Determination of Natural Killer Cell Function by Flow Cytometry

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Natural killer cells (NK cells) are a subset of peripheral blood lymphocytes that mediate non-major histocompatibility complex-restricted cytotoxicity of foreign target cells. The “gold standard” assay for NK cell activity has been the chromium release assay. This method is not easily performed in the clinical laboratory because of difficulties with disposal of radioactive and hazardous materials, short reagent half-lives, expense, and difficulties with assay standardization. We describe a flow cytometric assay for the clinical measurement of NK cell activity. This study compared the chromium release assay and the flow cytometric assay by using clinically relevant specimens. There were no significant differences between the two assays in the measurement of lytic activity for 17 peripheral blood specimens or in reproducibility in repeated samplings of healthy individuals. We also established a normal range of values for NK activity in healthy adults and identified a small cluster of individuals who have exceptionally high or low levels of NK activity. The flow cytometric assay was validated by testing specimens from subjects expected to have abnormally low levels of NK activity (pregnant women) and specimens from healthy individuals in whom the activity of NK cells was enhanced by exposure to interleukin-2 or alpha interferon. Treatment with these agents was associated with a significant increase in NK activity. These results confirm and extend those of others, showing that the flow cytometric assay is a viable alternative to the chromium release assay for measuring NK cell activity.

Natural killer cells (NK cells) are a subset of non-B, non-T peripheral blood lymphocytes that appear to play a crucial role in the human innate immune response (9, 16, 30). They mediate a cell contact-dependent cytolysis of target cells, including those expressing foreign major histocompatibility complex (MHC) molecules, virally infected cells, and tumor cells. NK cells are unusual among lymphocytes in that their function is not restricted by cells expressing self MHC molecules (MHC restricted) (9, 16). NK cells compose up to 15% of peripheral blood lymphocytes (24, 30) and are not dependent on the thymus for development, as evidenced by their normal expression and function in patients with severe combined immunodeficiency (9, 16, 24). They are large granular lymphocytes which by definition fail to express the T-cell receptor or surface immunoglobulin (9, 24, 30). NK cells are hypothesized to express an antigen receptor capable of recognizing and binding foreign cells, but the identification and characterization of this putative receptor are incomplete (9, 16, 30). Recent work has identified a number of cell surface proteins which may be involved in recognizing self MHC molecules and protecting autologous cells from cytolysis (16).

The function of NK cells is important for the clearance of tumor cells, for the removal of immunoglobulin-bound antigens, and for the control of viral infections (24, 27, 30). NK function has been reported to be decreased in certain people, including those with primary immunodeficiencies, those with late-stage human immunodeficiency virus infections, and pregnant women (5, 6, 11, 14, 21, 30). As more is learned about NK cells and their function, and more simplified and precise means to quantitate their numbers and function become available,

their analysis may become of more routine practical use to certain clinical specialties. A low level of NK cell activity may be useful in predicting patient outcome as well (6, 10, 11, 31). Thus, a suitable clinical assay for NK cell activity is necessary.

The standard method for the determination of NK activity is the chromium release assay (⁵¹Cr assay) (4, 6, 24, 32). 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, and entails concerns in handling and disposal. Also, because of the short half-life of chromium, this reagent must be continuously replaced. Lastly, interlaboratory variability in this and other functional assays is also of potential concern, especially since proficiency testing programs are not routinely in place for these assays.

For these reasons, investigators have developed new flow cytometry-based, nonradioactive methods for measuring NK cell activity (4, 6, 23, 28). Flow cytometric assays avoid the problems associated with the use of radioactivity and are rapid and more amenable to standardization. We compared a flow cytometric assay with the ⁵¹Cr assay by using peripheral blood lymphocytes from healthy human control subjects and patients. In addition, individuals with decreased levels of NK activity were analyzed to validate the usefulness of the flow cytometric assay as a clinical assay. The modified flow cytometric function assay gave results similar to those of the ⁵¹Cr assay and was less expensive and less time-consuming.

MATERIALS AND METHODS

Blood donors. Donors were selected from laboratory volunteers ranging from 20 to 55 years of age, including both males and females. These subjects were determined by interview to be healthy, free of medication, and not pregnant.

