Application Note 8

Isolation of Natural Killer (NK) Cells for Functional Studies

SORTING

FACSCALIBUR, FACSORT

Introduction

Cell sorting is commonly used to isolate cells for functional studies.*
In this application note we use the FACSort™ to sort natural killer cells from peripheral blood mononuclear cells (PBMCs) based on surface phenotype, then assay the isolated cells in an in vitro cytotoxicity assay. We show that the isolated population is not only active in the cytotoxicity assay, but is enriched for NK activity when compared to unfractionated cells obtained from the same donor.

* For research use only. Not for use in diagnostic or therapeutic procedures.

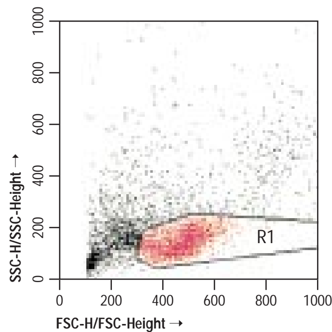

Figure 1a

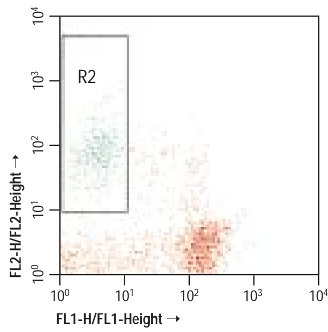

Figure 1b

Figure 1 *Dot plots of forward scatter versus side scatter (1a) and FL1 versus FL2 gated on lymphocytes (1b) before sorting. A logical combination (R1 AND R2) of the lymphocyte gate (R1) and a fluorescence gate (R2).*

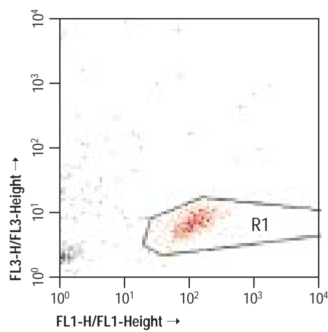

Figure 2a

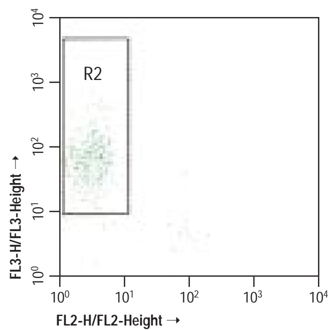

Figure 2b

Figure 2 *Dot plots of forward scatter versus side scatter (2a) and FL1 versus FL2 gated to exclude debris (2b) after sorting the CD5⁻CD56⁺ NK cells.*

Materials and Methods

Cell Preparation and Staining

Twenty mL of whole blood was collected into a VACUTAINER® K_3 EDTA blood collection tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). The PBMCs were isolated by the use of two 10-mL LeucoPREP® separation tubes (Becton Dickinson Labware [BDL], Lincoln Park, NJ) as instructed in the package insert. The PBMCs were washed with Ca^{++}/Mg^{++}-free Dulbecco's phosphate-buffered saline (DPBS) (ICN Biomedicals, Costa Mesa, CA) with 0.5% bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO). The cells were then resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 mM HEPES buffer at a concentration of 10^7 cells/mL, and stored overnight at 4°C. The cells were stained with 400 µL (20 µL per 10^6 cells) each of CD5 (Leu™-1) fluorescein isothiocyanate (FITC) and CD56 (Leu-19) phycoerythrin† (PE) (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) for 20 minutes on ice. The stained cells were washed twice and resuspended at 2.5 x 10^7 cells/mL in DPBS with 0.5% BSA for sorting.

Flow Cytometry

The FACSort was set up using BDIS AutoCOMP™ software and CaliBRITE™ beads. The amplifiers were set with the scatter channels in linear mode and the fluorescence channels in log mode. The instrument settings were adjusted to optimize the resolution of the cell population in all of the scatter and fluorescence parameters after applying the AutoCOMP settings. The cells were sorted using a logical combination (R1 AND R2) of the lymphocyte scatter region (R1) and the $CD5^-CD56^+$ fluorescence region (R2), as shown in Figure 1. This population comprised approximately 11% of the sample.

The cells were sorted in the Exclusion mode set on the LO flow rate using PBS as sheath fluid. Approximately 4 x 10^5 cells were sorted at a rate of approximately 80 cells/s into 50-mL BDL polypropylene conical tubes coated with 4% BSA-PBS and rinsed with 0.5% BSA-PBS immediately before sorting. When the tubes were filled, 500 µL of FBS was added and the tubes were stored on ice until the sort was complete.

The sorted cells were concentrated by centrifugation at 300 x *g* for 5 minutes, and all but approximately 300 µL of the supernatant was removed. The cell pellets were then resuspended and pooled. A sample of this suspension was analyzed on the FACSort. The recovered cells were counted, centrifuged, and resuspended at 5 x 10^5 cells/mL in RPMI 1640 with 10% FBS, 2 mM L-glutamine and 25 mM HEPES buffer for the chromium release assay. Cytocentrifuge slides of the unsorted and sorted cell suspensions were prepared and stained using the DiffQuik (American Scientific Products, McGaw, IL) method (see *Sorting Application Note 1*).

