

ABSTRACT

NK effector function has traditionally been examined using ⁵¹Chromium release assays. Recently, to avoid costs associated with disposal of radioactive waste, a number of fluorescent based flow cytometric assays have been developed as alternatives. Most of these assays use one dye that is irreversibly incorporated into the cell and propidium iodide as the DNA dead cell staining dye. We describe here a novel assay in which target cells are first labeled or "painted" with CFSE, incubated with effector cells and then monitored for the percentage killed using a membrane impermeant dye, 7AAD. Both live and dead targets retain the signal from the CFSE dye, as it is covalently linked to intracellular amine groups upon internalization and does not leach from cells into the media nor is transferred to adjacent cells. Dead target cells in addition take up the 7AAD. Although propidium iodide has been traditionally used to detect the dead cells, 7AAD is preferable as it better defines the dead cell population, allowing more accurate determination of cell killing. Using this assay, increasing amounts of dead target cells can be detected and quantified as increasing amounts of NK effectors purified from whole blood are added. In addition effects of IL-2 incubation and treatment with anti-perforin antibodies on cell death are readily detectable.

INTRODUCTION

One of the functions of the immune system is to recognize and destroy targets, such as tumor cells or cells infected with microbes (bacteria or viruses). This is accomplished by effector cells, such as T cells, NK cells, macrophages or granulocytes, which employ cell mediated cytotoxicity (CMC), natural killer activity (NK) or antibody-dependent cellular cytotoxicity (ADCC), respectively. CMC and NK activities are commonly monitored in order to assess the potency of immune cell stimulation by small molecules, antibodies or other regulatory molecules, whereas ADCC activity is used to assess the potency of antibodies for therapy.

Cellular cytotoxicity has traditionally been measured using ⁵¹Chromium release assay¹, although alternative assays (including LDH², MT³, and AlamarBlue⁴, among others) have been used recently due to concerns about the handling and disposal of radioactive materials. Another approach to measure cytotoxicity involves the detection and quantification of dead target cells by flow cytometry. Live and dead target and effector cells can be distinguished through the use of differential staining by fluorescent dyes.^{5,6}

The Guava CellToxicity[™] assay uses two dyes, a cell painting dye and a cell impermeant DNA binding dye, to distinguish effector or cytotoxic cells from target cells, and to distinguish dead target cells from live target cells. The cell painting dye, CFSE (also known as CFDA SE), diffuses freely into cells where intracellular esterases cleave off the acetate groups, converting it to a fluorescent, membrane impermeant dye. The dye is retained in the target cell's cytoplasm even after the cell is killed due to covalent crosslinking to proteins through its succinimidyl groups. Painted target cells are subsequently added to unpainted effector cells at various ratios and incubated to allow effector cells to function. A dead cell dye, 7-AAD, is then added to determine which of the painted target cells have been killed.

MATERIALS & METHODS

Cultured Cells: A non-adherent human bone marrow cell line, K562 (ATCC Cat. No. CCL-243), and a non-adherent human B cell line, Daudi (ATCC Cat. No. CCL-231), were kept in log phase growth in complete medium designed to stimulate optimal growth. Iscove's modified Dulbecco's medium (Cellgro Cat. No. MT 10-016-CV) supplemented with 10% FBS (ATCC Cat. No. 30-2020) and 2 mM L-glutamine (Cellgro Cat. No. MT 25-005-CV) was used for culturing the K562 cells. RPMI 1640 (Cellgro Cat. No. MT-10-040-CV) medium supplemented with 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose (Sigma Cat. No. G8769), and 1 mM sodium pyruvate (Cellgro Cat. No. MT 25-000-CL) was used for culturing the Daudi cells.

