Natural Killer Cell Cytotoxicity: A Methods Analysis of ^{51}Chromium Release versus Flow Cytometry

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The purpose of this study is to describe design considerations for the use of flow cytometry (FC) compared to ^{51}chromium (^{51}Cr)-release assays utilizing cryopreserved peripheral blood mononuclear cells (PBMCs) to detect natural killer (NK) cell cytotoxicity. Subjects were 10 healthy women aged 18 to 39 years. Intra-assay variability between methods differed only at the lowest effector-target ratios evaluated. Interassay variability was wide but did not differ between methods. The relationship of lytic unit-10 between methods was strongly positive. Cytotoxicity detected by ^{51}Cr release was higher than that detected by FC for all 10 subjects. Cost was comparable. However, had more assays been performed, technician time would have been greater with flow cytometry. More whole blood was needed to perform the flow cytometry cytotoxicity assay than ^{51}Cr-release cytotoxicity assay. The authors found no compelling reason to adopt NK cell cytotoxicity by flow cytometry over ^{51}Cr release.

Key words: *natural killer cells, ^{51}chromium-release assay, flow cytometry, cytotoxicity*

Evaluation of immune function is a central component in the design of many nursing research studies (Zeller and others 1996). One important and frequently measured indicator of immune function is natural killer (NK) cell cytotoxicity. NK cells are the first line of defense against virus and tumor invasion (See and others 1997). Along with T cells, B cells, and monocytes, NK cells are a subpopulation of peripheral blood mononuclear cells (PBMCs). However, unlike T

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and B cells, NK cell percentage and cytotoxicity vary in response to many factors, including emotional, cognitive, and physiological stressors (e.g., Fawzy and others 1990; Sieber and others 1992).

There is substantial variability in NK cell cytotoxicity between individuals (Whiteside and Herberman 1989), with apparently healthy individuals categorized into high or low levels of NK cell cytotoxicity (Pross and Baines 1982). Moreover, some of the variability in the NK cytotoxicity results may be attributed to the fact that the assay involves interactions between 2 biological systems: the effector cells and the target cells. The effector cells are the subject's NK cells obtained from peripheral blood. The target cells most often used are K562 cells (an NK cell–sensitive cell line derived from a patient with chronic myelogenous leukemia). Steps in the NK cytotoxicity process include recognizing the target cell, binding, transmitting a lethal hit to the target cell, and lysing the target cell (Ellis 1993). Percent cytotoxicity (also called percent specific lysis [PSL]) is quantified by the percentage of target cells killed: The greater the number of cells killed, the higher the NK cell cytotoxicity.

There are 2 commonly used methods of evaluating in vitro NK cell cytotoxicity: ^{51}chromium (^{51}Cr) release and flow cytometry. The ^{51}Cr-release method is considered the gold standard cytotoxicity measure (Whiteside and Herberman 1994; Kane and others 1996; Derby and others 2001) and is more widely used in published reports of NK cell cytotoxicity (e.g., Pross and Baines 1982; Kiecolt-Glaser and others 1986; Whiteside and others 1991; Irwin and others 1992) than are other methods, such as flow cytometry (e.g., Radosevic and others 1990; Hatam and others 1994; Piriou and others 2000). In this assay, target cells are incubated with the radioactive ^{51}Cr and then mixed with effector cells. After a 4-h incubation, the amount of radioactivity released from the target cells is directly proportional to the number of target cells killed by NK cells (Whiteside and others 1990).

NK cell cytotoxicity by flow cytometry can distinguish effector cells and target cells on the basis of differential fluorescent probes. Furthermore, it can distinguish between live and dead target cells. The flow cytometry cytotoxicity method we utilized in the study described below used 2 fluorescent probes, propidium iodide (PI), a red nuclear stain, and 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO), a green fluorescent lipophilic membrane stain, to label cells. Target cells are labeled with DiO and then mixed with effector cells. After a 3-h incubation, PI is added to the mixed cell suspension. As soon as the target cell receives its "lethal hit" from the NK cell, the target cell is no longer viable, and it begins to take up PI. Normally, cell membranes are impermeable to PI. However, as the target cell begins to die, its cell membrane begins to disintegrate, allowing PI to enter and bind with its DNA. Flow cytometry is able to discriminate between effector cells (DiO negative) and target cells (DiO positive) and between viable target cells (DiO positive, PI negative) and nonviable target cells (DiO positive, PI positive). PSL is calculated from the ratio of nonviable target cells to total target cells (Ellis 1993; Hatam and others 1994).