† US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942

Natural Killer Cell Cytotoxicity Assay

Preparation of target cells

Cultured K562 (ATCC CCL 243) tumor cells were used as the target cells for this assay. Viable cells were identified by trypan blue exclusion, counted, then 2 x 10^6 cells were transferred to a 50-mL polypropylene conical tube. The cells were centrifuged at 800 x *g* for 5 minutes. After the spin, the supernatant was decanted from the cell pellet (approximately 100 µL of media remained). The cells were radiolabeled by adding 100 µL of 1.0 mCi/mL ^{51}Cr sodium chromate (Amersham Corp, Arlington Heights, IL) to the cell pellet, vortexing, and placing them in a 37°C, 7% CO_2 incubator for approximately 3 hours. The cells were then washed two times (to remove excess radioisotope) with RPMI 1640 with 10% FBS (heat-inactivated), 2 mM L-glutamine and 25 mM HEPES by centrifuging at 800 x *g* for 5 minutes. The radiolabeled target cells were resuspended at 1 x 10^5 cells/mL in the RPMI medium.

Chromium release assay

The assay was performed in a 96-well u-bottom microtiter plate. The stained, unsorted cells were added to wells in triplicate to make the effector to target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1 (by serial dilution in RPMI media). The sorted cells were added to wells in triplicate to make one E:T ratio of 10:1. Approximately 10^4 (100 µL) labeled target cells (~3000 cpm) were added to all the wells. To measure spontaneous release of chromium, 100 mL of RPMI was added to three wells in place of effector cells. To measure maximum release of chromium, 100 µL of 10% Triton-X 100 was added to three wells in place of effector cells. The plate was centrifuged at 200 x *g* for 3 minutes and placed in a 37°C, 7% CO_2 incubator for 4 hours. After incubation, the plate was centrifuged again at 200 x *g* for 3 minutes and 150 µL of the supernatant was removed from each well. The supernatants were transferred to plastic tubes containing an absorbent material and the radioactivity was measured using an LKB 1272 CliniGamma Counter.

The activity of the NK cells (cytotoxicity) was calculated from the radioactivity measurements using the following equation:

$$\% \text{ cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

Results

The dot plot from the analysis of the sorted cells shown in Figure 2 shows the high purity of the sorted sample (approximately 97%, excluding low FSC debris). Photomicrographs of the slide preparations from the sorted and unsorted cell suspensions show the heterogeneity of the population before sorting, and the homogeneity and retention of cell morphology after sorting (Figure 3).

Figure 4 shows a graph of the E:T ratio versus the percent cytotoxicity of the unsorted and sorted cells. As expected, the cytotoxicity decreased as the E:T ratio decreased from 40:1 to 5:1, using unsorted cells as effectors. At an E:T ratio of 10:1, the sorted cells exhibited increased cytotoxicity (47% vs 16%) when compared to the unsorted cells.

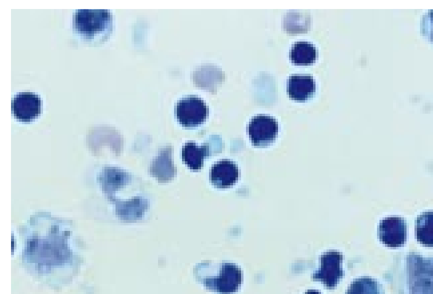

Figure 3a

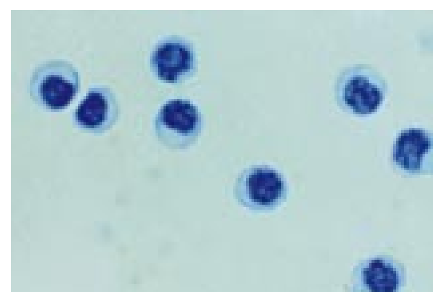

Figure 3b

Figure 3 *Photomicrographs of the unsorted cells (3a) and the sorted natural killer cells (3b). The cells were stained with DiffQuik.*

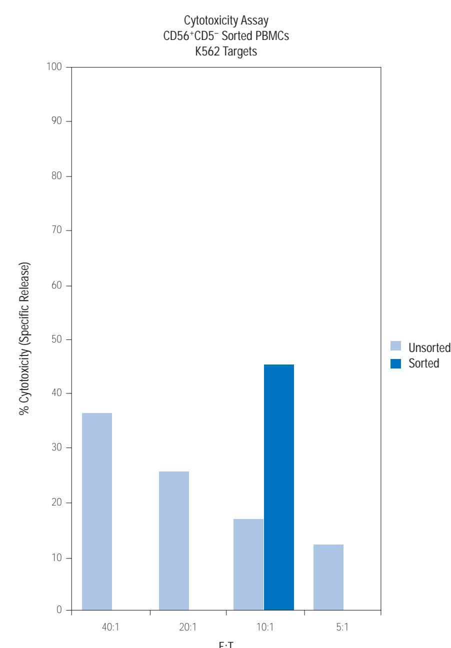

Figure 4 *Cytotoxicity data of the unsorted and sorted cell populations.*

BDIS publishes this method as a service to investigators. Detailed support for non–flow cytometric aspects of this procedure may not be available from BDIS.

Discussion and Summary

Lanier et al[1] demonstrated that the majority of NK activity resides in the $CD5^-CD56^+$ population in peripheral blood. In this note we showed that NK activity was indeed increased in this population compared to unsorted cells. The effect of staining the effector cells with antibody was controlled in that both the unsorted and sorted populations were stained with the same antibody combination. These results show that the FACSort can be used to isolate NK cells, which retain their functional activity when tested in an in vitro functional assay.

Hints

- Do not use sodium azide (NaN_3) in any washing solutions. This chemical alters the metabolism of the cells and may affect your functional study.
- DiffQuik was used to examine the general morphology of the cells. To visualize granules in NK cells, use a Wright-Geimsa stain.
- Avoid using CD3 or CD16 to mark populations that will be used in functional assays, since these antibodies may induce nonspecific cytotoxic activity.

References

1. Lanier LL, Le AM, Civic C, Loken MR, Phillips JM. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigens on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol*. 1986;136:4480–4486.

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