly stimulatory in the absence of any stimuli.
8. The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses such as most allergens will require a longer period of time (5–6 d) than those eliciting a higher frequency of responding T cells (3–4 d). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time of T-cell proliferation. Addition of thymidine on days 2, 3, 4, 5, and 6 will provide a useful test. Further extension of culture periods will not yield any improvements, because of exhaustion of nutrients in the medium (yellow color indicating acidic condition).

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