Pregnant donors with a gestational age greater than 26 weeks and considered healthy by the referring physician were selected. Informed consent was obtained from all donors in accordance with the policies of the University of North Carolina Internal Review Board.

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FIG. 1. Dual-parameter dot plot (FL1 [FITC fluorescence] and FL2 [PI fluorescence]) of PBMCs (effector cells) and DiO-labeled target cells. Quadrant 1 contains nonviable effector cells, quadrant 2 contains viable target cells, quadrant 3 contains viable effector cells and viable target cells without any dye uptake, and quadrant 4 contains viable target cells.

Reagents. K562 cells were purchased from American Type Culture Collection (Rockville, Md.). 3,3'-Diocetadecyloxycarbocyanine perchlorate (DiO) (Molecular Probes, Eugene, Oreg.), was dissolved in dimethyl sulfoxide (Sigma, St. Louis, Mo.) to a concentration of 30 mM. Aliquots were frozen at -20°C and thawed each day. RPMI media containing penicillin, streptomycin, and gentamicin with and without phenol red were obtained from Gibco (Grand Island, N.Y.). Complete medium (CM) consisted of RPMI medium supplemented with 10% fetal bovine serum, 100-U/ml penicillin, 100-mg/ml streptomycin, and 50-mg/ml gentamicin. Propidium iodide (PI) (Sigma) was dissolved in CM to obtain a working solution of 10 mg/100 ml. Fetal bovine serum was obtained from HyClone (Logan, Utah), and lymphocyte separation medium was obtained from Organon-Teknika (Durham, N.C.). Alpha interferon (IFN- α) (Roferon) was obtained from Roche (Nutley, N.J.), and recombinant interleukin-2 (IL-2) was purchased from Boehringer Mannheim (Indianapolis, Ind.).

The antibodies for flow cytometric analysis, obtained from Becton Dickinson Immunocytometry Systems (BDIS) (San Jose, Calif.), were as follows: CD2, CD3, CD4, CD7, CD8, CD16, CD25, CD45, CD56, CD57, and CD69. These antibodies were directly conjugated with fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein and were used at concentrations recommended by the manufacturer.

Preparation of target cells. K562 cells were grown as stationary cultures in CM and maintained at 37°C in 5% CO_2 . For the 3 days prior to the assay, cells were subcultured every 24 h to ensure that they were in log phase. K562 cells were washed and resuspended at a concentration of 10^6 cells per ml in phosphate-buffered saline (PBS). Prior to use in the assay, these cells were labeled by adding 10 μl of 30 mM DiO per ml of K562 cells, and the cells were incubated at 37°C in 5% CO_2 for 20 min. They were washed twice in PBS and resuspended to a final concentration of 10^6 cells per ml in CM without phenol red.

Effector cell preparation. Peripheral blood mononuclear cells (PBMCs) were used in the ^{51}Cr and flow cytometric assays and were separated from heparinized blood by using lymphocyte separation medium as previously described (25). The PBMCs were washed twice with unsupplemented RPMI medium and resuspended to a final concentration of 3.85×10^6 cells per ml in CM without phenol red.

^{51}Cr assay. K562 cells were grown and washed as described above and further prepared as previously described (4). Briefly, 100 to 200 mCi of ^{51}Cr was added, and the cells were returned to the incubator for 1 h. The cells were washed twice and resuspended to 5×10^4 cells per ml and kept in the incubator until needed. PBMCs were used in effector-to-target (E/T) ratios of 50:1, 25:1, 12.5:1, and 6.25:1, except where indicated. These ratios were based on previously published results (4) and were modified following optimizing titration experiments. Target and effector cells were mixed in a 96-well plate which was then briefly centrifuged and placed in a CO_2 incubator for 4 h. An aliquot of 100 μl of supernatant was removed, and the amount of radioactivity was measured with a gamma counter. Each ratio was determined in triplicate. Percent cytotoxicity was calculated as follows: percent cytotoxicity = [(experimental release - spontaneous release) / (maximum release - spontaneous release)] $\times 100$.

