STIMULATION WITH PBMC PULSED WITH KNOWN SPECIFIC CTL EPITOPES
Method 2 (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.

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 mL whole heparinized blood
3.2 RPMI (Bio-Whittaker, Walkersville, MD)
3.3 RPMI + 10% FBS (Biocell, Rancho Dominguez, CA; lot #2262) + penicillin 100 U/mL; streptomycin 100 mg/mL and L-glutamine 1 mM/mL (R10)
3.4 recombinant vaccinia viruses: vvLac (vSC-8) and vDK1 (HIV-1 gag, D. Kouritzkes)
3.5 HIV peptides: SLYN, ILKE, KIRL, QVPL, TLYC, AIPQ, TPGP, GGKK, GPKV, PLKE, NSSK, PPIP, and VPLR (QCB, Inc., Hopkinton, MA)
3.6 Lymphocult T (Biotest, Dreieich, Germany)
3.7 96-well round-bottom microtiter plates (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 Isolate PBMC from whole blood using Ficoll-Hypaque density centrifugation:

5.1.1.1 Layer whole blood on Ficoll; spin at 2000 rpm for 20 min. with no

brake.

5.1.1.2 Wash once with PBS + 4 mM EDTA at 1500 rpm for 10 min with brake

5.1.1.3 Wash second time with PBS + 4 mM EDTA at 1200 rpm for 10 min with brake.

5.1.2 Preparation of Stimulator Cells

5.1.2.1 Take 2 x 10 cells from PBMCs isolated from whole blood for 6 stimulator cells; wash by centrifugation at 1500 rpm for 5 minutes.

5.1.2.2 Discard supernatant, resuspend cell pellet in 500 ml working peptide solution (100 ml peptide/mL)

5.1.2.3 Incubate for 2 h at 37°C; 5% CO2

5.1.2.4 Irradiate cells at 3000 rad

5.1.2.5 Add 2 mL 100 U/mL lymphocult T + 8 mL R10

5.1.2.6 Add 50 ml/well in 96-well plates (50,000 cells/well)

5.1.3 Preparation of Effector Cells

5.1.3.1 Prepare 3 mL of 1.6 x 10 cells/mL in R10 5

5.1.3.2 Add 50 ml R10 to rows "4000 cells/well" through "63 cells/well"

5.1.3.3 Add 50 mL of cell suspension to rows "8000 cells/well" and "4000 cells/well"

5.1.3.4 Take 50 mL from "4000 cells/well" and add to "2000 cells/well." Repeat through "63 cells/well." See Figure 1 for plating sample.

5.1.3.5 Final volume is 100 ml (including stimulator cells) in all wells; incubate at 37°C; 5% CO2

5.1.3.6 Microcultures are fed at 5 and 10 days by the addition (no removal of media) of 50 ml lymphocult T-containing media (1 mL 100 'U'/mL lymphocult T + 9 mL R10). Total volume after day 10=200 ml.

5.2 CTL assay

5.2.1 After 14 days of culture, the effector cells are assessed for cytotoxicity against Cr-labeled target cells. 51

5.2.2 The evening before the addition of targets (Day 13) the effector cells are mixed in their individual wells and equal volumes are transferred to 96-well round bottomed plates (depending on the number of targets; each target is tested against the entire plate: i.e. if 4 targets, then each well of effectors is split to 5 new wells in 5 new 96-well plates to include control). See Figure 2 for plate setup.

5.2.3 Add 50 ml R10 to each well of target 96-well plate.

5.2.4 Add 100 ml of appropriate target cells to each well (105/ml).

5.2.5 Add 100 ml of target cells to 24 wells of separate Min/Max plate (24 wells for each target begin tested).

5.2.6 Add 100 ml R10 to the first 12 wells for each target in the Min/Max

plate. Add 100 ml 5% Triton solution to the final 12 wells for each target. See Figure 3 for plate setup.

5.2.7 Spin plates at 500 g for 2 min with no brake.

5.2.8 Incubate the plates for 5 hours at 37 C, 5% CO . o

5.2.9 Spin plates again before collecting the supernatants.

5.2.10 After 5 hours, harvest 30 ml supernatants, and count Cr. 51

5.3 Preparation of Targets

5.3.1 For each target to be tested: centrifuge 2.5 X 106 autologous BLCL in a 15cc tube, discard the supernatant, and resuspend cells in 200 ml R10.
5.3.2 Add 100 mCi 51Cr.
5.3.3 Add 200 ml 100 mg/ml working solution peptide (for peptide-infected targets; 100 ml/ml)
5.3.4 Infect with recombinant vaccinia (MOI=1; for vaccinia-infected targets)
5.3.5 Incubate at 37°C in 5% CO overnight (12 ~ 16h). 2
5.3.6 Wash target cells by centrifugation 3X
5.3.7 Resuspend cells at 10 /ml 5
5.3.8 Add 100 ml of target cells to each well
e.g. 1 1' plate control peptide
2 2' plate TLYC (p17)
3 3' plate AIPQ (RT)
4 4' plate vLac (control construct)
5 5' plate vDK-1 (gag)

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 2

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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