Rapid Communication

Cell-Mediated Cytotoxicity Evaluation Using Monoclonal Antibody Staining for Target or Effector Cells With AnnexinV/Propidium Iodide Colabeling by Fluorosphere-Adjusted Counts on Three-Color Flow Cytometry

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Background: In addition to ^{51}chromium release assay, flow cytometric methods have been described to assess in vitro cell-mediated cytotoxicity. In this report, we describe a new flow cytometric approach for determination of in vitro cell-mediated cytotoxicity utilizing three-color flow cytometric assay.

Methods: This method is based on monoclonal antibody staining of either effector or target cells to evaluate cytotoxicity with increased accuracy by utilizing fluorospheres for calibration. The basic strategy involves labeling effector or target cells with a specific fluorescent-conjugated monoclonal antibody, in addition to staining with annexinV-FITC and propidium iodide to identify apoptotic/dead cells. The effector and target cell populations as well as conjugates were clearly and easily identified by this approach.

Results: We obtained significant correlation between cytotoxicity calculated by this technique and ^{51}chromium release assay results. The integration of fluorospheres allowed us to determine the absolute number of events reflective of the cumulative cell death rather than a cross-sectional, percentage-based cytotoxicity assessment in the target cell population at the time of analysis.

Conclusions: This method provides additional advantages to other methods and enables the study of target cell fate in more detail, as well as providing a potential contribution to understanding the mechanisms of cell elimination. Cytometry Part A 56A:53–60, 2003.

Key terms: flow cytometric cell-mediated cytotoxicity assay; monoclonal antibody; annexinV; propidium iodide; fluorosphere-adjusted counts; cell-mediated cytotoxicity; NK cytotoxicity; LAK cytotoxicity; target cell labeling; effector cell labeling

^{51}Cr release assay (CRA) has been the standard and most popular method to study cell-mediated cytotoxicity in vitro. In this method, target cells are loaded with Na^{51}CrO, which passively enters cells and binds to intracellular proteins. Upon target cell lysis, ^{51}Cr is released into the supernatant and the amount of ^{51}Cr is quantified using a beta or a gamma counter. Though this method has the benefits of being reproducible and relatively easy to perform, it has several drawbacks, including: the difficulty in labeling cells with low cytoplasm–nucleus ratio; increasing spontaneous release of ^{51}Cr from target cells (>15%) over time, especially in long-term assays; a delay between actual cell damage and release of ^{51}Cr-bound intracellular proteins into the supernatant; and the measurement of cytolysis at the population versus single-cell level. Chromium release assay reflects the changes in the entire target cell population, i.e., partial ^{51}Cr release from the entire target population or total ^{51}Cr release from a fraction of the targets cells. Also there is need for specific care and

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handling associated with radioactive isotope usage, with the high cost and the short half-life of the radioisotope (1,2).

Nonradioactive methods, such as fluorescent labeling of effector or target cell populations, have also been used. Initially, these methods were used to measure increases in target cell membrane permeability for the assessment of cell-mediated cytotoxicity. Fluorescent dyes such as carboxy-fluorescein diacetate can be loaded into the cell cytoplasm with relative ease. Upon cytolysis, the release of these dyes into the supernatant or their retention in target cells is measured using a microfluorimeter. However, spontaneous release of these dyes can be quite high, giving rise to spurious results and limiting their use in short-term assays. Alternately, two-color flow cytometry has been used to measure increases in target-cell membrane permeability. Target cells are labeled with a variety of fluorescent reagents such as calcein-AM prior to the cytolytic assay (3). Lysis is measured by two-color flow cytometry as an increase in membrane permeability sufficient to allow access of DNA-binding dyes to the target cell nucleus. This approach has proven useful, especially in the context of longer-term assays, in which use of CRA is problematic because of leakage from target cells. There has been a similar dye-leakage problem with cell membrane binding fluorochromes, such as PKH-2, PKH-26, or F-18, which has resulted in artifactual staining of other populations during the coincubation (2). In recent years, several groups have developed multicolor flow cytometry-based methods using the dyes listed above, and others, to study cell-mediated cytotoxicity (4–7).

An essential element of the multicolor flow cytometric assays is the ability to discriminate effector cells from target cells by further characterization of the target cell fate. Several different methods have been suggested to identify target and effector populations, such as the use of differences in light scattering properties. Recently, Zamai et al. (8) developed a flow cytometric assay using preculture, direct staining, with a CD56-antibody and light scatter changes for the detection of cytotoxicity against K-562 cells, which can be useful, although not easily adapted to other systems (8,9). In later studies, researchers utilized mAb staining in combination with either PI or annexinV-FITC (AnnV) (2,8).

