Indian Journal of Experimental Biology
Vol. 52, October 2014, pp. 983-988

Rapid flow cytometry based cytotoxicity assay for evaluation of NK cell function

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Received 30 September 2013; revised 7 August 2014

Assessment of natural killer cells (NK-cell) cytotoxicity is used not only in research settings but is also important in diagnosis of various diseases. NK-cell cytotoxicity assays are based on measurement of target cells killed by cytotoxic cells analyzed either by chromium (^{51}Cr) release assay or flow cytometry. Both these methods use peripheral blood mononuclear cells (PBMC) or pure NK-cell population and hence require large volume of blood sample which is difficult to obtain in pediatric patients and patients with cytopenia. Hence, a flow cytometric assay was designed to determine NK cell activity using whole blood, eliminating the need for isolation of PBMCs or pure NK cells. This assay is based on a dual fluorescent staining of target cells (K562 cell line). The DIOC18 dye labeled K562 cells are incubated with whole blood and then counterstained with 7-AAD enabling the measurement of dead target cell and then percent cytotoxicity is calculated. This study compared the NK cell cytotoxicity using PBMC and whole blood in clinically relevant samples. There was no significant difference between two assays in the measurement of lytic activity or in reproducibility in the repeated samplings of healthy individuals. The whole blood assay required less volume of blood and also less processing time as compared to PBMC assay. It was also validated by testing patients diagnosed with familial hemophagocytic lymphohistiocytosis expected to have low NK-cell activity. This assay is rapid, sensitive and reproducible and requires significantly less volume of blood which is important for clinical evaluation of NK-cell function.

Keywords: Flow cytometry, Natural Killer cell cytotoxicity, Whole blood

NK-cells are a subset of non-B, non-T peripheral blood lymphocytes that play a crucial role in the human innate immune response[1]. NK cells possess relatively large numbers of cytolytic granules, which are secretory lysosomes containing perforin and various granzymes. These granules traffic to the contact zone with the susceptible target cell via so-called immunological synapse and their contents are extruded to effect lysis. This perforin-dependent cytotoxicity is the major mechanism for clearance of cells expressing foreign major histocompatibility complex (MHC) molecules, virally infected cells, and tumor cells by NK cells. Defect in this cytotoxic mechanism causes impaired NK cell activity reported in many diseases[1]. Patients with primary immunodeficiencies like familial hemophagocytic lymphohistiocytosis (FHL) are reported to have low NK cell activity[2,3], whereas patients with chronic viral infections like congenital cytomegalo virus (CMV)[4] or pregnant women[5] are reported to have enhanced NK cell activity. NK cell activity detection is also important in human inflammatory diseases including atopy, asthma and autoimmunity[6].

As our understanding of NK cell function and their role in disease and health improves, the indications for comprehensive evaluation of NK cell function will also increase. Enumerating NK cell numbers is not adequate and one requires assessment of the NK cell activity. Thus a suitable clinical assay for NK cell activity is required.

The conventional method for the determination of NK-cell cytotoxicity is the radioactive chromium (^{51}Cr)-release method. However, this assay is not a preferred method for use in the clinical laboratory for a variety of reasons; e.g., it requires the use of radioactive chromium, which is inherently expensive, entails concerns in handling and disposal and also has lot of inter laboratory variability[7]. Hence in last few years it is largely replaced by flow cytometry based assays. These assays avoid the problems associated with the use of radioactivity and are rapid and more amenable to standardization. It also allows the detection of cytotoxicity at single cell level and also simultaneous measurement of several parameters

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which may be more advantageous for clinical monitoring[8-10]. However all the flow cytometric assays for NK-cell cytotoxicity described till date uses either peripheral blood mononuclear cells (PBMC) or pure NK-cell population and hence require large volume of blood sample. This is difficult to obtain especially from pediatric patients below the age of 1 year and patients with cytopenia. Cell separation procedures are time consuming and may also lead to loss of physiological conditions, such as soluble factors like cytokines, growth factors or chemokines[11].

Keeping above reasons in view a rapid flow cytometry-based assay has been designed for measuring NK-cell cytotoxicity using whole blood. The results were compared with PBMC obtained simultaneously from healthy human control. In addition, reproducibility in the repeated samplings of healthy individuals was analyzed to validate the usefulness of the assay in a clinical laboratory.

