LYMPHOCYTE PROLIFERATION ASSAY (LPA)

1. PRINCIPLE, CLINICAL APPLICATIONS AND OVERVIEW OF THE ASSAY

1.1. Lymphocyte proliferation assay (LPA) measures the ability of lymphocytes placed in short-term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a foreign molecule, antigen or mitogen. $CD4^+$ lymphocytes proliferate in response to antigenic peptides in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). This proliferative response of lymphocytes to antigen *in vitro* occurs only if the patient has been immunized to that antigen, either by having recovered from an infection with the microorganism containing that antigen, or by having been vaccinated. Therefore, some normal individuals may not respond to a given antigen, but most people will respond to at least one of several common microbial antigens.

1.2. Antigen-specific T-cell proliferation is a major technique for assessing the functional capacity of $CD4^+$ lymphocytes to respond to various stimuli. In the AIDS Clinical Trials Group (ACTG) it is used to measure improvements in immunological function following antiretroviral therapy, to measure the development of anti-HIV immune responses following the administration of an HIV-vaccine, and to detect the presence of immune responses against specific opportunistic pathogens [1, 2]. This assay has been used in numerous adult and pediatric ACTG protocols.

1.3. Almost everyone's lymphocytes can be stimulated to proliferate nonspecifically by stimulating them *in vitro* with the mitogens phytohemagglutinin (PHA) or pokeweed mitogen (PWM), or the antibody anti-CD3. However, these substances provide strong stimuli that are not antigen specific, and usually do not discriminate as well as antigens in reflecting different levels of immunodeficiency. Antigen-specific T-cell proliferation can be measured *in vitro* using such antigens as cytomegalovirus (CMV) antigen, tetanus toxoid (TT), varicella zoster virus (VZV) antigen, and HIV-1 antigens (e.g., gp120 and p24), if an individual has been previously exposed to these agents.

1.4. Our consensus method involves isolating peripheral blood mononuclear cells (PBMCs), placing 100,000 of the cells in each well of a 96-well plate with or without various stimuli, and allowing the cells to proliferate for six days at 37°C in a CO_2 incubator. The amount of proliferation is detected on the sixth day by adding radioactive ^3H (tritiated) thymidine for six hours, which is incorporated into the newly synthesized DNA of the dividing cells. The amount of radioactivity incorporated into DNA in each well is measured in a scintillation counter and is proportional to the number of proliferating cells, which in turn is a function of the number of lymphocytes that were stimulated by a given antigen to enter the proliferative response. The readout is counts per minute (cpm) per well.

2. SPECIMEN REQUIREMENTS

2.1. For most ACTG trials, peripheral anticoagulated blood, obtained in an acid citrate dextrose (ACD, yellow top) tube, will be used. Heparinized blood (green top tubes) may be used, but in general, PBMCs prepared from heparinized blood tend to clump and may incorporate slightly less H^3-thymidine than cells from ACD-

anticoagulated blood. However, if blood is to be shipped, the proliferative capacity may be preserved to a greater extent in heparin or an acid citrate dextrose cell preparation (CPT) tube [3, 4]. The use of EDTA as an anticoagulant is not acceptable for the LPA. For a given study, all samples from all sites must employ the same anticoagulant. The LPA can also be used to assess lymphocyte function of cells from lymph nodes, spleen, thymus, gut, and tissue-infiltrating lymphocytes. Refer to the Protocol Immunology Information Sheet (PIIS) for a given protocol for specific instructions regarding which source of lymphocytes will be used.

2.2. For most Adult ACTG protocols, PBMCs should be prepared and placed in culture on the day that the blood was drawn from the patient. If whole blood is being shipped (as is the case with most Pediatric and a limited number of Adult protocols), the PBMCs should be isolated within 30 hours of draw. The use of shipped whole blood for the LPA may only be used in Adult protocols with the approval of the Protocol Immunologist. While normal PBMCs can be frozen, stored for months, thawed and placed in culture with little loss of ability to proliferate, preliminary data indicates that cells from AIDS patients do not survive as well when frozen or shipped [3, 4]. Once PBMCs are isolated, they should be plated within 1 hour to avoid the loss of adherent APCs that may stick to plastic ware.

