

INDUCTION OF TNF- alpha and IFN- gamma (WHOLE BLOOD METHOD)

1. PRINCIPLE

- 1.1 The capacity for production of cytokines is an important functional measure of immunologic status. Production of IFN-gamma in response to soluble antigens is mainly a reflection of CD4 cell function, and is progressively lost in HIV infection. Production of TNF-alpha is mainly a reflection of monocyte and macrophage activation. The capacity for production of TNF-alpha is generally maintained in HIV infection, and may be increased during certain opportunistic infections. Production of both these cytokines may be affected by immunomodulatory drugs such as prednisone.

RBCs and PMNs neither contribute to nor inhibit production of these cytokines. Thus, it is not necessary to remove these cells prior to stimulation with antigens or mitogens in culture. This is the basis for the "whole blood" method.

Other cytokines can potentially be studied by this method, including IL-2, IL-6, IL-10, IL-12, RANTES, and TGF. These have not yet been studied systematically by the ACTG, however. It may also be possible to study CD4 and CD8 responses to HIV gp120 with this method, by stimulating, respectively, with soluble gp120 protein, or by infection with a gp120 vaccine construct. These methods are under development.

2. SPECIMENS

- 2.1 1 x 7mL heparinized vacutainer tube (green top) per subject. The actual volume required is only 1mL, so if necessary, a pediatric collection tube may be used. If the specimen is to be divided among several tests, it is critical that this be done under sterile conditions! Regular heparinized vacutainer tubes are fine - background TNF-alpha production due to contamination has not been a problem. Vacutainers which contain EDTA (e.g. "pyrogen free" Terumo tubes) are not advised, because the EDTA is carried over into the assay, and may inhibit cytokine production. The use of citrate tubes has not yet been studied.
- 2.2 The specimen should be kept at room temperature after collection, and should be transported to the lab for culture within 4 hours. The effect of overnight shipping is not yet known.

3. EQUIPMENT, REAGENTS, AND OTHER SUPPLIES

- 3.1 Equipment
- 3.1.1 CO2 incubator, 37°C, 5% CO₂, 100% humidity, with glass pan of water with 0.1% SDS or other detergent at bottom to minimize contamination
- 3.1.2 Pipetting devices (10 - 100ml and 100 - 1000ml)

3.1.3 Laminar flow hood or clean work bench

3.1.4 Freezer, -70°C

3.2 Reagents

3.2.1 PHA: Sigma , catalog number L9132, Dilute to 500 ug/ml in medium. Store frozen at -70oC. Use 10 ul/well, for a final concentration of 5 ug/ml, as a stimulus for IFN-gamma.

3.2.2 Candida antigen: Greer Labs (704-754-5327), catalog listing CASTA, lot XPLM73-7-X8 (for ACTG Induced Cytokine Group), price, \$22.00 / 400 ug vial. This is a lyophilized research reagent, not the liquid material used for skin testing. Resuspend the 400 ug in 0.5 ml medium. Store frozen at -70oC. Use 50 ul/well, for a final concentration of 40 ug/ml as a stimulus for IFN-gamma.

3.2.3 LPS (E.coli 026:b6): Sigma, catalog number L2654, price \$14.95 / 1 mg. Dilute to 10 ug/ml in medium. Store frozen at -70oC. Use 10 ul/well for a final concentration of 100 ng/ml as a stimulus for TNF-alpha. NB: results of pilot study currently underway may change this concentration.

3.2.4 MAC filtrate (optional, from M. avium LR114): Wallis lab (216-368-4844). Availability must be arranged in advance. Dilute to 100 ug/ml in medium, store frozen at -70oC. Use 10 ul/well for a final concentration of 1 ug/ml for induction of TNF-alpha and IFN-gamma.

3.2.5 M. tuberculosis PPD (optional, from H37Rv): Wallis lab. Supplied at 1 mg/ml. Use 10 ul/well for a final concentration of 10 ug/ml for induction of TNF-alpha and IFN-gamma.

3.2.6 Tetanus toxoid (optional): Connaught, (800-822-2463). 2000-3000 Lf/ml, preservative free, (may be approximately 2-10 mg/ml), price \$630/5 ml vial. The final concentration is approximately 1 ug/ml for induction of IFN-gamma. (This has not been tested through this committee!)

3.2.7 Medium [RPMI 1640 medium, liquid 1x with and 25 mM HEPES, endotoxin tested (Sigma # R 5632, available in 500 mL bottles)], store at 4oC. NOTE: USE ONLY ENDOTOXIN-FREE PREPARED MEDIUM. DO NOT USE POWDERED MEDIUM.

3.2.8 Pen/Strep/L-glutamine solution 10,000 U PCN, 10 mg strep, 200 mM L-glutamine/mL, sterile, endotoxin tested, in NaCl (Sigma # G 6784, 100 mL), store at 0oC, add 5 mL/500 mL.