Materials and Methods

Blood samples

Normal samples: Fresh heparinized peripheral blood samples were collected from 75 healthy donors 20-55 years of age, including both males and females. These subjects were determined by interview and physical examination to be healthy and free of medication.

HLH patient samples: Hemophagocytic lymphohistiocytosis (HLH) diagnosis was carried out based of diagnostic criteria proposed by Histiocyte society which includes fever, splenomegaly, cytopenia, hemophagocytosis, hyperferritinemia, hypertryglyceridemia, hypofibrinogenemia and high levels of sCD25[4]. In these patients perforin estimation and granule release assay was performed to differentiate familial hemophagocytic lymphohistiocytosis (FHL) patients from acquired HLH patients. FHL patients have inherited NK cell defects which is irreversible whereas in acquired HLH patients NK cell defect is usually temporary. So, for validation of proposed assay, FHL patients were evaluated. Fresh heparinized peripheral blood samples were collected from 20 FHL patients.

Each subject gave informed consent for participation in the study after the nature and possible consequences of the studies were fully explained.

***NK cell enumeration*—**NK cell enumeration was done using dual platform. Absolute white blood cells (WBC) count and lymphocyte absolute count was determined using Sysmex XS-800i. NK cell population among lymphocytes was determined by staining 50 µL blood samples with CD45 (PerCP-Cy5.5), CD56+ CD16 (PE) and CD3 (FITC) (BD Multitest 6-color TBNK reagent, BD Biosciences) for 20 min in dark and then lysing erythrocytes using FACS Lysing solution (BD Biosciences). Samples were analyzed on FACSAria using FACSDiva software (Becton Dickinson, San Jose, CA, US). Lymphocytes were gated by CD45 gating and NK cell percentage was defined as $CD45^+/CD56^+/CD16^+/CD3^-$. The absolute NK cell number was determined as:

NK cell absolute count = [NK cell percentage of lymphocytes × Absolute lymphocyte count]/100

Flow cytometry for NK cell activity

Preparation of target cells and target cell labeling: K562 cells (a human erythroleukemia cell line) cultured in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), L-glutamine i.e. complete medium (CM) at 37 °C under 5% (v/v) CO_2, were used as target cells. Cells were cultured for 72 h before being assayed by flow cytometry. Target cell labeling was carried out by labeling 10^6 cells/mL with 20 µL of 2 mg/mL DIOC-18 (3,3′-Dioctadecyloxacarbocyanine perchlorate) (Sigma) in DMSO. The cells were incubated for 30 min at 37 °C under 5% (v/v) CO_2, washed thrice with PBS and resuspended to a final concentration of 10^5 cells/mL in complete RPMI media. Before using for the test, cell labeling and viability using 7- amino-actinomycin D (7-AAD) was confirmed by flow cytometry. The test was performed when cells were labeled homogeneously and cell viability was more than 80%.

Preparation of effector cells (PBMC and blood samples): PBMC were isolated from 10 mL of heparinized blood by centrifugation with Ficoll-Hypaque. Cells were resuspended at a final concentration of 10^6cells/200 µL in complete RPMI media. For whole blood assay, blood volume was adjusted to obtain lymphocyte count of 10^6cells/200µL in RPMI containing 10% (v/v) FBS.

***Controls for spontaneous lysis*—**Target cells (100 µL, 10^4 cells) were incubated at 37 °C under 5% (v/v) CO_2 for 4 h without effector cells. After incubation 7-AAD was added to this control tube to determine the spontaneous cell death.

***Flow cytometric assay of NK cell activity*—**The method for the NK-cell flowcytometric assay was

modified from previously published methods[10,11]. For purpose of comparison, flow cytometric NK cell activity assay was performed on both whole blood and PBMCs simultaneously using different E:T ratios (6.25:1, 12.5:1, 25:1, 50:1,100:1 and 200:1) in 10 peripheral blood samples of normal controls. The tubes were centrifuged at 500 rpm for 5 min to increase the cell contact and the mixture was incubated for 4 h at 37 °C under 5% (v/v) CO_2. After incubation, 10 µL 7-AAD (BD biosciences) was added to each tube and incubated for 20 min in dark, at 4-8 °C. Samples were then analyzed on flow cytometer (FACS Aria I; Becton Dickinson, San Jose, CA, USA).

