LIMITING DILUTION ANALYSES TO DETERMINE HIV-SPECIFIC PCTL
Method 1 (of 2)

1. PURPOSE AND DESCRIPTION

1.1 These methods measure precursor frequencies recognizing a specific HIV-1 gene product (method 1) or CTL epitope (method 2) by limiting dilution assay.

1.2 The data are useful in determining quantitative changes in HIVspecific effector responses in a given individual over time. This should provide a better estimate of the effects of a given intervention on CTL responses than qualitative measurements of specific lysis to a given gene product.

1.3 Method 1 involves the stimulation with autologous monocytes infected with recombinant vaccinia containing HIV-1 ENV/GAG/POL.

2. SPECIMEN

2.1 Anticoagulated blood (ACD or heparin) fresh blood, processed within 24 hours of collection, can be shipped overnight at room temperature (Preliminary)

3. MATERIALS AND REAGENTS

3.1 20-30 ml of heparinized blood irradiated autologous PBMC and adherent monocytes (irradiated at 3300 rad)
3.2 human recombinant interleukin-2 (rIL-2) (Cetus, Emeryville, CA)
3.3 PBS without calcium, magnesium, EDTA, pH 7.4
3.4 Hanks buffered salt solution with 2% FCS
3.5 RPMI (Bio-Whittaker, Walkersville, MD)
3.6 RPMI + 10% human serum (Biocell, Rancho Dominguez, CA) + penicillin
3.7 100U/ml; streptomycin 100 mg/ml and glutamine 1mM/ml (R10)
3.8 recombinant vaccinia viruses: vvEGP (vAbt408, Therion) vvLac (vSC-8), vPE-16 (HIV-1 gp160), vRT (vCF21), vDK1 (HIV-1 gag, D. Kouritzkes) LAI
3.9 cell scraper (Baxter)
3.10 24-well plates (Costar)
3.11 96-well round-bottom microtiter plates (Costar, Cambridge, MA)
3.12 6-well plate (Costar, Cambridge, MA)

4. INTRUMENTATION

4.1 Radioactive isotope counter capable of counting Cr release, calibration 51 according to manufacturer's directions.

5. PROCEDURE

5.1 Day 0:

5.1.1 Preparation of stimulator cells.

5.1.1.1 Isolate PBMC from 15-20 ml blood (yield about 15-25 million cells in HIV+ sample).

5.1.1.2 Resuspend 10 million PBMC in 3 ml RPMI, place into one well of a 6-well flat-bottom plate. (Note: You can start with less PBMC and should expect to recover 5-10% adherent monocytes.)

5.1.1.3 Incubate for 2-3 h at 37 C, 5% CO , then remove nonadherent cells by washing 3 times with PBS and resuspend adherent cells in 2 ml R10.

5.1.1.4 Infect adherent cells (monocytes) with vvEnv-Gag-Pol (MOI=10) adding the virus in a small volume directly to the well.

5.1.1.5 Incubate for 14-16 h in 37C with 5% CO2.

5.1.2 Preparation of responder cells.

5.1.2.1 Plate fresh PBMC in a 96-well round-bottom plate with serial dilutions of cells from 100,000 to 3,125 cells/well in 100 8 R10, with 24-well replicates for each cell concentration.

5.1.2.2 Add irradiated autologous PBMC as feeder cells, plating 50,000 cells per well in 50 ul R10. (Note: Use the nonadherent cells from step 5.1.1.3 above as feeder cells).

5.1.2.3) Incubate at 37 C, 5% CO . o

5.2 Day 1:

5.2.1 Stimulation of responder cells

5.2.1.1 UV-inactivate the vaccinia-infected adherent cells by removing the plate lid and placing the plate 10 cm from the UV light in the biosafety cabinet for 10 min.

5.2.1.2 Detach the adherent cells with the cell scraper, combine in a 15 cc tube. Add 2 ml PBS into the well and repeat scraping 2-3x to remove all adherent cells.