3. REAGENTS

3.1. Pooled human AB serum, seronegative for HIV, must be pretested for its ability to support vigorous proliferative responses to recall microbial antigens with low spontaneous incorporation of thymidine. PBMCs from several different individuals must be used for screening sera. This must be done by ACTG labs. Several companies supply pooled AB sera. One source that has provided sera for several AACTG studies is Gemini Bio-Products, although some of their lots are not satisfactory for LPA. NABI has provided several lots of sera for several PACTG protocols. It should be noted that most companies screen sera only for their ability to support fibroblasts or human cell lines.

3.1.1. Gemini Bio-Products
1301 East Beamer Street
Woodland, CA 95776
Phone 1-800-543-6464
Gemcell Human serum AB, catalog #100-512

3.1.2. NABI
16500 NW 15th Ave.
Miami, FL 33169
Phone 1-888-345-6224
Human serum AB, catalog #2120

3.2. Specific ACTG protocols will require a specific vendor or lot of serum. Verify with protocol immunologist or check the PIIS for each protocol found on the ACTG website.

3.3. Serum must be thawed and heat inactivated, unless the manufacturer has already done this. Immerse the bottle of *thawed* serum in a 56°C water bath for 30 minutes, making sure that the level of the water is the same as the level of the serum in the bottle. Allow the serum to cool. Small aliquots can then be frozen for

future use. Serum should be filtered before use. The easiest way to do this is to add the appropriate amount of RPMI 1640 (with other components, if needed), and filter the medium through a 0.22µM filter. A polyethersulfone (PES) membrane for fast flow and low protein binding is recommended. A glass fiber pre-filter may be required to aid in filtering.

3.4. Medium: RPMI 1640 with L-glutamine should be used. If not used within 30 days, fresh L-glutamine must be added before use.

3.4.1. *Recommended vendor:* Gibco BRL Life Technologies
Grand Island, NY
Phone 1-800-828-6686
Catalog #11875-093, 500mL, RPMI 1640 with 2mM L-glutamine

3.5. RPMI without glutamine can also be purchased, and fresh L-glutamine added at the time medium is used.

3.6. L-glutamine: 200mM (100X), liquid. For a 2X concentration, add 2 mL of 200mM L-glutamine to 98mL media.

3.6.1. *Recommended vendor:* Gibco BRL Life Technologies
(address and phone number above)
Catalog #25030-081, 100mL, 200mM L-glutamine, 100X concentration

3.7. Penicillin-Streptomycin: 10,000 units of penicillin G (sodium salt) and 10,000µg of streptomycin sulfate (yields a 100X stock solution) should be used. Add 2mL of Pen-Strep solution to 98mL media to yield a 2X concentration.

3.7.1. *Recommended vendor:* Gibco BRL Life Technologies
(address and phone number above)
catalog #15140-122, 100mL Pen-Strep liquid, 100X concentration

3.8. Dulbecco's Phosphate-Buffered Saline (D-PBS), without calcium and magnesium.

3.8.1. *Recommended vendor:* Gibco BRL Life Technologies
(address and phone number above)
Catalog #14190-144, 500mL, 1X liquid

3.9. Ficoll-hypaque. Many brands are available, such as Ficoll-Paque Plus (Amersham Pharmacia Biotec Inc.) or Histopaque-1077 (Sigma Chemical Co.).

3.10. 96-well, *round-bottom* sterile tissue culture plates with lids. Ninety-six wells are essential. Many brands are available and may be purchased through Fisher, VWR, or other laboratory supply companies.

3.11. Recall, microbial antigens. Check the PIIS or with the protocol immunologist for antigens or mitogens required for a given protocol. Specific lot numbers and concentrations should be indicated for each protocol. The PIIS should also specify the order of priority for the antigens or mitogens in the event that less than an optimal number of PBMCs are obtained.