Data acquisition and analysis—Analysis was performed with FACSAria using DIVA software (Becton Dickinson, San Jose, CA, USA). During sample acquisition, DIOC18 [λabs= 484 nm and λem=501 nm] was detected on the FITC channel and 7-AAD [λabs= 488 nm and λem=655 nm] was detected on PerCP channel. A threshold was set on FITC channel, so that only cells labeled with DIOC18 i.e. labeled target cells were acquired and rest of the cells including RBCs are excluded. The data acquisition and analysis is displayed in Fig 1. Target cells were gated on the DIOC18 positivity and then examined for cell death by up-take of 7-AAD. The percentage of cytotoxic activity was then calculated using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{[\text{Dead target cell (\%)} - \text{spontaneous death (\%)}]\times 100}{[100 - \text{spontaneous death (\%)}]}$$

Statistical analysis—Data are presented as median ± SE. Two tailed t-test was used to analyze the results between whole blood assay and PBMC assay and also between normal controls and FHL patients. The results were reported to be statistically significant if the ***P*** value was < 0.05.

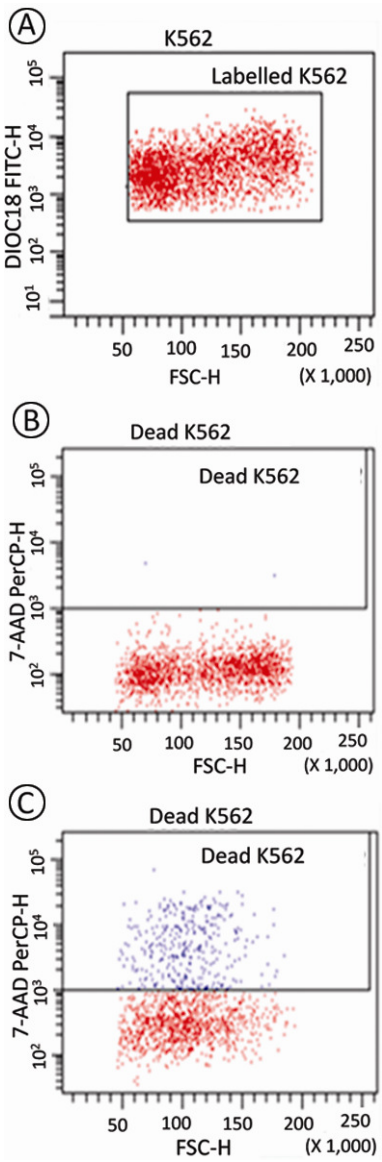


Fig. 1—Flowcytometric analysis of NK cell cytotoxicity assay: (A) FITC vs. FSC dot plot, gates the DIOC18 labeled K562 cells (target cells). (B) Displays Spontaneous death (C) Represents NK cell activity in a normal sample at E:T ratio of 100:1.

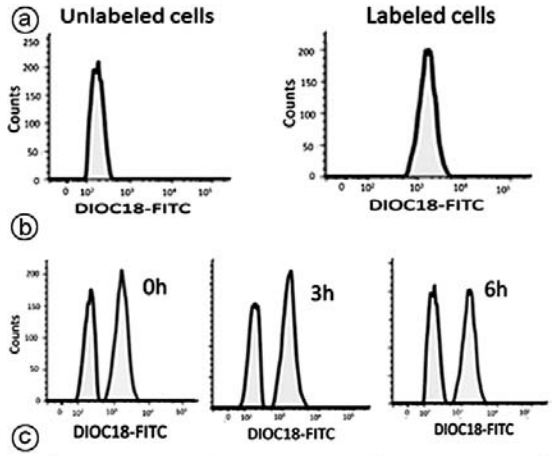
Results

Optimization of target cell labeling and viability staining—For K562 cells (target cells) labeling, 2 fluorescent dyes viz. DIOC18 and CFSE were compared. As compared to DIOC18, CFSE dye had a significant spectral overlap in the adjacent channels. Labeling with DIOC18 allowed homogenous staining of more than 90% of K562 cells without affecting viability. Also, there was no leakage of dye into unlabeled cells even after 6 h of co-incubation of DIOC18 labeled and unlabeled K562 cells (Fig. 2), indicating stability of dye. Hence DIOC18 was found to be suitable for target cell recognition. 7AAD (viability dye) was used to detect dead target cells.