3.3 Other supplies

3.3.1 Tissue culture coated, 24-well plastic tissue culture plates (Sigma M 9655, or Corning 25820-24)

- 3.3.2 50 mL conical screw top vials (Sigma C 8171, or Corning 25330-50)
- 3.3.3 1mL screw-top cryogenic storage vials, sterile
- 3.3.4 Sterile pipette tips, 100ul and 1000ul
- 3.3.5 Gloves (powderless are preferable for less contamination of cultures)

4. METHOD

4.1 General

4.1.1 Dilution of blood in medium: 1/10

4.2 Duration of culture:

- 4.2.1 TNF-alpha: 20 hr
- 4.2.2 IFN-gamma: 3 days (72 hrs) for PHA
- 4.2.3 7 days (168 hrs) for antigens

4.3 Reagents for induction of TNF-alpha:

4.3.1 LPS, PPD, MAC filtrate, (and unstimulated control)

4.4 Reagents for induction of IFN-gamma:

4.4.1 PHA, CASTA, PPD, MAC filtrate, tetanus toxoid, (and unstimulated controls)
Note that two unstimulated controls are necessary for IFN-gamma (3 days and 7 days).

4.5 Work should be done in a laminar flow hood (or on a clean benchtop using sterile technique if a laminar flow hood is not available). When a new bottle of medium is opened, add antibiotics (2.5 mL/500 mL medium), write date and initials on label. Discard bottle for yellow color or any particulate matter. Pour medium with antibiotics from bottle into 50 mL tube for use. Discard remainder from 50 mL tube when done.

4.6 Suggested template for cultures

A	B	C	D	E	F
20h unstim	3d unstim	7d unstim			
20h LPS	3d PHA	7d CASTA			
20h MAC		7d MAC		etc	
20h PPD		7d PPD			

You may need to modify this depending on the number of reagents you study.

5. PROCEDURE

5.1 Add 900 ul medium with antibiotics to each well. Mix blood by inversion for 1 minute - this is critical. Immediately uncap and add 100 ul blood to the well.

- 5.2 Add appropriate volumes of stimuli. Label the plate cover appropriately and place in incubator.
- 5.3 At appropriate intervals, remove plate from incubator. Remove cover from plate, placing it on the table in the same position it was on the plate. It is not critical to entirely remove the supernatants, however, you should be able to easily remove approximately 800 ul of supernatants. Label each of the tubes (minimum of 3) with IDNO, phlebotomy date, and well number/letter (e.g. 2A) or stimulus and duration. Place 200 ul of supernatant into each of 4 labeled tubes, taking care to leave cells at bottom of well. Freeze the specimens. Return plate to incubator and repeat until culture is complete.

6. CALCULATIONS

- 6.1 None. Note that this protocol deals with production of supernatants. Cytokines in supernatants will be measured by commercial ELISA in which a standard curve using recombinant cytokine is generated. A comparison of several ELISAs and selection of a single kit is currently underway.

7. CONTROLS

- 7.1 Control wells
 - 7.1.1 Control (unstimulated) wells should be included for each subject's cytokine and day of harvest.
- 7.2 Normal subjects
 - 7.2.1 A healthy control subject with known skin test reactivity to one of the tested antigens (or with known positive responses on previous in vitro testing) should be studied once monthly.

8. EXPECTED VALUES

- 8.1 Control wells
 - 8.1.1 TNF-alpha: Values for unstimulated wells are generally >30 pg/ml. Values above 60 pg/mL generally indicate some type of contamination.
 - 8.1.2 IFN-gamma: Values for unstimulated wells are generally undetectable. Values above 40 pg/mL indicate likely carryover of circulating IFN-gamma.
- 8.2 Stimulated wells
 - 8.2.1 Values from stimulated TNF-alpha wells are generally from 800 -2400 pg/ml. immune status of donor. Responses to antigens in healthy sensitized donors will be above 600 pg/mL. With CD4 counts <200, this will decline to about 100 pg/ml. In general, the values are 60-80% of those expected from conventional mononuclear cell cultures. Background production of TNF-alpha in whole

blood cultures is generally less than 20% of what would be expected in conventional cultures.

9. PROCEDURE NOTES

- 9.1 Supernatants should grossly appear clear at the time of harvest. Specimens which are turbid or grossly hemolysed may be bacterially contaminated, which in turn may give erroneous values.
- 9.2 The culture method is simple enough that it should be possible for each site to generate supernatants locally. Cytokine Elisa's will be done at QA/QC'ed sites, most likely one cytokine per site. The pilot study will determine whether samples can be held and shipped overnight.

10. LIMITATIONS

- 10.1 Subjects with marked neutropenia may show low responses in this method. This may or may not reflect the actual immune status of the subject. Subjects with < 50 CD4 cells may not have detectable IFN-gamma responses in this method.

11. VALIDATION

- 11.1 In progress.

12. REFERENCES

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13. EFFECTIVE DATE/REVIEW DATE

13.1 November 12, 1996

14. AUTHOR

14.1 RS Wallis, and members of the ACTG Induced cytokines

ARCHIVE

Procedure: ACTG Lab Man Induction of TNA-alpha and IFN-gamma- Whole Blood Method

Prepared by: ACTG Laboratory Technologist Committee

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Updated on:

Reviewed by:

Date:

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