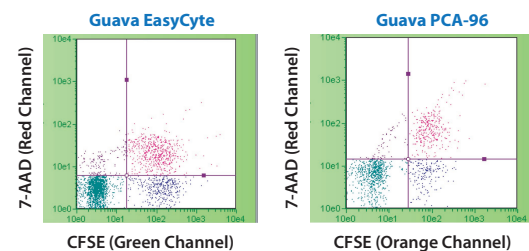
Purification of the NK cells: Peripheral blood mononuclear cells (PBMCs) from anonymous healthy adult blood donors were obtained by gradient centrifugation over Histopaque Plus (Amersham BioSciences Cat. No. 17-1440-02)⁹ of day old blood. NK cells were purified from PBMCs using the NK Cell Isolation Kit II (Miltenyi BioTech Cat. No. 130-091-152) following the manufacturer's protocol and resuspended in the target cell medium. NK cells were typically >90% pure.

Guava CellToxicity Assay: The Guava EasyCyte[™] CellToxicity Assay (Guava Cat. No. 4500-0230) was conducted according to the manufacturer's instructions. Briefly, target cells were seeded two days prior to each experiment as follows: the K562 cells were split to a final cell concentration of 300,000 cells/mL and the Daudi cells were split to a final concentration of 400,000 cells/mL. The K562 and Daudi cells were incubated with 0.5 μM CFSE and washed to remove excess dye. For those samples to be acquired on the Guava PCA-96[™] they were painted with 5 μM CFSE (Cat. No. 4500-0200) due to the decreased excitation of CFSE with 532 nm laser versus 488 nm laser light and detection of CFSE fluorescence at 580 nm. The painted target cells were then mixed with the purified NK cells at various effector to target ratios as described below (included target cells only and effector cells only) in 96-well plates and incubated for 4 hours at 37°C humidified incubator with 5% CO₂. In some experiments Interleukin 2[™] (IL-2; CalBiochem Cat. No. 407623) or purified anti-perforin mAbs (Becton Dickinson Cat. No. 556434) was added to each well along with the effectors and targets prior to incubation.

After incubation, 7-AAD was added to all wells, unless otherwise stated. For samples to be run on the Guava PCA-96, one-half the amount of 7-AAD was used in comparison to the samples that were run on the Guava EasyCyte or Becton Dickinson FACSCalibur[™]. In some cases, propidium iodide was used in place of 7-AAD.

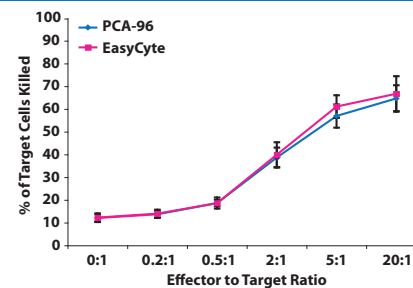
In some cases, the Molecular Probes Live/Dead[™] Cell Mediated Cytotoxicity Kit (Cat. No. L-7010) was also used according to the manufacturer's instructions.

Figure 1. Detection of Live and Dead Target Cells on the Guava EasyCyte and PCA-96



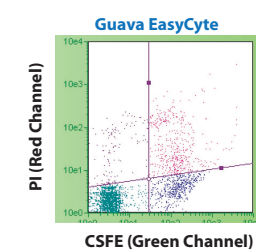
These dot plots show a clear distinction between the live and dead target and effector cells, from a sample acquired on a Guava EasyCyte or a PCA-96, with each cell population displayed using a unique color code. Unpainted, 7-AAD negative, live effector cells appear in the lower left quadrant (teal green), painted, 7-AAD negative, live target cells appear in the lower right quadrant (blue), unpainted, 7-AAD positive, dead effector cells appear in the upper left quadrant (purple), and painted, 7-AAD positive, dead target cells appear in the upper right quadrant (pink).