The purpose of this study is to describe design considerations for the use of flow cytometry versus ^{51}Cr-release NK cell cytotoxicity assays utilizing cryopreserved cells from a sample of 10 healthy women. The design considerations included intra-assay reliability (percent coefficient of variation [CV] of replicate cytotoxicity values within each assay), interassay reliability (percent CV of PSL between assay control samples over time), sensitivity (level of cytotoxicity and relationship with known percentage of NK cells), and cost. Cryopreservation of PBMCs is a method often used by smaller laboratories where staff scheduling may not match the timing of sample acquisition. Cryopreservation is also used when individual subjects have multiple blood samples drawn at disparate times to batch and analyze those samples together. Other investigators have addressed some of these design issues, but none have compared data using cryopreserved PBMC samples. Therefore, specific aims were to (1) estimate the intra- and interassay reliabilities of NK cytotoxicity for both methods; (2) describe, correlate, and compare the ^{51}Cr-release and flow cytometry NK cytotoxicity levels; (3) examine the bivariate relationships between each ^{51}Cr and flow cytometry cytotoxicity value and the percentage of NK cells; and (4) compare costs for the use of the flow cytometric method versus the ^{51}Cr-release method of quantifying NK cytotoxicity.

Understanding these design implications may assist an established investigator who uses ^{51}Cr-release as-

says in deciding whether to adopt the newer flow cytometry assay and may help a new investigator interested in NK cell cytotoxicity research in deciding which method to utilize. Interassay reliability is of particular interest because if interassay reliability were clearly superior using one method compared to the other, a greater effect size would result in enhanced statistical power with the same or fewer subjects.

Materials and Methods

This study received Human Subjects Review Committee approval prior to subject recruitment and participation. A repeated-measures design was used to address the study aims. A convenience sample of 10 healthy women between the ages of 18 and 39 years (M = 31.7 years, SD = 7.1), already participating as control subjects in an ongoing study of immune function in women, participated in this study. Data from these 10 subjects were used for all study aims except for estimating interassay reliability. Interassay reliability was estimated using consistent assay-control samples obtained from 2 additional healthy individuals.

As part of the ongoing study, a total of 30 ml of whole blood was collected for all immune function assays during the midfollicular menstrual cycle phase (days +5 to +7 postmenses). A 100-ml assay-control sample of whole blood was collected from each of 2 assay-control subjects. The circadian pattern associated with NK cell activity (Gatti and others 1987) was accounted for by scheduling all blood draws within 2 h after the normal wake-up time.

Standard Ficoll-Hypaque separation of whole blood was used to obtain PBMCs. The subjects' PBMCs were divided into 3 equal aliquots and cryopreserved, with the plan of using 2 aliquots for the ongoing study and 1 aliquot for this flow cytometry study. For the assay-control subjects, PBMCs were divided after Ficoll-Hypaque separation into 20 equal aliquots and cryopreserved. Ficoll-Hypaque separation and cryopreservation protocols are described elsewhere (Motzer and others 2002). Briefly, blood collected in sodium-heparin Vacutainer tubes (Becton-Dickinson, San Jose, CA) was diluted 1:1 with Dulbecco's phosphate buffered saline (PBS) (Gibco, Grand Island, NY), layered over Ficoll-Hypaque solution (Pharmacia, Piscataway, NJ), and centrifuged 30 min at 2000 rpm at 18 °C to 20 °C (Model TJ-6, Beckman, Irvine, CA). The PBMCs were collected, washed 3 times with Hanks balanced salt solution (HBSS) (Gibco), and counted. Finally, the cell pellet was resuspended with freezing media (10% dimethyl sulfoxide, DMSO, Sigma, St. Louis, MO, with fetal calf serum [FCS] Hyclone, South Logan, UT), aliquoted into Nunc CryoTubes (Intermed, Roskilde, Denmark), placed in a Nalgene Cryo 1 °C freezing container (Nalge Company, Rochester, NY), frozen at –70 °C, and then moved to liquid nitrogen storage. Ficoll-Hypaque centrifugation always began within 1 h of the blood draw.

For each subject, the 2 different NK cell cytotoxicity assays were performed on separate days. Both assay-control samples A and B were run with each of the 10 ^{51}Cr-release assays and the 7 flow cytometry assays. The day prior to each assay, cryopreserved PBMC samples were thawed quickly in a 37 °C water bath, washed twice in RPMI 1640 (Gibco) plus 5% FCS, and counted. Samples were resuspended in complete media (CM) (RPMI 1640, nonessential amino acids 1%, sodium pyruvate 1%, L-glutamine 1%, and penicillin/streptomycin 1%, all from Gibco, and FCS 10%) and set up for culture per the individual protocols. All cultures were incubated 18 h at 37 °C, 5% CO_2. The following day, K562 cells in their log phase of growth were harvested and counted before implementing the assay protocols. For ^{51}Cr release, we used triplicate samples at 6 effector-target ratios (E:T) (100:1, 50:1, 25:1, 12.5:1, 6.25:1, 3.125:1). However, for the flow cytometry assay, 1 cryopreserved aliquot of PBMCs was not adequate to obtain triplicate values of 4 E:T at a starting ratio of 100:1. Therefore, we performed the flow cytometry assay using duplicate samples at 4 E:T with a starting ratio of 75:1 or 50:1. Cell phenotyping was performed concurrently with the ^{51}Cr-release assay.