Comparison of flow cytometric assay for NK cell cytotoxicity using PBMCs and whole blood at different effector cells—Target cells (E:T) ratios To determine the performance of this assay, the assay using PBMC and whole blood samples from same individual was compared at different effector:target ratio (6.25:1, 12.5;1, 25:1, 50:1, 100:1, 200:1). No significant difference was found in NK cell cytotoxicity obtained by using whole blood and PBMCs at different E:T ratios (P=0.786) (Fig. 3).

With increase in effector cells, the NK cell cytotoxicity increased.

NK cell cytotoxicity assay using PBMC requires approximately 10 mL of blood. In many patients, obtaining this large volume of blood is not feasible and thus performing this assay becomes difficult. The whole blood assay requires only 500 µL blood, which can be easily obtained and also this assay eliminates the cell separation step, thus reducing the processing time by almost an hour.



Time of co-incubation	MFI of unlabeled cells	MFI of labeled cells
0h	172	1504
3h	172	1477
6h	173	1511

Fig. 2—Stability of DIOC18 dye: (a) DIOC 18 labeled and unlabeled K562 cells, (b) Labeled K562 cells were mixed with unlabeled cells. Immediately after mixing, the stained and unstained cells are clearly distinguishable, (c) displays the MFI (mean fluorescence intensity) of labeled and unlabeled K562 cells. There was no significant change in MFI even after 6 h of co-incubation.

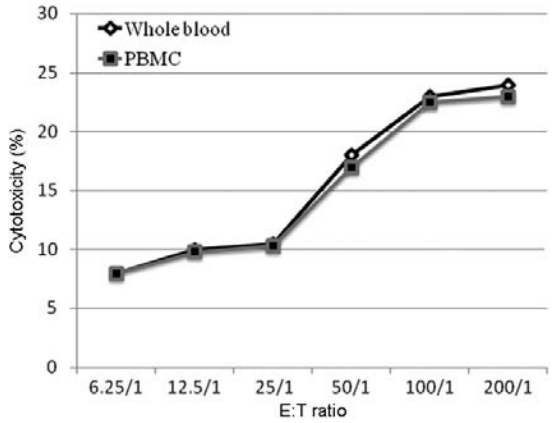


Fig. 3—Comparison of NK cell cytotoxicity obtained from Flow cytometry-based assay using whole blood and PBMCs at different E:T ratio: NK cell cytotoxicity obtained at different E:T ratio using whole blood and PBMCs simultaneously on the same sample from 10 normal healthy individuals was compared. There was no significant difference observed in both the assays.

Amongst the different effector:target ratios used, maximum NK cell cytotoxicity was obtained using 100:1 and 200:1 ratio. Since, no significant difference between cytotoxicity at effector:target ratio 100:1 and 200:1 was observed, for evaluating further samples 100:1 (E:T) ratio was used during the study.

***Reproducibility*—**Reproducibility of whole blood flow cytometric method for detecting NK-cell activity was determined by performing repeated analyses for the same individuals over a period of 6 months. Three healthy adult individuals donated blood on 3 separate occasions. There was no significant differences (P=0.97) in the mean percent cytotoxicity determined between time periods when 100:1 E/T ratio was used (Fig. 4).

***Range of NK cell activity for healthy adults*—**The median (±SD) cytotoxicity at the 100:1 (E/T) ratio in healthy normals (n=75) was 18±5.7% (range 9-32%).

***NK cell activity in FHL patients*—**To validate the flow cytometric assay for detecting low levels of NK cell activity, this assay was performed on 20 FHL patients. These patients have inherited NK cell defect and are thus expected to have low NK cell activity. When these FHL patient samples were processed simultaneously with healthy controls, all of these patients had low or absent NK cell activity (2.6±2.9%; range 0-8.4%) compared to healthy controls. However, there was no significant difference in the NK cell percentage and absolute count (Table 1).

Discussion

Evaluation of NK cells is essential whenever an autoimmunity, immunocompetency or immunodeficiency

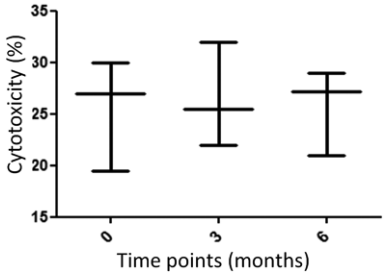


Fig. 4—Stability of the assay at different time points for same individuals: Percent cytotoxicity for 3 individuals analyzed by the whole blood flow cytometry assay at three different time points (in 6 months) at 100:1 (E/T) ratio. The upper and lower horizontal lines indicates the range and the middle line indicates the mean of the cytotoxicity. No significant difference was seen (***P***=0.97).