3.12. Tritiated thymidine ([^{3}H]TdR)

3.12.1. *Recommended vendor:* NEN Life Science Products, Inc.
549 Albany Street
Boston, MA 02118
Phone 1-800-551-2121
Catalog # NET027. Thymidine (Methyl-H^3), 6.7 Ci/mmol sterile aqueous

solution, at 1mCi/mL (37MBq/mL).

3.12.2. Use at a final concentration of 1.0 µCi/well (see Section 5, Preparation of [^{3}H]TdR). It is important to use [^{3}H]TdR of this relatively low specific activity.

4. INSTRUMENTATION

4.1. Centrifuge capable of spinning at 300-400 x *g* (check the radius of the centrifuge to determine the mid-tube rpms (r_{av}) for a given *g* force) for Ficoll-Hypaque centrifugation, or 200 x *g* for washing cells. Centrifugation should be done at room temperature. Whole cells should not be chilled. A refrigerated centrifuge can simply be set at *room temperature* (20° - 25°C).

4.2. Microscope and hemocytometer for counting cells.

4.3. 37°C, CO_2 incubator with greater than 95% humidity.

4.4. Cell harvester for quantitatively transferring and washing cells from a 96-well plate onto glass fiber filter mats. If the generation of aerosols during harvesting is a concern, the cell harvester should be in a biosafety cabinet or hood. If this is not possible, then Triton X-100 detergent should be added to the 96-well plate just before harvesting to inactivate HIV (see Section 5.d, Harvesting and Counting).

4.4.1. The harvester must be kept in excellent working order by checking for the uniformity of cell transfer and washing between wells every six months. This can be done by stimulating a large number of cells in a tube and then plating them out in a 96-well plate just before harvesting. Normal PBMCs are set at a concentration of 5 x 10^5 cells/ml in complete RPMI media containing 10% human AB sera. Five mLs of the cell suspension are then placed in a 17 x 100mm culture tube (e.g., Fisher 14-956-6B), and 5mL is placed in a second tube containing 5µg/mL PHA. Both tubes are then placed in a CO_2 incubator for six days (make sure the cap is loose). [^{3}H]TdR is added for the last six hours of the culture period at a final concentration of 5µCi/mL. Just before harvesting, 200µL of cells are aliquoted into each well of a 96-well plate. Five mLs of cultured cells are enough to do two rows (24 wells). Cells are then harvested. Individual counts for each well (control and PHA-stimulated wells are analyzed separately) should be within two standard deviations of the mean counts per minute. Documentation that these quality control checks have been done should be kept as part of the laboratory quality control records.

4.5. Scintillation counter for measuring the incorporation of ^{3}H-thymidine. It is highly desirable that data from the counter be directly accessible on a computer disk or on line. Preventative maintenance must be performed once per year to insure the machine is working properly. Filters or vials should be counted without isotope every three months to insure a low background cpm. During normal counting, blank wells (no media, no isotope) should also be harvested and counted to insure low background and to measure cross-talk (i.e., the counts in blank wells that are adjacent to wells with high cpms).

4.6. Calibrated pipettes and multichannel pipettes.

5. METHOD PREPARATION

5.1. NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS

2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING THE USE OF GLOVES AND LAB COATS).

5.2. Preparation of Culture Medium, Antigens/Mitogens, and Plates

5.3. Preparation of two types of media: RPMI-10% and RPMI-0.

5.4. RPMI -10%

5.5. RPMI 1640 containing L-glutamine and supplemented with 100U/mL penicillin, 100µg/mL streptomycin (Pen-Strep), and 10% heat inactivated human AB serum. HEPES buffer (10mM final) may also be added to the media.

5.6. RPMI -0

5.7. RPMI 1640 supplemented with Pen-Strep and L-glutamine only, NO SERUM.

5.8. Media is stored at 4^{o}C. Media must be re-supplemented with 2mM L-glutamine if stored for more than 1 month.

5.9. *Note:* Some laboratories prefer to make RPMI containing 20% serum and 2X concentrations of Pen-Strep and L-glutamine. This media is used in the preparation of antigens and mitogens for the LPA plates that are stored frozen. PBMCs that are added to the plate are prepared in RPMI-0, resulting in a final concentration of 1X Pen-Strep, 1X L-glutamine, and 10% serum.