Measures

A standard 4-h ^{51}Cr-release cytotoxicity assay was performed. Details of these methods have been previously reported (Motzer and others 2002). PSL was calculated for each E:T according to the following formula (Whiteside and others 1990):

$$PSL = \frac{\text{experimental cpm - spontaneous release cpm}}{\text{maximal release cpm - spontaneous release cpm}} \times 100,$$

where experimental counts per minute (cpm) is the amount of radioactivity released in wells containing the subject's effector cells mixed with ^{51}Cr-labeled target cells, spontaneous release cpm is the amount of radioactivity released by target cells incubated without effector cells, and maximal release cpm is the amount of radioactivity released from target cells incubated with an agent that lyses all target cells. LUs were calculated by linear regression analyses of the log E:T versus PSL using at least 4 E:T per sample (Pross and others 1981) and reported per 10^6 effector cells. Friberg and colleagues (1996) advocated for use of LU, as they posited that reporting single PSL values can be misleading. LU_{10} is defined as the quantity of effector cells needed to kill 10% of a predetermined number of target cells. The higher the LU, the greater the killing capacity of the NK cells.

The flow cytometry cytotoxicity assay was performed using the LIVE/DEAD Cell-Mediated Cytotoxicity Kit (Molecular Probes, Eugene, OR). After thawing the PBMCs, 100 µl of the cell suspension was aliquoted per well into 4 wells of a 96-well, U-bottom tissue culture plate (Linbro, ICN Biomedicals, Aurora, OH). Each sample was serially diluted with CM to obtain 4 E:T in a total volume of 200 µl and incubated overnight.

The next day, the K562 cells were washed twice with PBS. Then 10 µl of 3 mM DiO in anhydrous dimethyl sulfoxide (LIVE/DEAD kit, Molecular Probes) was added to each of two 15-ml tubes that contained 10^6 K562 cells in 1 ml of PBS. Tubes were incubated for 15 to 20 min in a 37 °C incubator. These cells were washed twice with PBS, counted, and resuspended in CM at 2×10^6 cells/ml. Then 10 µl of labeled K562 cells were added to each well, including 2 wells with 200 µl of CM alone, to measure spontaneous release. Plates were then incubated at 37 °C for 3 h. Following the incubation, 130 µl of diluted PI at 0.15 mM in CM from the LIVE/DEAD kit was aliquoted per tube into the same number of racked titer tubes (Bio-Rad Laboratories, Hercules, CA) as experimental wells. Samples were transferred from the plate wells into the tubes and then taken to a Becton-Dickinson FACScan. Events were collected for 3 min or until 200,000 total events (the number of cells, cell fragments, or debris detected by the FACS) were collected per sample, whichever occurred first. Samples were analyzed using Windows Multiple Document Interface for Flow Cytometry (WinMDI) software (Scripps Research Institute, La Jolla, CA). A K562 gate was set on the large, green-staining cells, eliminating fragments and debris. To obtain PSL, the percentage of PI-positive cells in the spontaneous control was subtracted from that of each sample (see Fig. 1).