5.2.1.3 Wash the cells with PBS by centrifugation and resuspend in 2 ml R10.

5.2.1.4 g-irradiate (3300 rad) the infected monocytes.

5.2.1.5 Add irradiated autologous monocytes (1000 cells/well in 50 ul R10) to each well containing the responder cells.

5.2.1.6 Incubate at 37 C, 5% CO .

5.3 Day 2:

5.3.1 To each well, add 20 ml of R10 containing 100 U rIL-2 (final concentration of 10U/mL).

5.4 Days 4 and 7:

5.4.1 Remove 90 ml of supernatant from each well (without removing the cells) and replace with 100 ml of fresh media (R-10 + 10 U/ml IL-2, final).

5.5 Day 9: Preparation of Targets for CTL

5.5.1 To prepare 4 sets of target cells, infect 3-4 million autologous BLCL with recombinant vaccinia (MOI=1): vLac, vDK-1, vP16, vRT (2 million cells/200 8 volume total).

5.5.2 Incubate for 90 min at 37 C, 5% o CO .

5.5.3 Wash cells once by centrifugation and resuspend the cells in 2 ml R10.

5.5.4 Transfer cells into one well of a 24-well plate.

5.5.5 Pulse with radioactive chromium (0.1 mCi/2x10 cells).

5.6 Day 10:

5.6.1 CTL assay To test the effector cells against 4 sets of target cells, the responder cell suspension from each well is equally divided among 4 wells in 4 separate assay plates (i.e., 1 well/plate). Thus, cells from the two responder plates (#1 and #2) are transferred to the corresponding 4 assay plates (for plate#1, transfer to #1A, #1B, #1C, #1D; and for plate #2, transfer to #2A, #2B, #2C, #2D).

5.6.2 Wash target cells 3x with HBSS with 2% FCS and resuspend in 2 ml R10.

5.6.3 Add 5,000 of the designated target cells in 100 1 to each well containing responder cells. Set up 16 target replicates for media alone to measure spontaneous chromium release and 16 target replicates with Triton X or NP-40 for maximum release.

5.6.4 Centrifuge the plates for 2 min at 500 rpm.

5.6.5 Incubate for 4 hr at 37oC, 5%CO .

5.6.6 Harvest 30 l of culture supernatant (not cells) and count chromium release.

6. CALCULATIONS

6.1 Determine the number of negative wells for each concentration of responder cells and for each target. A positive response is arbitrarily defined as greater that 3 standard deviations above the mean spontaneous release.

6.2 Plot the fraction of negative wells (y axis, log scale) for each cell concentration (x axis). The frequency of antigen-specific pCTL is estimated by the cell concentration in which 37% of the wells are negative for lysis. This is calculated using a chi-square minimization method (Ref: Clouse et al. 1989, J Clin Microbiol 27: 2316-2323). (Program for these calculations can be provided on disk upon request).

7. FREQUENCY/TOLERANCE/CORRECTIONS

Studies preliminary, this information pending

8. EXPECTED VALUES

pCTL frequencies varying from 0-1000 per 106 PBMC

9. PROCEDURE NOTES

Pending, analysis too preliminary

10. LIMITATIONS OF METHOD

10.1 Lack of recognition of epitopes included in peptide panel in method 2.

10.2 Sensitivity of method 1.

10.3 Need for lab workers to be immune to vaccinia (Method 1)

10.4 Lysis of control targets excessive (preexisting vaccinia or EBV responsiveness)

10.5 Viability and quantity of EBV-transformed B-LCL sufficient to perform the assay.

11. METHOD VALIDATION: INTRA ASSAY VARIABILITY AND INTRA-SUBJECT VARIABILITY

11.1 Work in progress for these determinations.

12. REFERENCES

To be provided

13. EFFECTIVE DATE/SCHEDULE

9/30/96

14. AUTHORS

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Procedure: ACTG Lab Man Cytotoxic T-Lymphocyte Method 1

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