5.10. Preparation of antigen/mitogen working solutions:

5.11. Antigen/mitogen solutions can be prepared in RPMI-10% (or RPMI-20, see note above). For each antigen/mitogen in the assay, prepare a solution at twice (2X) the intended final concentration in the assay wells. Prepare sufficient volume in order to fill the requisite number of assay wells (number of assay wells x 100 µL/well = minimum volume to prepare).

5.12. Refer to the PIIS for the final concentration of antigen or mitogen. An example of how to prepare antigen dilutions is illustrated below:

5.13. The protocol calls for a final (1X) concentration of CASTA at 10ug/mLand of CMV antigen at a dilution of 1:10.

5.14. Determine how many plates you will need for the protocol (how many patients your site expects to enroll times the number of visits that call for an LPA to be done). Labs usually make up about 20 plates at a time.

5.15. Determine if quadruplicate or triplicate wells for each antigen will be run.

5.16. For this example, we will run quadruplicates. I want to set up about 23 plates. I will need 10mL of 2X the intended concentration (100uL/well x 4 wells x 23 = about 10mL). I need 10mL of 20ug/mL CASTA and 10mL of a 1:5 dilution of CMV for a 2X concentration.

5.17. The CASTA antigen from Greer Laboratories comes in a 400ug vial. I add 1mL of RPMI to the vial to give a concentration of 400ug/mL. I need 200ug total (10mL at 20ug/mL), so I add 0.5mL of the CASTA stock to 9.5mL of RPMI-10% (400ug/mL x 0.5mL = 200ug in 10mL, or 20ug/mL).

5.18. The CMV antigen is a liquid that comes in a 5mL vial. I need 10mL of a 1:5 dilution, so I add 2mL of the CMV stock to 8mL of RPMI -10%.

5.19. The remaining CASTA and CMV antigen from the stock vials can be transferred aseptically to small cryovials and stored at –70°C.

5.20. Preparation of plates:

5.20.1. Tissue culture plates should be prepared in advance and stored in sealable plastic bags at –70°C. The preparation of a large number of plates in advance increases the uniformity of antigen addition and facilitates the rapid processing of a large number of blood specimens.

5.20.2. Obtain plate diagram (i.e. "worksheet", "template") for the LPA from the PIIS. The PIIS should also specify whether assays will be run in triplicate or quadruplicate. It is essential that all sites performing a given study place antigens in the same positions on the plate.

5.20.3. Dispense 100µL/well of each 2X concentration antigen/mitogen in strict accordance with the plate diagram provided. For control wells, dispense 100µL of RPMI -10% (see note in Section 5, Detailed Protocol, if RPMI -20% is preferred).

5.21. Preparation of [^{3}H]TdR:

5.21.1. The final concentration of [^{3}H]TdR should be 1µCi/well. Calculate the volume of diluted [^{3}H]TdR needed to dispense 25µL/well, equaling 1µCi/well (number of assay wells x 25µL/well = minimum volume to prepare). For example, the [^{3}H]TdR stock is at 1mCi/mL. A working solution of 40µCi/mL in RPMI-0 (or D-PBS) should be prepared. Dilute the [^{3}H]TdR stock 1:25 with RPMI-0 (0.5mL of stock + 12mL media). This is enough to pulse 500 wells. Add 25µL/well to give a final concentration of 1.0µCi/well. The diluted [^{3}H]TdR can be stored at 4°C for a month.

5.22. Preparation of PBMCs

5.22.1. Check the consensus protocol for preparation of PBMCs found in the Virology Manual for HIV Laboratories, "Preparation of PHA-stimulated uninfected donor peripheral blood mononuclear cells" on the web at: http://www.niaid.nih.gov/daids/vir_manual/.

5.22.2. If the blood collection tube has been spun to obtain the plasma fraction, D-PBS or RPMI-0 equal to the original volume of the plasma must be added to the tube. Now add sterile D-PBS or RPMI -0 equal to the volume of blood so that the volume is 2X the original volume.