Figure 2. Accuracy of Percent of Target Cells Killed on the Guava PCA-96 vs the Guava EasyCyte



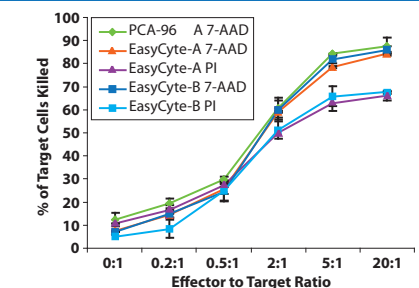
K562 target cell killing was assessed using the Guava PCA-96/PCA-96 CellToxicity Kit and the Guava EasyCyte CellToxicity Kit. Each cell population is displayed as the mean ± the standard deviation of sixteen samples. K562 cells painted with CFSE were mixed with unpainted NK cells at the indicated ratios and stained with 7-AAD following a 4 hour incubation in a 96-well round bottom plate. The results obtained from both assays were very similar with very variation as shown by the small error bars.

Figure 3. Detection of Live and Dead Target Cells on the Guava EasyCyte with Propidium Iodide as the Dead Cell Stain



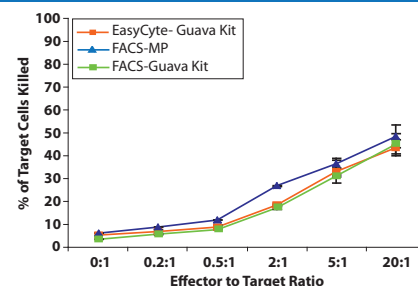
The dot plot shows a clear distinction between the target and effector cells while the distinction between the live and dead target cells is not as clear. Each cell population is displayed using a unique color code from a sample acquired on a Guava EasyCyte. Unpainted, PI negative, live effector cells appear in the lower left quadrant (teal green), painted, PI negative, live target cells appear in the lower right quadrant (blue), unpainted, PI positive, dead effector cells appear in the upper left quadrant (purple), and painted, PI positive, dead target cells appear in the upper right quadrant (pink).

Figure 4. Comparison of Propidium Iodide and 7-AAD as the Dead Cell Stain



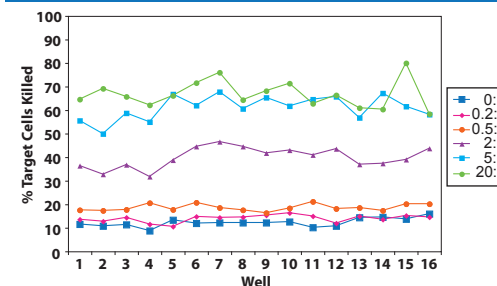
Detection of K562 target cell killing with 7-AAD versus PI. The K562 cells were painted with CFSE, mixed with unpainted NK cells at the indicated ratios and stained with 7-AAD or propidium iodide following a 4 hour incubation in a 96-well round bottom plate. Each point on the graph is the mean ± the standard deviation of three samples. Data derived from the same experiment are shown in figures 1 and 3. At moderate to high effector to target ratios the results obtained with the PI staining consistently showed lower percents of targets killed than the samples stained with 7-AAD.

Figure 5. Accuracy of Guava EasyCyte CellToxicity Kit vs. a Traditional Flow Cytometer



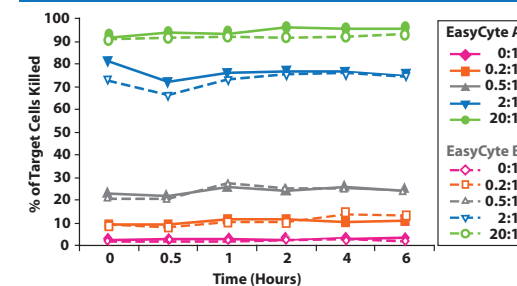
K562 target cell killing was assessed using the Guava EasyCyte CellToxicity Kit and the Molecular Probes Live/Dead Cell-Mediated CytoToxicity Kit (MP). The Guava EasyCyte CellToxicity Kit samples were run on both the Guava EasyCyte and a traditional flow cytometer. The Live/Dead Cell-Mediated CytoToxicity Kit was run on a traditional flow cytometer. K562 cells were painted with CFSE or DIOC₁ (Molecular Probes kit), mixed with unpainted NK cells at the indicated ratios and stained with 7-AAD (Guava kit) or PI (Molecular Probes kit) following a 4 hour incubation in round bottom 96-well plates. Each point is shown as the mean ± the standard deviation of three samples. The results obtained from all three assays were very similar with very low variation, as indicated by the small error bars.