NK flow cytometric assay. The method for the NK flow cytometric assay was modified from and is a composite of two previously published methods for measuring NK activity (4, 6). Target cells (10 μl) at the standard concentration and 130 ml of PBMCs (effector cells) at 1:1 (neat), 1:2, 1:4, and 1:8 dilutions were added to tubes (12 by 75 mm) to create E/T ratios within a range from 50:1 to 6.25:1. A total of 130 μl of PI was added, and the tubes were centrifuged for 30 s at $1,000 \times g$ to pellet the effector and target cells together in the presence of the PI. These cell mixtures were incubated for 2 h at 37°C in 5% CO_2 . Controls consisted of target cells only plus PI and effector cells only plus PI and were set up to detect spontaneous lysis and nonviable effector cells, respectively. After the cells were incubated together, they were analyzed by flow cytometry. For potentiation experiments, IL-2 (250 U/ml) and IFN- α (40 U/ml) were added to the PBMCs and target cells, and the mixtures were incubated overnight at 37°C in 5% CO_2 . Controls contained cells that had been incubated overnight in CM. These cells were then used in the flow cytometric assay as described above. For flow cytometric analysis, the samples were gently mixed and analyzed on a FACScan flow cytometer (BDIS). The flow cytometer was operated in accordance with the manufacturer's recommendations after daily analysis of BDIS Calibrite Autocomp beads and fine adjustments for optimization. The forward- and side-scatter parameters were adjusted to accommodate the inclusion of both target and effector cells within the acquisition gate. No cells were excluded from the analysis, and 10,000 cells were counted. Data were collected by using the Consort30 (BDIS) program and analyzed with Lysis software (BDIS). Data are presented in Fig. 1 as a dot plot of FL1 (FITC fluorescence) versus FL2 (PI fluorescence) with quadrant markers drawn to distinguish DiO-labeled cells and cells which had incorporated PI. Percent lysis was calculated from the following equation: percent lysis = [quadrant 2 events / (quadrant 2 + quadrant 4 events)] $\times 100$. The same equation was used to calculate the percent spontaneous lysis for a sample containing only target cells. The spontaneous lysis was subtracted from the actual lysis for each sample.

Flow cytometric immunophenotyping. To determine the immunophenotype of the activated NK cells, the cytokine-pretreated cells and control cells were centrifuged following overnight incubation with the cytokines and were resuspended at 10^7 cells per ml in PBS. A total of 10^6 cells were incubated with the appropriate fluorescently labeled monoclonal antibodies for 30 min on ice. The cells were washed twice with PBS and were resuspended to 10^6 cells per ml in PBS and analyzed by flow cytometry on the FACSort flow cytometer (BDIS) by following the procedure described above.

Statistical analysis. Data are presented as means $\pm$ standard errors of the means at the indicated E/T ratios. Comparisons of the ^{51}Cr and flow cytometric assays, the reproducibility studies, and the effects of cytokine stimulation were all performed by using the Wilcoxon signed-rank test. The paired t test was used for comparisons of surface marker expression between control and cytokine-treated PBMCs.

RESULTS

Comparison of ^{51}Cr and flow cytometric NK assays. Seventeen peripheral blood specimens from eight healthy adult volunteers were obtained, and the ^{51}Cr and flow cytometric assays were performed on each. The correlation ($r = 0.85$) in percent cytotoxicity between the two methods was high (Fig. 2A), and the dose-response curves were similar (Fig. 2B). In addition, there was no statistically significant difference between the two methods ($P > 0.05$) when the data from each were converted to lytic units (144 ± 39 and 170 ± 17 lytic units for the flow cytometric and ^{51}Cr assays, respectively).

Reproducibility of the flow cytometric method. The reproducibility of the flow cytometric method for detecting NK activity in human peripheral blood lymphocytes was determined by performing repeated analyses for the same individuals over time. Six healthy adult individuals donated blood on three separate occasions over a 1-month period, and PBMCs were tested by the flow cytometric and ^{51}Cr assays. There were no significant differences ($P > 0.05$) in the mean percent cytotoxicity determined by either method between time periods or between the methods at each time period (Fig. 3) when a 50:1 E/T ratio was used.

