BLOOD / BONE MARROW FOR CMV ANTIGENEMIA

I. Introduction

Human cytomegalovirus (CMV) can cause severe, life-threatening disease in immunocompromised patients such as transplant patients and patients with AIDS. Systemic infections are characterized by carriage of CMV in the polymorphonuclear leukocytes (PMNL) of peripheral blood (viremia). Infected PMNL can be detected by direct detection of CMV pp 65 antigen (CMV antigenemia) using an indirect immunofluorescence (IFA) technique. CMV antigenemia can also be used to monitor the course of CMV infection during and after treatment. Antigenemia will be performed on EDTA or heparinized blood requesting CMV. Antigenemia and culture will be performed on bone marrow samples requesting CMV.

II. Collection and Transport

A minimum of 5 mL of blood is collected in an EDTA Vacutainer® tube (purple top). Samples should be transported to the laboratory as soon as possible at room temperature. Smaller volumes of blood from infants will be accepted and the procedure will be completed despite a small number of PMNL's. Blood samples received 14:00–13:00 hrs will be processed as far as preparation and fixing of slides. Staining and reading will be done the next day. Samples received after 15:00 hrs will be refrigerated and processed the next working day or a fresh sample requested (except on Fridays, consult Charge technologist).

Note:
Toronto Hospital Division of UHN: Reject (Transplant patients, In or Out patients)

1. Specimens received after 2 p.m., before 12 a.m. (previous night)
2. Specimens received over the weekend (Friday after 2 p.m. to Sunday midnight)

Phone Ward if specimen is rejected!!

Refer complaints to Dr Atul Humar (pager no. 416-664-8211) or Dr. Mazzulli @17-4695.

Other sites (PMH, MSH): Only reject specimens received over the weekend.

III. Materials:

i) Phosphate Buffered Saline:

For 1000 mL - 10 mM PBS powder, pH 7.4 (Sigma p-3813) in a glass vial
- 1000 mL dH_20

Autoclave and store at room temperature.

OR

Obtain sterile PBS from Rm. 977, ext. 8257.

ii) Erythrocyte Lysing Solution:

For 2000 mL -16.6 gm NH_4CL power, M.W.53.49 (Sigma A-0171)
-2.0 gm Potassium bicarbonate
-2000 mL dH_20

Filter sterilize and store at 4°C. Stable for 2 months.

iii) Fixative Solution:

For 500 mL -25 mL Formaldehyde (Sigma Cat. F-1268)
-10 gm Sucrose, M.W. 342.3 (Sigma S-1888)
-500 mL PBS buffer

Store at room temperature. Stable for 1 month.
Use fresh aliquot of fixative each day.

iv) Wash Solution

For 500 mL -5.0 mL FCS
-500 mL PBS

Store at 4°C. Stable for 1 week.

v) Permeabilization Solution

For 500 mL
- -2.5 mL NP40 (ICN cat# 198596 formerly Nonidet, sigma)
- -50 gm Sucrose (Sigma S-1888)
- -5.0 mL Fetal Calf Serum (FCS)
- -500 mL PBS

Store at 4°C. Stable for 1 month.

vi) Antibody 1

Monoclonal Anti-HCMV pp65, (Biotest Clonab CMV, Cat No: P/N 912600). Mix 1 mL of pp65 with 4 mL of PBS. Aliquot and store at 4°C. Test with a CMV positive control slide. Enter lot no. and QC slide results in Reagent History Form.

vii) Antibody 2

Monoclonal Anti-Mouse – FITC Conjugate with Evans Blue (Baxter B1029-86B). Store at 4°C.

viii) Control slides

Double-well slide with CMV positive and negative wells are stored at 4°C and at -70°C.

IV. CMV Antigenemia Procedures:

A. Cell Separation Procedure

1. Invert blood tube several times to mix.
2. Transfer ~ 2.0 mL of blood to a 15 mL graduated centrifuge tube which contains 10 mL of ELS.
3. Mix and put on a rotator for 5 minutes (or until RBC's are lysed).
4. Spin at 1300 rpm for 7 minutes.
5. Gently pour supernate into a discard container.
6. Add ELS to 10 mL level.
7. Mix and spin at 1300 rpm for 7 minutes.
8. Gently pour supernate into a discard container.
9. Using transfer pipette, carefully remove excess red blood cells from suspension above the WBC deposit.

10. Add PBS solution to 10 mL level.
11. Mix and spin at 1300 rpm for 7 minutes.
12. Gently pour supernate into a discard container.
13. Gradually add PBS to cell pellet until it reaches a turbidity of ~ 0.5 to 1.0 MacFarland Standard.
14. Proceed to cell counting procedure

B. Cell Counting Procedure

1. Before using Coulter Counter:
   - Replace the blue Cleaner Solution with Isotonic Diluent in an ACCUVETTE II vial.
   - Run the Isotonic Diluent 3 times using the Start/Stop key.
2. Dispense 10 mL of Isotonic Solution into an ACCUVETTE II vial for each sample.
3. Invert cell suspension, dispense 50 ul of cell suspension into each ACCUVETTE II vial. Pipette up and down several times.
4. Add 3 drops of ZAP-OGLOBIN (which will lyse any remaining RBC's).
5. Stir with transfer pipette to mix. Avoid introducing air bubbles.
6. Read immediately two times and calculate the average WBC count (e.g. Reading of 2,200 E6 means the cell suspension has a WBC count of 2.2 x 10^6 /ml). Write down the average WBC count on the centrifuge tube.