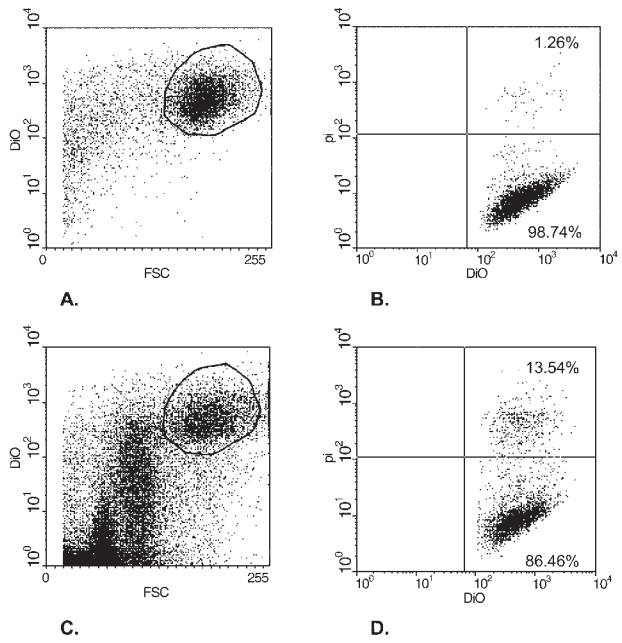

Figure 1. An example of calculating natural killer (NK) cell cytotoxicity by flow cytometry. (A) Dot plot of K562 target cells only. The *x*-axis, FSC, is forward scatter, a cell-size indicator. The *y*-axis is log fluorescence intensity of DiO, the membrane stain. The K562 gate, shown as a circle around the large green-staining cells, is used in all percent specific lysis (PSL) analyses. (B) Spontaneous control dot plot of K562 target cells only. The percentage of nonviable K562 cells that have incorporated the propidium iodide (PI) (nuclear) stain spontaneously is shown in the upper-right quadrant (1.26%). The lower-right quadrant represents the viable K562 cells (98.74%). The *x*-axis is log fluorescence intensity of DiO; the *y*-axis is log fluorescence intensity of PI. (C) A dot plot of effector cells incubated with target cells at a 75-to-1 effector-target ratio. The gate shown on the K562 target cells and both the *x*-axis and *y*-axis are the same as shown in plot A. (D) Dot plot of NK cell cytotoxicity. The nonviable K562 cells are in the upper-right quadrant (13.54%). The viable K562 cells (86.46%) are in the lower-right quadrant. To calculate PSL, the percentage of spontaneously lysed K562 cells from plot B (1.26%) is subtracted from the total nonviable K562 cells in plot D (13.54%), for a PSL value of 12.28%.

NK cell phenotyping was done to enumerate the percentage of NK cells. Phenotyping was performed using 3-color flow cytometry and a FACScan (Becton-Dickinson). Each sample of PBMCs used for phenotyping was cultured overnight at 10^5 cells/well in 200 µl CM in a 96-well, U-bottom plate. For each staining sample, PBMCs from 5 wells were combined into 1 well and stained using a pretitered amount of monoclonal antibodies (MAb) specific for cluster of differentiation (CD) 3, CD56, and/or CD69, or mouse IgG1 (as a control; all MAb from PharMingen, San Diego, CA). MAb were directly labeled with either fluorescein isothicyanate, phycoerythin, or Cychrome™ fluorescent dyes, which emit green, orange-red, and red light, respectively, when excited by an argon or helium-neon laser (Winkelstein and Donnenberg 1997). Samples were incubated on ice for 30 min, washed once with HBSS, once with PBS, and then fixed in paraformaldehyde (J. T. Baker Inc., Phillipsburg, NJ) diluted in PBS to 1%. These samples were stored in the refrigerator until run on the FACScan. Control samples were used to assess non-specific staining and autofluorescence. Refer to previously published description of the staining protocol for greater detail (Motzer and others 2002). A total of 10,000 events per sample were collected by flow cytometry. Percentages of NK cells ($CD3^-/CD56^+$) in the lymphocyte gate were obtained using Reproman Software (TrueFacts Software, Seattle, WA).

Data Analysis

Descriptive statistics, histograms, box-and-whiskers plots, scatterplots, and line graphs were used to examine the data. To equitably compare findings, because triplicate samples were run in ^{51}Cr release and duplicate samples were run for flow cytometry, 2 of 3 ^{51}Cr-release values were randomly selected and then used in all calculations. Random selection was conducted blindly using a random feature in SPSS. Spearman's rho and scatterplots were used to examine the relationship between ^{51}Cr LU_{10} and flow cytometry LU_{10}. LU_{10} was used rather than the more commonly reported LU_{20} because it was more normally distributed and had a wider range. Intra-assay reliability was estimated by examining the percent CVs (*SD*/*M*) of the cytotoxicity values within each subject's assays. Percent CVs were calculated using ^{51}Cr cpm and flow cytometry PSL values per standard laboratory protocols. These values are the lowest common denom-inator of both assays and are the levels of evaluation in our laboratory as well as the laboratories of others. A box-and-whiskers plot was used to visually evaluate intraassay variability, and a Wilcoxon signed rank test was used to test differences in intraassay variability. Interassay reliability was determined by examining the percent CVs of PSL from the 2 assay control samples across assays. For each matching E:T (i.e., 50:1-to-6.25:1), a Levene test was used to compare homogeneity of variances of PSL. Sensitivity of the assay was evaluated by comparing levels of LU_{10} and examining the relationships of LU_{10} with percentage of NK cells. A Wilcoxon signed rank test and a line graph were used to compare LU_{10} values for both methods. Spearman's rho and scatterplots were used to examine bivariate relationships between percentage of NK cells and both ^{51}Cr and flow cytometry LU_{10}. Costs per assay were calculated for both methods, including technician time, supplies, radiation and biohazard disposal, and hourly equipment charges.