Range of NK activity for healthy adults. For the implementation of the flow cytometry assay in a clinical laboratory setting, values for healthy individuals should be determined. To establish a normal range, 87 PBMC specimens were obtained from healthy adults, and the NK activity was determined by flow cytometry for each. The mean percent cytotoxicity at the 50:1 E/T ratio was $41.4\% \pm 15.0\%$.

FIG. 2. (A) Scatter plot demonstrating the correlation of percent cytotoxicity values for 17 samples analyzed by the flow cytometric and ⁵¹Cr assays. Data indicate percent cytotoxicity at all E/T ratios. (B) Dose-response curves (means $\pm$ standard errors of the means) of the flow cytometric ($\diamond$) and ⁵¹Cr ($\circ$) assays.

NK activity in late pregnancy. To validate the flow cytometric assay for detecting low levels of NK activity, blood was obtained from pregnant women and tested for NK activity. Pregnant women, especially those in a late stage of gestation, have been shown by others to have abnormally low NK activity levels (5, 14, 15, 19, 24, 25). The mean NK activity (50:1 E/T ratio) of the pregnant subjects ($28.6\% \pm 14.2\%$) was significantly lower ($P < 0.05$) than that of 22 healthy adult volunteers who were assayed concurrently (mean NK activity, $41.7\% \pm 15.0\%$).

Augmentation of NK activity by cytokine addition. The validity of the flow method was also analyzed by determining whether a change in NK activity to levels greater than typically expected could be detected. Previous reports have suggested that the cytokines IL-2 and IFN- α increase the activity of NK cells against tumor cell targets (2, 3, 7-9, 18, 21, 24, 30). To test this effect by the flow cytometric assay, PBMCs from healthy

individuals were treated with these cytokines, and the change in NK activity compared with the activity of nontreated cells was determined. Figure 4 shows that treatment with these cytokines caused a significant increase ($P < 0.05$) in the lytic activity of both IL-2- and IFN- α -treated cells.

Immunophenotyping of NK cells. Distinct patterns of surface marker expression on NK subsets may be related to the activation state of the cells. To determine if activated NK cells express surface markers different from those expressed by resting NK cells, flow cytometry was used to immunophenotype NK cells of low normal individuals (individuals who are considered low normal responders for NK activity) incubated with and without IL-2 or IFN- α . Changes in activation marker expression (CD69 and CD25) were observed in the treated specimens. Cells that had been incubated with the cytokines showed a significant increase in NK activity. However, the testing of eight individuals was unable to show any consistent changes in NK phenotype which paralleled the changes observed in NK activity (Table 1).

DISCUSSION

NK cells, a small subset of peripheral blood lymphocytes that mediate non-MHC-restricted cytotoxicity of foreign target cells, appear to play a crucial role in immunologic defense and regulation (9, 24, 30). There has been an increasing interest by clinicians in accurately measuring in vitro NK cell activity because of possible correlations between NK cell activity and disease outcome or progression (6, 10, 11, 13, 31). It was our intention to find a simple and reproducible method for determining NK cell activity that could be incorporated into the evaluation of immune parameters in patients with immunodeficiencies and psychiatric disorders.

The "gold standard" assay for NK cell activity has been the ⁵¹Cr assay (4, 6, 24, 32). In this assay, target cells internalize radioactive chromium which is subsequently released upon cytotoxicity by NK cells. The amount of radioactivity released can be measured with a gamma counter. However, many difficulties with this assay prevent it from being routinely used in

FIG. 3. Percent cytotoxicity (mean $\pm$ standard error of the mean) for six individuals analyzed by the flow cytometric ($\blacksquare$) and ⁵¹Cr ($\blacksquare$) assays at three different time points. Measurements 2 and 3 were obtained from 1 to 6 weeks after measurement 1. Data are for the 50:1 E/T ratio for each method.