   Acceptable limit is between 1.0 x 10^6 to 2.0 x 10^6/ml
7. Increase the volume added to the cytospin funnel if a specimen has a very low WBC count (maximum 300 ul). Each slide should have 200, 000 WBC's.
8. At the end of the day:
   - Immerse the Aperature Tube of the Coulter Counter into an ACCUVETTE II vial full of blue Cleaner Solution.
   - Run the Cleaner Solution 3 times using the Start/Stop key.
   - Leave the Aperature Tube immersed in the Cleaner Solution overnight.

C. Preparation of Slides

1. Dispense 200 ul of cell suspension from ACCUVETTE II vial (see above) into a single cytospin funnel.
2. Spin at 900 rpm for 3 minutes in a SHANDON Cytocentrifuge.
3. Air dry slide for 5 minutes inside the Laminar Flow Hood.

D. Fixation and Permeabilization of Slides

1. Immerse prepared slides in Fixative Solution for 10 minutes.
2. Rinse in Wash Solution for 5 minutes.
3. Immerse in Permeabilization Solution for 5 minutes.
4. Rinse in Wash Solution for 5 minutes.
5. Let slides dry in Laminar Flow Hood for 5 minutes and proceed to staining, or store dried slides in fridge at 4°C for up to 72 hr. Store in -70°C freezer if slides cannot be stained within 72 hr.

E. Staining of Slides

1. Place one double-well control slide with CMV positive and negative cell spots at a random position within each batch of slides to be stained.
2. Add 20 µl of working solution monoclonal antibody # 1 (anti-pp65) onto the sample and control slide. Incubate in a humidified chamber at 37°C for 30 minutes. FROM THIS POINT, DO NOT ALLOW THE CYTOPREP TO DRY AT ANY TIME DURING THE STAINING PROCESS.
3. Wash by immersion in fresh PBS 3 times for 1 minute each.
4. Wipe excess PBS off slide and add 20 µl of fluorescein conjugated antibody # 2 to the cytoprep. DO THIS ONE SLIDE AT A TIME, DO NOT ALLOW CELL SPOT TO DRY IN BETWEEN.
5. Incubate for 30 minutes at 37°C in a humidified chamber.
6. Wash by immersion in fresh PBS 3 times for 2 minutes each. Wash in fresh dH_2O briefly.
7. Allow slides to dry under hood about 5 minutes.
8. Coverslip slides with FA mounting media.

F. Reading of Slides

a. Reading is performed with the fluorescence microscope using the #4 filter (λ 490/575). Scan the entire surface of the cytoprep with the 40x objective, counting the number of infected cells.

The fluorescent, green polylobate nuclei are the infected and stained PMNL's.
A single fluorescent PMNL is sufficient to indicate a positive antigenemia result.

The following appearances **DO NOT** constitute a positive:

- cytoplasmic fluorescence in large granules (eosinphils)
- slightly greenish periphery of PMNL
- all of the PMNLs appear greenish
- peri-nuclear staining of PMNLs

b. Calculation of positive CMV antigenemia cell count performed in LIS:

Under DPP 65, at the media screen:
WBC # : WBC count from Coulter Counter, written on each centrifuge tube after counting.
PREP : # of positive cells seen in cytospin slide
DIV2 : # of positive cells divided by 2
POS # : DIV2/ WBC#.
F7, put 'V' in isolate # field, Enter, Enter, F2 under 'Org id', F12, choose 1 for CMV, F8, V,and '8' , F12, F12.

G. Daily Quality Control:

a. Check reagent expiratory date and verify that Reagent QC is satisfactory for the reagent lot/kit being used

b. Appropriate control slide with positive and negative CMV wells (commercial; home-made slide with ATCC strain or buffer coat of known CMV positive are acceptable) must be stained with each batch. Place QC slide at a random position within each batch

c. Examine the negative control well first to establish the dull red colour (Evans blue counterstain) and to determine if there is any nonspecific staining.
The positive control must be clearly distinguishable from the negative control or the test is invalid.

d. Record daily QC results in LIS (under procedure VPP65D, CMV pp65 - daily QC STN Staining Reaction).

Failed Daily QC:

a. Do not release patient results pending resolution of QC error.
b. Inform charge/senior technologist.
c. Record in Reagent Log, Instrument Log if microscope/incubator is involved in the failure and file incident report as appropriate.
d. Re-run failed controls in parallel to fresh controls (and/or external QC) to evaluate the QC material itself.
e. If the re-run shows the old QC material still fails, fresh QC passes and nothing else is wrong with the batch (only the old QC material failed, patient results valid) patient results may be released.

Marked decrease/absence in fluorescence can be due to:

a. Reagent deterioration/skipping (did not apply primary/secondary stain)
b. Microscope (filter, bulb, alignment)
c. Other equipment, reagents or technique

IV. Reporting Results

CMV Antigenemia: Negative Report: "Negative for Cytomegalovirus."

Positive Report*: "POSITIVE, - # positive cells/100,000

***Telephone all antigenemia results to Bone Marrow Transplant Clinic. Telephone all positive results to UHN (TGH & TWH).**
When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window.

V. References

1. Clonatec Co.: Detection of HCMV 65 Kda protein kinase in peripheral blood polymorphonuclear leukocytes by indirect immunofluorescence. Clonatec, Biosoft Department Siege social: 60 rue de Wattignies, 75580, Paris Cedex 12 Tel. (1) 43 42 38 30, Fax (1) 43 40 48 86.

2. Gerna, G. et al. Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp65) for optimal quantitative of human cytomegalovirus antigenemia. J. Clin. Micr. 1992; 30: 1232 – 1237.

3. Niubo J. et al. Association of quantitative cytomegalovirus antigenemia with symptomatic infection in solid organ transplant patients. Diagn. Microbiol. Infect. Dis. 1996; 24: 19-24.

4. Ho S. et al. Rapid cytomegalovirus pp65 antigenemia assay by direct lysis and immunofluoresence staining. J. Clin. Micro. 1998; 36;638-640.