Figure 6. Precision of Percent of Target Cells Killed



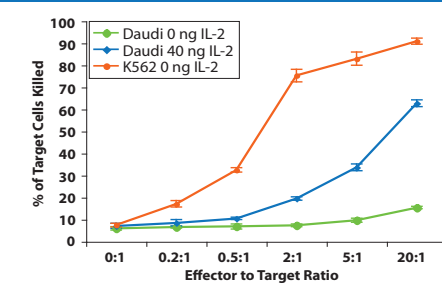
Data are derived from the same experiment as shown in Figure 2, except here the data for each individual well are shown. The effector to target ratios are listed in the legend. The percentage of target cells killed for each sample at a given effector to target ratio was consistent over the time it took (approximately 1 hour) to acquire the entire plate. The %CVs for all Percent of Target Cells Killed were under 15% at effector to target ratios that resulted in killing above background, showing consistent results across an entire 96-well plate. The %CVs of the MFI ratios for both the CFSE and 7-AAD fluorescence were under 25% which shows consistent signal to background MFI ratios over the plate.

Figure 7. Assessing the Stability Over Time of the NK Assay



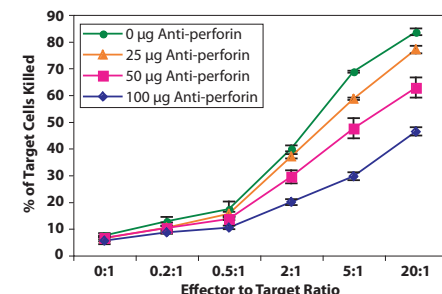
Samples were prepared at six different effector to target ratios and acquired at the indicated times after completion of the assay. Each point on the graph was derived from 3 samples. The percentage of target cells killed was stable for at least 6 hours for all E:T ratios. Similar results were obtained for 5:1 effector to target ratio (data not shown). The MFI values for the populations also remained stable, only varying 15% over the 6 hour period (data not shown).

Figure 8. Effect of IL-2 on NK Activity Against Daudi Cells



Painted K562 and Daudi cells were incubated with purified NK cells. IL-2 at 2.5, 10, and 40 ng was included in some of the wells during the incubation. Each point on the graph is shown as the average ± the standard deviation for 3 replicate samples. Significant lysis of K562 cells was observed at all E:T ratios whereas very little lysis of Daudi cells was observed unless the NK cells had been activated with IL-2. Lower concentrations of IL-2 resulted in less activation and hence less lysis (data not shown).

Figure 9. Effect of Anti-Perforin mAbs on NK Activity Against K562 Cells



Painted K562 cells were incubated with purified NK cells. Purified anti-perforin mAb at 25, 50, and 100 μg was included in some of the wells during the incubation. Each point on the graph is shown as the average ± the standard deviation for 3 replicate samples. Significant lysis of K562 cells was observed at all E:T ratios where no perforin was present. The anti-perforin mAbs decrease the killing of target cells in a dose dependent manner.

References

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SUMMARY AND CONCLUSIONS

The Guava EasyCyte Cell Toxicity Assay was shown to:

- ◆ Allow the user to easily distinguish between the live and dead target cells in a single sample;
- ◆ Demonstrate better detection of target cells killed with 7-AAD than with PI
- ◆ Accurately determine the percent of target cells killed at a variety of effector to target ratios as compared to Guava PCA/PCA-96 CellToxicity assay and predicate flow cytometry methods;
- ◆ Precisely determine the percent killing of target cells and the MFI of the target cells, with CVs of typically less than 15% across a 96-well plate;
- ◆ Yield stable results for up to 6 hours; and
- ◆ Detect both enhanced and decreased NK activity when effector cells are co-cultured with IL-2 or anti-perforin mAbs.