FIG. 4. NK activities (means $\pm$ standard errors of the means) of PBMCs from six individuals pretreated with CM (none) or 250 U of IL-2 per ml or 40 U of IFN- α per ml. *, $P < 0.05$ for cytokine pretreatment versus no pretreatment.

clinical settings. It is difficult to standardize, expensive, time-consuming, uses reagents with short half-lives, and requires disposal of radioactive and hazardous materials. Therefore, another method for measuring NK cell activity would be desirable.

Although many methods for the detection of NK cell activity have been reported, two reports have recently documented flow cytometry-based methods for measuring NK cell activity (4, 6). Our method utilized the incorporation of a green lipophilic fluorescent dye, DiO, into the membranes of target cells grown in culture. This dye allows the easy discrimination of target cells from effector cells with a flow cytometer. Additionally, dead cells are identified by the intercalation of PI into the nuclear DNA (20). Since PI fluoresces in the orange-red spectrum, the percentage of target cells that have been damaged by the effector NK cells can be accurately determined by dual-fluorescence analysis.

Our method utilizes components of previously published work (4, 6). However, our method of analysis differs from that published by Chang et al. (4) in the way the cells are evaluated and the way the percent lysis is calculated. By setting a threshold on forward scatter to exclude debris and a threshold on fluorescence detector 1 to exclude effector cells, their method included only the acquisition of target cells. We felt it would be more appropriate to include all events in the analysis of NK activity, rather than narrowing our analysis to include only target cells. This may be especially important because as cells become damaged, their scatter properties change and target and effector populations may become less distinct (20). The calculation of percent cytotoxicity performed by Chang et al. also included dimly DiO-positive cells which fell in quadrant 1 of the dual-fluorescence dot plot. In our analysis, target cells were defined as those retaining bright DiO staining (no quadrant 1 events). It was reasoned that NK cells may also show weakened membrane integrity following incubation and that the percent lysis of targets might be overestimated if cells which were stained with PI but not with DiO were included (quadrant 1). Likewise, the total target population (quadrants 2 and 4) was used as the denominator when determining the

percent lysis by NK cells. This analysis is similar to that used by Hatam et al. (6), who performed similar experiments with PKH-26 as the membrane-intercalating dye instead of DiO. The choice of either PKH-26 or DiO is most likely influenced the most by personal preference in terms of emission spectra, since both dyes label cells quickly and efficiently and do not leak appreciably within the time frame of the flow cytometric assay (4, 6).

Our method was compared with the ^{51}Cr assay in a clinical setting to determine if the flow cytometric method would be a suitable alternative for measuring NK activity. The flow cytometric assay gave results comparable to those obtained by the ^{51}Cr assay and was found to be faster and easier to perform than that assay.

NK activity normally may be inconsistent within an individual because of the many possible immunological and endocrine influences on NK cells (1, 3, 5, 13, 18, 21, 24, 30). Therefore, minor changes in NK activity may be expected when repeatedly testing healthy individuals. However, our data demonstrate that the flow cytometric assay is as reproducible as the ^{51}Cr assay when used to assay individuals repeatedly over time. In fact, the flow cytometric assay was less variable than the ^{51}Cr assay.

To validate the results generated in the flow cytometric assay we tested PBMCs from individuals who were expected to have low levels of NK activity and PBMCs from individuals who were expected to have increased levels of NK activity. Previous results (5, 14, 19, 24, 25) support the conclusion that women in late pregnancy have reduced levels of NK activity. Therefore, this group was used as a representative group of individuals with low-level NK activity. The flow cytometric method showed significantly lower levels of NK activity in this group compared with healthy individuals, as would be expected. We also evaluated the validity of the assay by comparing levels of NK activity for the same individuals before and after treatment with cytokines reported to enhance NK activity in vitro (7, 8, 18, 21). This was done to determine the ability of the flow cytometric assay to detect higher levels of NK activity than might normally be expected. Using the flow cytometric assay, we were able to show significantly increased NK activity in cells treated with either IL-2 or IFN- α . These cytokines were also added together to test for a synergistic effect, but the dual stimulation was not significantly different from that produced by IL-2 alone (data not shown). Thus, the observations that the flow cytometric assay accurately identifies increases and decreases in NK activity support its use for clinical determinations of NK activity.