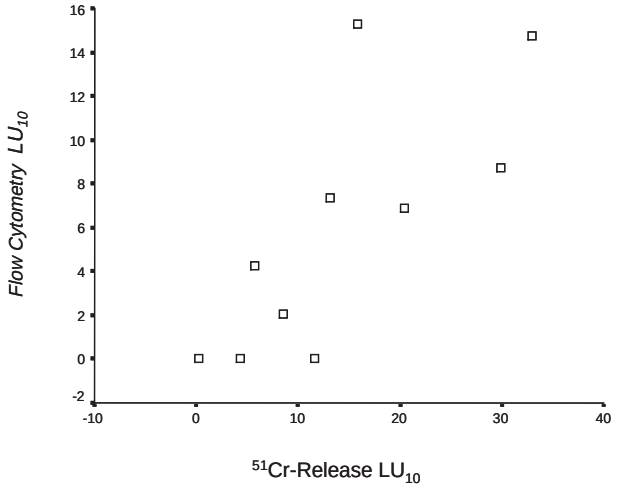

Figure 2. Scatterplot of the relationship between ^{51}Cr-release and flow cytometry methods of cytotoxicity for lytic unit-10 (LU_{10}) in 10 subjects (r_s = 0.79, *P* = 0.006).

Results

The 2 methods were strongly and positively related to each other (see Fig. 2). Intraassay variabilities on duplicate samples across 4 E:T for ^{51}Cr-release cpm and flow cytometry PSL were low with similar vari-

Table 1. Interassay Reliabilities (CV) and Descriptive Statistics of NK Cytotoxicity (PSL) by ^{51}Cr-Release and Flow Cytometry Assays for Assay Control Subjects A and B

	^{51}Cr Release (n = 10)				Flow Cytometry (n = 5)			
E:T	**50:1**	**25:1**	**12.5:1**	**6.25:1**	**50:1**	**25:1**	**12.5:1**	**6.25:1**
Assay control subject A								
CV (%)	34.60	51.30	34.93	60.74	41.58	41.00	46.26	43.62
PSL								
M (%)	10.20	9.26	6.70	4.61	10.51	7.10	4.41	2.98
Median (%)	10.06	9.56	6.29	4.00	9.19	6.60	4.13	2.84
SD	3.53	4.75	2.34	2.80	4.37	2.91	2.04	1.30
Assay control subject B								
CV (%)	31.89	63.49	36.02	54.37	34.61	29.11	35.84	33.64
PSL								
M (%)	9.50	6.82[a]	3.22[a]	2.06[a]	14.10	7.66	4.13	2.14
Median (%)	9.00	5.30	2.65	1.75	14.61	7.94	4.20	2.14
SD	3.03	4.33	1.16	1.12	4.88	2.23	1.48	0.72

NOTE: CV = coefficient of variation; PSL = percent specific lysis; E:T = effector-target ratio. All PSL calculated from duplicate samples. Levene's test for homogeneity of variance indicated that PSL variances were not different between assay methods.
a. n = 9.

ability for each method. Only the CVs calculated at the lowest E:Ts evaluated here for each method (12.5:1 for ^{51}Cr and 9.375:1 for flow cytometry) were statistically different from each other (z = –2.55, P = 0.011, Wilcoxon signed rank test), with the flow cytometric variability being greater than the variability for ^{51}Cr release (see Fig. 3). Interassay variabilities of PSL across 4 E:T for ^{51}Cr-release and flow cytometry methods for assay control subjects A and B were high (see Table 1). PSL values for the 2 assay-control subjects also are presented in Table 1. Levene's test for homogeneity of variance indicated that the PSL variances were not different between assay methods (F = 0.05 to 1.1, P = 0.32 to 0.82).

Median ^{51}Cr LU_{10} values were significantly higher than median flow cytometry LU_{10} values (see Table 2). In fact, ^{51}Cr-release LU_{10} values were greater than the flow cytometry LU_{10} values for all 10 subjects (see Fig. 4). The mean percentage of NK cells ($CD3^-$/$CD56^+$) was 7.7% (SD = 5.23, median = 9.42%). The relationships between percentage NK cells and both cytotoxicity methods were strongly positive (^{51}Cr release, r_s = 0.79, P = 0.007; flow cytometry r_s = 0.75, P = 0.012).

Excluding PBMC separation costs, cost for supplies and disposal for each ^{51}Cr assay was $67, and for each flow cytometry assay, it was $54. Technician time for performing and analyzing each ^{51}Cr and flow cytometry assay was the same—16 h—and is not included in the total cost. There were no fees associated with use of our gamma counter. A user fee of $45 per hour (2 h, or $90) was required for the FACScan, for a