5.22.3. Overlay diluted blood on Ficoll-hypaque.

5.22.4. Centrifuge at 300-400 x *g* for 30 minutes in a room temperature centrifuge (20° to 25°C). Make sure the centrifuge brake is turned off.

5.22.5. Harvest the PBMC from the interface and immediately wash two or three times in D-PBS or RPMI -0, centrifuging at 200 x *g* for 12 minutes at room temperature. Minimize delay time between washes. After washing, PBMCs are re-suspended in RPMI -10% (or RPMI -0 if RPMI -20% was used to prepare the antigen plates) and should not sit for more than one hour at room temperature. Do not place the cells on ice.

5.22.6. Count ≥300 cells for white blood count and ≥100 cells for viability. Adjust PBMC concentration to 1x10^6 viable cells/mL for dispensing into culture

wells. For Pediatric protocols, some wells may be omitted if not enough PBMCs are obtained; follow guidelines in the protocol or the PIIS.

6. ASSAY SETUP

6.1.1. Thaw antigen/mitogen culture plates at 37°C in an incubator. Label the tissue culture plate with patient identification (PID), date plated, date to be pulsed with isotope and harvested.

6.1.2. Dispense 100µL of the PBMC suspension (10^6 cells/mL or 100,000 cells per well) into culture wells. Be certain to keep cells suspended, by gentle mixing, while the cells are being dispensed. Cells should be dispensed as soon as possible in order to avoid the medium becoming alkaline. This is day 0. It may be necessary to store LPA plates in bags inside the incubator if lack of humidity is a problem.

7. HARVESTING AND COUNTING

7.1. On the morning of day 6, pulse plates with 25µL/well (1µCi/well) of [H^3]TdR.

7.2. After six hours, harvest on glass fiber filters using a cell harvester. If the cell harvester is not in a biohazard hood or splashing during harvesting is a concern, then add 25µL/well of a 5% Triton X-100 detergent solution to inactivate HIV. If harvesting can not be done immediately after the six-hour pulse, plates may be stored at –20°C until harvesting. Do not store plates at –20°C for more than a few days. Do not add Triton X-100 to plates prior to freezing because it causes bubbles during thawing.

7.3. After harvesting, filters are allowed to dry at room temperature on the bench top, punched-out into scintillation vials, or the filter sheets are processed for counting in a betaplate counter. Add scintillation fluid to vials/filters and leave filters in scintillation fluid according to the manufacturer's directions prior to counting. Count vials/filters on a beta scintillation counter at laboratory-determined settings to measure counts per minute (cpm).

8. CALCULATIONS AND REPORTING OF RESULTS

8.1. LPA data can be expressed and calculated in two different ways following determination of mean values (e.g. from triplicates, quadruplicates); both methods make biological sense. Because the stimulation index (SI) is a ratio, it is greatly influenced by biologically insignificant variations in the low cpm levels of background, unstimulated wells.

8.2. SI = (cpm experimental/ cpm background unstimulated)

8.3. Net counts or cpm = (cpm experimental - cpm background unstimulated)

8.4. For multicenter trials, agreement is slightly better if the SI calculation is used, since the efficiency of counting of different scintillation counters varies. Raw data (cpm) from each culture well shall be entered electronically onto Excel®-based spreadsheets provided for the study. Individual technicians should not censor the data unless gross contamination is evident. ALL raw data should be reported to the statisticians. It should also be reported if the specimen was QNS (quantity not sufficient/no PBMCs obtained) or the assay was not set up for any reason, but blood was obtained. Data will be exported to the Data Management Center at Frontier Science & Technology Research Foundation and the Statistical Data Analysis Center will perform the calculations based on the raw data.

9. CONTROLS

9.1. The vast majority of ACTG protocols that include LPA measure changes in the response to a specific antigen over time in a given patient. An appropriate control for these measurements in patient's assays is to perform (in parallel) sequential measurements of LPA to recall antigens using PBMCs from normal donors. This will serve both a positive control to assure that the assay is working well, and also as an assessment of intra-assay variability.