TABLE 1. Immunophenotypes of cytokine-treated cells

Surface antigen	% Positively staining cells with ^a :		
	No pretreatment	IFN- α	IL-2
CD2	79.1	78.1	78.0
CD3	71.5	69.8 ^b	71.6
CD7	83.4	82.7	83.6
CD8	30.0	29.0 ^b	28.1 ^b
CD16 ⁺ /CD3 ⁻	8.1	7.3 ^b	7.0
CD19	8.0	7.8	8.3
CD25	14.6	17.5 ^b	15.5
CD45	97.1	97.3	97.3
CD56 ⁺ /CD3 ⁻	3.3	3.0	3.1
CD57	13.6	13.3	12.5
CD69	6.8	21.7 ^b	21.3 ^b

^a Cells were pretreated with 40 U of IFN- α per ml or 250 U of IL-2 per ml.

^b $P < 0.05$ for pretreated cells versus control cells not pretreated.

To use the flow cytometric NK assay clinically, a range of normal results should be established. By testing a large number of healthy adults, it was determined that the majority of individuals were within 1 standard deviation of the mean of our normal range for NK activity (data not shown). There are a few individuals who were consistently outside 1 standard deviation. These results suggest that there are low, normal, and high responders for NK activity. These categories may be useful to clinicians who are trying to compare patient results with results for healthy controls. As more test results are collected, a more precise definition of each of the three categories may be obtained. Having a suitable and rapid flow cytometry-based assay for the measurement of NK activity would be very advantageous for the evaluation of patients with immunodeficiencies, malignancies, viral infections, and psychiatric illnesses because an improved prognosis (6, 10, 11, 13) in each of these disease states has been associated with good NK activity.

NK cells express the leukocyte common antigen (CD45) and some combination of CD2, CD7, CD8, CD16, CD56, and CD57 on their surfaces (24, 30). CD2 and CD7 are present on most T cells and NK cells, while CD8 and CD57 mark some cytotoxic T cells and some NK cells. CD16 is an Fc receptor for immunoglobulin G and is present on NK cells and granulocytes. CD56 is found on cells of neural origin, but it is thought to be on NK cells only in peripheral blood. NK cells also express a number of adhesion molecules and may express activation markers, including CD25, CD69, and HLA-DR (24, 30). While NK cells can be identified by their combination of surface markers, there is not a unique phenotype characteristic of all NK cells. There is conflicting evidence regarding whether the different NK phenotypes might be representative of the various activation states of the cells (3, 11, 12, 17, 22, 23, 27–30). Thus, at this time, it is not possible to use immunophenotyping as a surrogate assay for NK cell function. Nevertheless, when NK cells are activated there are consistent changes in the surface phenotype, primarily in the level of expression of typical activation markers, such as the IL-2 receptor (3, 29, 30). We attempted to identify changes in phenotype that paralleled changes in activity by measuring the percentage of cells expressing various NK surface markers with and without cytokine enhancement of activity (Table 1). While changes in the activation markers CD25 and CD69 were detected, other consistent changes in specific NK phenotypes were not clearly established. Therefore, we were unable to identify a definite correlation between immunophenotype and activity of NK cells. The absence of an appropriate surrogate test further supports the use of the rapid flow cytometry-based assay for clinical evaluations of NK cell activity.

The advantages of the flow cytometry assay are numerous. It is less expensive than other assays, and because many clinical immunology laboratories are already using flow cytometry for other assays, the hardware and software for NK analysis are currently in place. By eliminating the use of radioactivity in the laboratory, the disposal of used supplies and old reagents would no longer be a concern. Many laboratories not licensed for radioisotope use would thus be able to perform the non-radioactive assay. This assay can be effectively performed in a few hours, similar to the more rapid 4-h chromium assay that has also been described (4, 30). Another advantage of this assay is that flow cytometry is being included in the proficiency testing profile at many clinical facilities. By testing flow cytometry results in an interlaboratory manner, the flow cytometric NK assay may be suitable for standardization, which would enhance its usefulness in the clinical laboratory. All of these benefits of the flow cytometric assay provide reasonable justification for evaluating its usefulness.

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