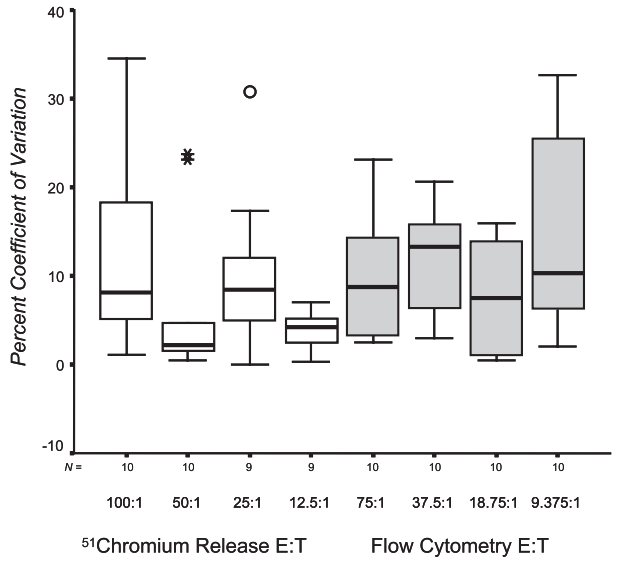

Figure 3. Box-and-whiskers plots of intraassay variability of duplicate coefficients of variation (CV) for 10 subjects at 4 effector-target ratios (E:T). ^{51}Cr release CV values (white boxes) were calculated from counts per minute at 100:1 to 12.5:1 E:T. Flow cytometry CV values (gray boxes) were calculated from percent specific lysis at 75:1 to 9.375:1 E:T. Only the CVs calculated at the smallest E:Ts (i.e., 12.5:1 for ^{51}Cr and 9.375:1 for flow cytometry) were statistically different from each other (z = –2.55, P = 0.011, Wilcoxon signed rank test).

Table 2. Natural Killer (NK) Cytotoxicity (LU_{10}) by ^{51}Cr-Release and Flow Cytometry Assays (n = 10)

Assay	Median	Range
^{51}Cr release	12.42	0.18–33.01
Flow cytometry	5.58	0.00–15.30

NOTE: LU_{10} = lytic unit$_{10}$. All ^{51}Cr-release LU_{10} values were greater than flow cytometry LU_{10} values, z = –2.80, P = 0.005, Wilcoxon signed rank test.

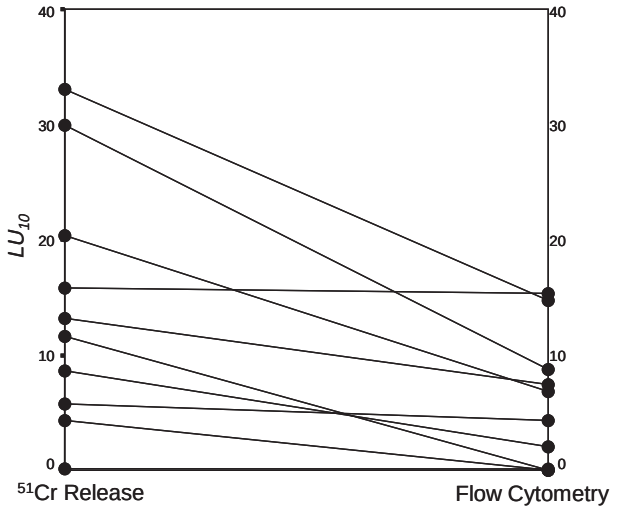

Figure 4. Line graph of lytic unit-10 (LU_{10}) by ^{51}Cr-release assay and flow cytometry assay. ^{51}Cr-release assay LU_{10} values are higher than flow cytometry LU_{10} values for all 10 subjects (z = –2.80, P = 0.005, Wilcoxon signed rank test).

total of $144 per flow cytometry assay versus the $67 total cost per ^{51}Cr-release assay set up in the manner outlined above.

Discussion

As expected, the ^{51}Cr-release and the flow cytometry cytotoxicity assays were strongly and positively related to each other. Our findings support the relationships of 0.81 to 0.96 ($P < 0.001$) reported by others using fresh PBMCs (e.g., Chang and others 1993; Godoy-Ramirez and others 2000; Piriou and others 2000).

Intraassay reliabilities of both methods were comparable and fell within an acceptable range. The standard procedure in our laboratory as well as in other laboratories (e.g., Whiteside and others 1990) is to use triplicate samples for the ^{51}Cr-release assay. Using triplicate ^{51}Cr samples is relatively easy and requires only slightly more cells than running duplicate samples, with only slightly increased technician and analysis time. If samples are run in triplicate, a single cpm outlier can be discarded, resulting in improved intraassay reliability. Previously, Motzer and colleagues (2002) reported intraassay variability of less than 6% for ^{51}Cr-release assays. In the current study, we did not obtain adequate amounts of whole blood for flow cytometric analysis of NK cell cytotoxicity as specified in our original design and had to adjust the number of replicates from 3 to 2. Using duplicate samples is an accepted practice for flow cytometry cytotoxicity assays (e.g., Hatam and others 1994). By reporting randomly selected duplicate ^{51}Cr values to achieve an equitable methods comparison, we may have underestimated reliabilities of ^{51}Cr-release assay.

Interassay variability was high but not different between methods. Interassay CVs of both methods were well within the 31% to 92% range of LU_{20} ^{51}Cr-release CVs reported by Pross and Maroun (1984), who studied 13 subjects up to 62 times each over 3 years, using fresh PBMCs for all assays. Some of their variability likely was due to changes in NK cell cytotoxicity in response to life events. Since we used cryopreserved PBMCs from a single blood draw for each assay-control subject, there was no effect of life events on our variability.

