Benchmark

Flow Cytometric Assay for the Simultaneous Analysis of Cell-Mediated Cytotoxicity and Effector Cell Phenotype

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A growing number of clinical vaccine trials involve monitoring of the patient immune response (7,8). One of the main obstacles for comprehensive monitoring is usually a limited amount of patient blood available. It would be beneficial to use methods that allow measurement of different parameters in one test tube. A number of flow cytometric methods to measure cell-mediated cytotoxicity, particularly based on the uptake of propidium iodide (PI),

have been offered recently (2–6). Excellent correlation has been found between 7-aminoactinomycin D (7-AAD) inclusion in flow cytometric cytotoxicity assay and the results of the ^{51}Cr release assay, with both fresh and cryopreserved effector cells used. The correlation coefficient R^2 was higher than 0.9 in all experiments performed (1).

We developed a method based on flow cytometry that would allow us to simultaneously analyze effector cell phenotype and target cell death using the same sample under conditions that ensure reliable discrimination of the target and effector cells.

K562 (human myelogenous leukemia cell line; ATCC, Manassas, VA, USA) and C1R.A2 (human plasma leu-

kemia cell line that expresses a transfected genomic clone of HLA-A2.1) were cultured in complete medium (CM) consisting of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM pyruvate, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 50 $\mu\text{g}/\text{mL}$ gentamycin (BioWhittaker, Walkersville, MD, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of normal human volunteers by buoyant density centrifugation over Ficoll-Paque[®] (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Aliquots of effector cells were cryopreserved in the vapor phase of liquid nitrogen for future use in functional testing and fluorescence-activated cell sorting (FACS) analysis. For cytolytic T lymphocyte

Figure 1. Principle of simultaneous analysis of cell-mediated cytotoxicity and effector cell phenotype. (A) Target and effector cells together. R1, target cells labeled with PKH 67; R2, unlabeled effector cells. Y axis, FL1 (PKH 67); X axis, side scatter. Gating was based on the difference in fluorescence (FL1) of target cells before and after labeling. (B) PKH 67-labeled target cells (R1 region of panel A) after incubation with effector cells, followed by treatment with 7-AAD. Y axis, side scatter; X axis, FL3 (7-AAD). Dead cells are 7-AAD positive. PKH 67-labeled target cells incubated without effector cells served as a control for setting the regional gates. (C) Non-PKH 67-labeled effector cells (R2 region of panel A) analyzed for the expression of CD8. Y axis, side scatter; X axis, FL2 (CD8). Isotype Ig-treated cells served as a control for setting regions.

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(CTL) induction, PBMCs from normal HLA-A2-positive donors were used. C1R.A2 were employed as target cells. 3×10^6 PBMC in 2 mL CM supplemented with 1000 U/mL IL-7 (Pepro- tech, Rocky Hill, NJ, USA) were seeded in 24-well plates (Costar, Corning, NY, USA). Flu-Matrix peptide (FMP, $\text{NH}_2\text{-G-I-L-G-F-V-F-T-L-COOH}$; Genosys Biotechnologies, Woodlands, TX, USA) was added to the PBMC in final concentration of 3 μM . On day 2 and day 5, 20 U/mL rIL-2 were added. Cells were assayed on day 7. Before use, targets (C1R.A2) were pulsed with 3 μM FMP for 1 h at 37°C. To induce lymphokine-activated killer (LAK)

cells, PBMC (10^6 cells/mL) were incubated in RPMI 1640 supplemented with 10% pooled human serum, 2 mM glutamine, 1 mM pyruvate, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (Bio-Whittaker), and 100 U/mL human rIL-2 (Hoffman-La Roche, Nutley, NJ, USA), in 5% CO_2 and used on day 6 or 7.

To discriminate effector and target cells, target cells were labeled with PKH 67, a green fluorescent probe that becomes incorporated into the plasma membrane. PKH 67 was diluted (Sigma, St. Louis, MO, USA) according to the PKH67 Green Fluorescent Cell Linker Kit directions. Briefly, 3×10^6 target cells were washed in Dulbecco's

PBS (Life Technologies, Rockville, MD, USA) and resuspended in 1 mL solution C from the kit. The PKH 67 was diluted to 4×10^{-6} M in 1 mL solution C. Cells were combined with dye, and the tube was inverted several times over 3 min. FCS (2 mL) was added to the tube and inverted continuously for 1 min. Cells were then transferred to a 15-mL conical tube with 4 mL RPMI 1640 without Phenol Red (Sigma) with 10% FCS (BioWhittaker) and washed three times in the same medium. As our experience shows, PKH 67 labeled equally well various cell lines including such widely used cells as YAC-1, CEM, JY, and T2.