9.2. Set up a culture of antigens and mitogens with normal donor PBMCs as specified in the PIIS. Each site must identify a pool of five to eight of HIV-seronegative, healthy individuals who could be used as laboratory normal controls and are available for serial draws over the course of any ACTG protocol. These lymphocytes are evaluated for their proliferative response to recall/microbial antigens, not to HIV antigens (unless specified by the PIIS). The incorporation of thymidine into unstimulated wells containing PBMCs serves as the negative control.

10. EXPECTED VALUES

10.1. As previously noted, an HIV-seronegative individual may not respond to a particular microbial antigen but rarely will fail to respond to at least one of four commonly used microbial antigens. Stimulation indices may range from 3 to 200, and net cpms from 1,500 to 300,000. The cpms of control wells should be low (generally below 1000). It is important to remember that the LPA assay should be used as a qualitative test, not a quantitative one.

10.2. HIV-infected subjects in general have a decreased LPA response to microbial antigens. For patients with CD4 counts < 200/mm^3, there may be no response to antigens. However, a response may become detectable after effective antiretroviral therapy, or an existing response may increase in magnitude.

11. DETECTION LIMITS

11.1. Within an assay, it is straightforward to detect a significant response, since the variation between replicate unstimulated wells, and the variation between replicate stimulated wells can be measured. An SI of 3 to 5 is usually significant depending on the antigen and the control used.

11.2. Variability between sequential measurements on the same HIV-seronegative donor or AIDS patient is often large. This variability means that changes in LPA induced by therapeutic interventions also must be large to achieve significance. Because of the inherent variability between labs, quality control is very important. In addition, the statistician may call data into question if it is suspected that the data was entered incorrectly or if it is "noisy", with a large background. Guidelines for determining this include whether the maximum cpms divided by the minimum cpms for a given stimulant are greater than 6, or if the SI of the antigen-containing wells is less than one.

12. POTENTIAL ERRORS

12.1. Failure to detect incorporation of thymidine can occur for a variety of reasons:

12.2. Patient is immunocompromised; few circulating $CD4^+$ memory cells

12.3. Patient has not been sensitized to one specific antigen

12.4. No antigen or no isotope added to wells; incorrect specific activity of isotope used

12.5. Isotope added at incorrect time or for too short a period

12.6. Evaporation from wells because of failure to wrap plates or because of low humidity in incubator

12.7. Other sources of variability include:

12.7.1. Donor and donor status (e.g. recent infection, underlying disease, immunization)

12.7.2. Conditions of blood collection (e.g. clotting)

12.7.3. Conditions and duration for blood storage prior to isolation of PBMCs.

12.7.4. Potential for toxicity to PBMCs if left in Ficoll post-centrifugation for a prolonged period of time

12.7.5. Conditions for PBMC storage prior to plating of cells

12.7.6. Counting of cells is a MAJOR source of variation

12.7.7. Plating of PBMCs: variability in amount of cells plated per well, secondary to pipette and/or inadequate mixing of PBMCs during plating process

12.7.8. Incubation conditions (e.g., temperature, CO_2 level, humidity, contamination)

12.7.9. Cell harvesting: calibration and servicing of harvester; inadequate washing of wells and tubing; filter paper employed

12.7.10. Beta scintillation counting: duration filter paper is dried prior to addition of scintillation fluid; scintillation fluid employed; duration filter paper is incubated with scintillation fluid prior to counting

12.7.11. Static electricity may affect counting efficiency, especially in plate-format counters. Contact Customer Service Support for your model of counter to determine if this is a potential problem.

13. INTRA-ASSAY AND INTRA-SUBJECT VARIABILITY

13.1. It has been noted that subjects differ considerably in the specific antigens to which they will respond, and the magnitude of their responses.

13.2. Of greater concern is the variability between assays on the PBMCs from a given subject. A discussion of this subject was included in Sections 9, 10, 11 and 12.

14. REFERENCES

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AUTHORS: Fred Valentine and Howard Lederman with contributions from a large number of ACTG Immunologists and ACTG Laboratory Technologists.

Procedure: ACTG Lab Man Lymphocyte Proliferation Assay

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

_____

Reviewed by: Date:

Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997