Table 1—Comparison of NK cell activity, NK cell percentage and NK cell absolute count in normals and HLH patients: The NK cell activity in Familial HLH patients was significantly lower than the normals but not significant difference was seen in NK cell percentage and NK cell absolute counts.

[Values are median ± SD from 75 normals and 20 familial individuals]

	Normal	Familial HLH
NK cell activity (%)	18±5.7	2.6±2.9[a]
NK cells (% of lymphocytes)	8.2±6.25	8±7.5[b]
NK cell absolute count	170±121	176±111[b]

P values: [a]<0.0001; [b]>0.1

investigation is undertaken[6]. Although chromium release assay is recognized as 'gold standard' for measuring NK cell activity, it has disadvantages like use of radioactive compounds, poor loading and high spontaneous release. It is difficult to perform this assay in clinical laboratory because of difficulties with disposal of radioactive waste and standardization problems[7]. In last few years there has been increasing number of references using flow cytometry for measurement of NK cell cytotoxicity[8-10]. All these assays however have been performed on PBMC rather than whole blood and have compared the cytotoxicity using the flow cytometry based assays with the standard ^{51}Cr release assays and have found it to be comparable. The flow cytometry based assays used till now use either difference between Forward scatter (FSC) and Side scatter (SSC) for discrimination of cell subpopulations and distinction between live and dead cells[12] or use property of differential labeling of target cells with various fluorescent dyes such as DIOC18, CFSE, FITC, PKH26[9,10]. In the present whole blood assay, the recognition and acquisition of the target cells solely depends on the cell labeling, hence it becomes important to label the target cells with a dye which is stable, does not leak into other unlabeled cells, long lasting, has minimal spectral overlap and does not affect cell viability. In the present study, compared to CFSE, DIOC18 had minimal spectral overlap in adjacent channels. Also, DIOC18 did not affect the cell viability, and was found to be more stable. Finally, the low cost of DIOC18 with easy and quick staining protocol, makes it suitable dye for routine use in clinical laboratories. This flow cytometric assay enables monitoring of cell cytotoxicity at single cell level rather than indirectly by the release of a preloaded marker.

Normal range of NK cell activity in healthy individuals in different populations has been reported. These studies differ in target cells, effector cells, effective E/T ratio and also detection assay used[13-15]. NK cell activity in healthy normals obtained using PBMC at 100:1 (E:T) ratio by flow cytometry based assay by Chung ***et al***[5] was similar to the present assay.

This is for the first time a flow cytometry based assay using whole blood for NK cell cytotoxicity has been proposed. This assay not only requires less volume of blood, it is also simpler and less time consuming. This eliminates the need for purification of cells which may result in loss of soluble factors and other physiological conditions. Whole blood assay for ^{51}Cr release assay using whole blood has been proposed previously[11]. They reported that NK-cell cytotoxicity from whole blood samples showed slightly increased cytotoxicity compared to PBMC samples suggesting that the presence of additional factors in whole blood samples positively affects the cytolytic activity of NK cells which are likely to be removed by cell purification. But in the present study when whole blood assay was compared to PMBC there was no statistically significant difference between the two methods. It also showed an excellent reproducibility when compared in normals at 3 different time points.

Absent or reduced NK cell activity is a hallmark of FHL and it is used as one of the important criteria for diagnosis of these patients. Majority of the FHL patients are in pediatric age group[4] and hence obtaining large volume of blood samples required for NK cell cytotoxicity assays using PBMC is often difficult. This assay is likely to be most beneficial for these patients if it can be established in a clinical laboratory. Therefore to validate the assay, this group was used as the representative group of individuals with low-level NK cell activity. FHL patients showed significantly low levels of NK cell activity as compared to healthy individuals. This was also tested at different time points.

Conclusion

The flow cytometry based whole blood assay as compared to previously reported PBMC assay, requires very less volume of blood, is less time consuming and has comparable results. This rapid assay also avoids necessity for specialized laboratory and accurately measures the NK-cell activity, indicating its application in evaluation of NK-cell function in various clinical situations. As flow cytometer is now available at every clinical laboratory

this simple test can be employed more often for the benefits of patients.

Acknowledgement

The authors acknowledge the Department of Biotechnology (DBT) for the financial support and SM acknowledges the Lady Tata Memorial Trust for granting Junior Research Fellowship (JRF).

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