Figure 2. Simultaneous flow cytometric analysis of cell-mediated cytotoxicity and effector cell phenotype. (A–C) PBMC tested against K562 cells in 4 h cytotoxicity assay. E:T ratio, 10:1. (A) 7-AAD-positive cells among gated target cells stained with PKH67. (B and C) CD8- and CD69-positive cells among non-gated PBMCs. (D–F) Anti-FMP-CTL tested against FMP-pulsed C1R.A2 cells in 4 h cytotoxicity assay. E:T ratio, 10:1. (D) 7-AAD-positive cells among gated target cells stained with PKH67. (E and F) CD8- and CD69-positive cells among non-gated CTLs. (G–I) LAK cells tested against K562 cells in 4 h cytotoxicity assay. E:T ratio, 10:1. (G) 7-AAD-positive cells among gated target cells stained with PKH67. (H and I) CD56- and CD69-positive cells among non-gated LAK cells.

Effector cells were washed twice in Phenol Red-free medium and mixed with PKH 67-labeled target at various E:T ratios in 12 × 75 mm Falcon® 2052 tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The effector and targets were incubated at 37°C with 5% CO₂ for 4 h. At the end of the incubation period, cells were washed in PBS, resuspended in 100 µL PBS with 1% FCS, and 10 µL phycoerythrin (PE)-conjugated anti-CD69, -CD56, or -CD8 (Exalpha, Boston, MA, USA) was added to cell suspension for 30 min on ice. Isotype controls were included in all experiments. After incubation, the cell suspension was washed, and 10 µL of a 50 µg/mL solution of 7-AAD (Sigma) was added to the cell suspension for 10 min on ice to evaluate increased membrane permeability of target cells due to cytotoxic action of effector cells. 7-AAD is a DNA intercalator that can be excited by a 488-nm argon laser and emits in the far-red range of the spectrum ($\lambda_{em_{max}}$: 655 nm). Its spectral emission can be effectively separated from the emissions of FITC ($\lambda_{em_{max}}$: 517 nm) and PE ($\lambda_{em_{max}}$: 578 nm) (5); PKH 67 has $\lambda_{em_{max}}$: 502 nm, and this was the main reason we chose 7-AAD over the PI ($\lambda_{em_{max}}$: 625 nm). Excellent correlation between 7-AAD and PI uptake by dead cells have been found by Schmid et al. (5) as well as in our preliminary experiments (not shown).

Samples were analyzed on a FACScan™ flow cytometer (Becton Dickinson) with an air-cooled argon-ion, 488-nm coherent beam laser. The instrument contained three optical filters, FL-1 (PKH 67) at 530/30 nm, FL-2 (anti-CD-antibody-PE) at 585/42 nm, and FL-3 (7-AAD) at 650 nm Long Pass. Spectral overlap was electronically compensated using single-color controls. A minimum of 20 000 events were collected and analyzed with LYSIS II software from the instrument manufacturer. Percentage of dead cells (7-AAD-positive, red fluorescence) among PKH 67-stained target cells (green fluorescence) and percentage of effectors (non-PKH-stained cells) stained with PE-conjugated anti-CD56, -CD8, or -CD69 antibodies (red fluorescence) were calculated (Figure 1).

Anti-flu-matrix peptide (α FMP) CTL were incubated with FMP-pulsed

C1R.A2 targets for 4 h. After incubation, cells were treated with PE-anti-CD69, PE-anti-CD8, or PE-anti-CD56 and with 7-AAD. We found 62% of dead target cells when α FMP-CTL were tested against FMP-pulsed C1R.A2 cells and only 5.6% of dead cells when non-activated PBMC were used against the same targets (Figure 2). In preliminary experiments, we have shown that the killing of non-pulsed C1R.A2 cells was significantly lower than the killing of pulsed cells and in most cases was negligible (1). We also found that 64% of CTL were CD8 positive and 71% were CD69 positive. Corresponding numbers for PBMC were 5.6%, 28.6%, and 7.7%. Similarly, when human LAK cells were tested against K562 and 7-AAD and PE-anti-CD69 or anti-CD56 antibody were used, we found 57% dead target cells, 36.6% of CD56, and 64% of CD69-positive cells in cell suspension. PBMC in this experiment had 12% of CD56 positive cells (not shown).

In this report, we describe a new approach for the determination of in vitro cytotoxicity and effector cell phenotype in the same sample utilizing three-color flow cytometric analysis. The application of this method for immunological monitoring could help to perform the monitoring when the amount of clinical material is limited.

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