We expected that sensitivity of the flow cytometry assay to cell death would be superior to that of the ^{51}Cr-release assay. Specifically, spontaneous release of ^{51}Cr by the target cells (Whiteside and others 1990) cannot be differentiated from the ^{51}Cr released in response to lysis by effector cells, which contributes to its reduced sensitivity. To the contrary, target cell membrane binding with DiO is stable and, in conjunction with the DNA binding of PI, allows for accurate discrimination of live and dead target and effector cells (Kane and others 1996). However, our data suggest that ^{51}Cr-release assays may be more sensitive to cell death than flow cytometric cytotoxicity assays at low LU levels.

We examined the level of cytotoxicity as one way to estimate assay sensitivity to cell death. Cytotoxicity levels were low in both assays, but we found that median LU_{10} and all 10 pairs of individual LU_{10} were higher in the ^{51}Cr-release assay than in the flow

cytometry assay. Low cytotoxicity may have been related to overnight incubation in complete media as well as attenuation from cryopreservation (Kawai and others 1988; Whiteside and others 1990). This finding is in contrast to both Godoy-Ramirez and others (2000) and Hatam and others (1994), who reported higher cytotoxicity (PSL at 50:1 E:T, and PSL at 100:1, 50:1, and 25:1, respectively) with flow cytometry than ^{51}Cr release in fresh PBMCs. However, their PSL values also were higher (Godoy-Ramirez and others 2000: ~35% and 62% for ^{51}Cr and flow cytometry, respectively; Hatam and others 1994: 50% and 70% for ^{51}Cr and flow cytometry, respectively) than the PSL we obtained at the same or higher E:T for either method. It may be that the differences in the findings resulted from use of cryopreserved PBMCs incubated overnight in complete media versus fresh PBMCs.

Per standard assay protocols, we did use different incubation times for the 2 methods (4 h for ^{51}Cr release, 3 h for flow cytometry), which could have accounted for the differences in cytotoxicity. The ^{51}Cr-release assay requires target cell lysis to occur, spilling radioactive cell contents into the supernatant. Lysis is the last step in the NK cell cytotoxicity process. The standard incubation time for ^{51}Cr-release assay incubation is 4 h (Whiteside and others 1990). The flow cytometric cytotoxicity assay requires only that the dying target cell's membrane becomes permeable so that the PI can enter the cell. This change in membrane permeability occurs once the lethal hit has been delivered by the effector cell and prior to target cell lysis. The minimal recommended incubation time is 2 h (Molecular Probes Inc. 2001) but ranges from 2 to 4 h (Ellis 1993; Hatam and others 1994). Chang and colleagues (1993) tested a variety of incubation times (30 min to 6 h) for the flow cytometric cytotoxicity assay. They reported a plateau in PSL after 2 h incubation across all 4 E:T tested, with a strong relationship (r = 0.91) between cytotoxicity obtained from a standard 4-h ^{51}Cr-release incubation and a 2-h flow cytometric incubation. However, in another study (N = 10) comparing cytotoxicity by ^{51}Cr release and flow cytometry, using the same 4-h incubation for both methods, Godoy-Ramirez and others (2000) obtained a significantly higher PSL with flow cytometry ($P < 0.001$). Therefore, it is possible that the 1-h longer incubation time used with ^{51}Cr release resulted in higher cytotoxicity in this study.

We also examined the relationships between LU_{10} for each method and the percentage of NK cells as another way to estimate sensitivity of the assays. Since a change in the percentage of NK cells in relationship to other lymphocytes often but not always parallels NK cell cytotoxicity (Whiteside and Herberman 1989), it may be that the assay with the stronger relationship with a known percentage of NK cells also has the higher sensitivity. The ^{51}Cr-release assay had a slightly stronger relationship with percentage of NK cells than did the flow cytometry assay. However, the average percentage of NK cells was low, with 4 apparently healthy subjects having less than 5% NK cells and 2 of those 4 subjects having no detectable NK cells. For the other 6 subjects, mean NK percentage (11.4% ± 2.3%) was within the normal range (10%, with the middle 80% ranging from 5.4% to 16.3%) (Whiteside and others 1990). Cryopreservation should not have accounted for the low percentage of NK cells. In our laboratory, after PBMC cryopreservation, on average the cell recovery rate is 96% and the cell viability rate is greater than 90%. Friberg and others (1994) reported identical lymphocyte gating profiles after cryopreservation.

In this small-sample study, costs were comparable between methods. Although costs of supplies were slightly higher per assay for ^{51}Cr release, our major cost, technician time, was the same. However, as more samples are run, technician time increases at a much higher rate for flow cytometry than for ^{51}Cr release. Each flow cytometry sample must be monitored and run on the FACScan by a highly skilled technician. In contrast, a gamma counter unattended overnight can read hundreds of ^{51}Cr assay samples. There is also an economy of scale for technician time as well as for target cells if multiple subjects can be run simultaneously in 1 day. In our laboratory, 1 technician has tested up to 16 subjects in 1 day with ^{51}Cr release, which would not be feasible for flow cytometry cytotoxicity. For flow cytometry, using the same protocol, we estimate that only 4 subjects could be assayed. Thus, a clear understanding of technician time may be the most important cost decision. We did not evaluate purchase costs of a gamma counter or a FACS. Equipment access charges and maintenance costs vary by institution but may be

important cost considerations of individual investigators. Another consideration is ease of access to the requisite equipment. For small laboratories utilizing equipment operated by others, access to a FACS may be more difficult to schedule than access to a gamma counter, potentially influencing when assays may be performed.

Not evaluated here but worth noting are the safety concerns associated with radioactive and biohazardous markers. Use of ^{51}Cr to label target cells has implications for personnel safety, and its disposal is costly and raises environmental concerns. Although flow cytometry does not use radioactive labels, PI and DiO clearly are biohazardous. PI is a known mutagen (Fukunaga and Yielding 1978; Fukunaga and others 1981) and suspected carcinogen. Chemical, physical, and toxicological properties of DiO have not been fully investigated, although warnings to avoid inhalation, ingestion, and skin and eye contact are provided by supply companies (e.g., Sigma Chemical Company 1994; Molecular Probes Inc. 1999). However, in general, it is more difficult to protect personnel against radioactive materials than biohazardous reagents.

One design consideration that we did not intend to evaluate was the volume of whole blood needed per assay. We used 4 times as many target cells in the flow cytometry assay than in the ^{51}Cr-release assay, necessitating 4 times as many effector cells to attain proposed E:T. Increased numbers of cells were necessary to acquire 200,000 total events per sample, or 500 to 3000 target events. Thus, to keep samples sufficiently concentrated to run efficiently (i.e., 2-min running time per sample) on the FACScan, we found that the requisite amounts of whole blood to run 1 sample in triplicate at 4 E:T (starting at 100:1) was 14 ml for flow cytometry, compared to 3.5 ml for ^{51}Cr. (On average, our laboratory harvests 10^6 PBMCs from each milliliter of whole blood.) Clearly, the amount of blood needed depends on the number of samples to be assayed per day. For fewer samples, less blood would be needed. In addition, if the technician were able to spend more time at the FACS with a less concentrated sample, then fewer cells would be needed. Accurately predetermining the whole blood sample volume is critical for study logistics as well as for human subjects review approval. If proposed research included the study of children or anemic or ill adults, or involved frequently repeated blood draws, then using the ^{51}Cr-release assay or paring down replicates or numbers of E:T for the flow cytometry assay would be important design choices.

Assay specificity is another important design consideration, but it was not addressed in this study. With flow cytometry, multiparameter assessments can be carried out on the interactions between individual effector cells and target cells, including assessing the relative numbers of individual NK and target cells and the NK cells conjugated to viable and nonviable target cells (Godoy-Ramirez and others 2000). Functionally active NK cells can also be sorted based on their ability to bind to and kill target cells. Discrimination between single target cells and effector-target conjugates provides more insight into the NK cytotoxicity process. Specifically, a larger volume conjugate pair (DiO alone) is indicative of binding, a larger volume conjugate pair with DiO and PI is indicative of delivery of the lethal hit, and a smaller volume single target cell with DiO and PI demonstrates target lysis and effector cell recycling (Ellis 1993). The ^{51}Cr-release method does not examine cytotoxicity on a single-cell basis but rather in the aggregate. That is, the amount of ^{51}Cr that is released by a specific number of labeled target cells in response to incubation with a specific number of effector cells is quantified, but information about the cytotoxicity process is lost. Therefore, for investigators wishing to utilize a more sophisticated analysis to answer single-cell questions, flow cytometry is clearly the assay of choice.

Study Limitations

One possible limitation was overnight incubation in complete media, which may have attenuated cytotoxicity. Another limitation was the lower E:T and number of replicate samples used for the flow cytometry cytotoxicity assay compared to the ^{51}Cr-release assay.

Conclusion

^{51}Cr-release and flow cytometry NK cell cytotoxicity assays are comparable in terms of intra- and interassay reliability. At low cytotoxicity values, the ^{51}Cr-release assay may be more sensitive to cell death

than flow cytometry. Cost is comparable unless large numbers of assays are required. Technician time then becomes the major cost issue. Importantly, more whole blood is needed to perform the flow cytometry assay than the ^{51}Cr-release assay, especially if duplicate or triplicate values at multiple E:T are run. Therefore, based on the design issues evaluated in this study, there is no compelling reason to adopt NK cell cytotoxicity by flow cytometry over ^{51}Cr release unless single-cell questions about cytotoxicity are part of the research aims.

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