In this report, we describe a new approach for determination of in vitro cell-mediated cytotoxicity utilizing three-color flow cytometric analysis. Our assay is based on mAb staining of either effector or target cells to evaluate cytotoxicity with increased accuracy by utilizing fluorospheres (Flow-count™ fluorospheres, Beckman Coulter, Miami, FL) for calibration. The basic strategy involves labeling effector or target cells with specific fluorochrome-conjugated mAbs, in addition to staining with AnnV and PI to identify apoptotic/dead cells. Membrane phosphatidylserine (PS) externalization occurs during apoptosis; AnnV binds to the externalized PS, providing a convenient in vitro tool to measure apoptotic cell death. Externalization of PS has been demonstrated to precede changes in plasma membrane permeability as measured by PI uptake (1,2). The addition of fluorospheres allowed us to determine the absolute number of events reflective of the cumulative cell death, rather than a cross-sectional, percentage-based cytotoxicity assessment in the target cell population at the time of analysis.

MATERIALS AND METHODS

Purification of Human Peripheral Blood Lymphocytes and the Generation of Lymphokine-Activated Killer (LAK) Cells

Fresh PBLs were isolated after centrifuging fresh blood collected from healthy donors on Ficoll Histopaque-1077 (Sigma, St. Louis, MO). Fresh PBL were cultured for three days at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml gentamycin in the presence of IL-2 (50 U/ml) and IL-15 (10 ng/ml) (Biosource, Camarillo, CA). Cells were maintained in 25 cm^2 flasks and kept in the incubator (37°C, 5% CO_2).

Target Cells

The human erythroleukemia cell line K-562 is known to be NK-sensitive and has been used as the reference-standard cell line in cytotoxicity studies. Daudi is a malignant B-lymphoblastoid cell line and is LAK-sensitive. In a number of experiments we also used MEG-01 and U-937 myeloid leukemia cell lines. These cell lines were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 culture media, supplemented with 10% FBS. Before coincubation, target and effector cell viability were determined by the trypan blue exclusion test; a viability of >90% was required to proceed.

Labeling Effector Cells and the Use of mAbs

We initially utilized three different T-cell/NK-cell–associated mAbs: CD5-PE, CD7-PE, and CD2-PE, to mark the effector population. We obtained a better separation of the target cells from the effector population with CD2 staining in LAK cell samples. Both CD2 and CD7 are expressed in a great majority of LAK populations and CD2 expression in LAK cells was greater than 85% and mostly related to IL-2 induced upregulation (10). CD2 expression in LAK cells has an important role related to cytotoxicity in LAK cells (11). CD2 can also be used for marking fresh PBL and it was easy to separate CD2-negative Daudi and K-562 cells from CD2-positive effector cells. All monoclonal antibodies were purchased from Immunotech, Inc. (Westbrook, ME).

Marking Target Cells and mAbs

We utilized mAbs anti-CD33-PE for K-562 and anti-CD19-PE for the Daudi cell lines. mAbs were purchased from Immunotech, Inc. (Westbrook, ME).

Flow Cytometric Cell-Mediated Cytotoxicity Assay (FCM-CMCA) Setup

We performed the following stepwise approach in preparing and analyzing the samples:

Effector and target cells were coincubated in certain effector/target (E/T) ratios. Effector and target cells were added to 12 × 75 mm (5 mL) round bottom polystyrene tubes (Falcon, (Becton-Dickinson, Franklin Lakes, NJ) in various E/T ratios (0.5:1, 1:1, 2:1, 4:1, 8:1, 16:1, up to 128:1) for different incubation times. Control tubes including target or effector cells alone were studied to determine the location of populations on the histograms and spontaneous target cell apoptosis. Tubes were centrifuged at 115 *g* for 5 min and incubated at 37°C in 5% CO_2 for 2, 4, and 18 h.

Then cells were incubated with 10 μl of mouse anti-human PE-conjugated mAb (either CD2, CD33, or CD19) for 20 min at room temperature. In different experimental settings, either target or effector cells were stained with fluorescent-conjugated monoclonal antibodies.

To mark cell apoptosis/death, cells were incubated with FITC-conjugated AnnV and PI (TACS™ AnnexinV-FITC; R&D Systems, Minneapolis, MN) for 20 min before acquisition.

To determine the absolute number of viable or dead target cells and to standardize the analysis, 20 μl of Flow-count™ fluorospheres were added to cell suspensions (50 μl). Flow-count fluorospheres consist of 10 μm (nominal diameter) polystyrene fluorospheres in an aqueous suspension medium containing a surfactant and 1% formaldehyde. Each fluorosphere contains a dye, which has a fluorescence emission range of 525 nm to 700 nm when excited at 488 nm. Fluorosphere count has been used as a constant and independent means of halting data collection during analysis and to standardize samples to obtain accurate cell counts for T, B, NK lymphocyte, and lymphocyte subsets in whole blood analysis using flow cytometry (12). Since data acquisition time and sample flow rate varied between the samples, target cell numbers, and fluorosphere numbers, suspension volume, and the acquisition time were held constant in target-alone and in coincubation tubes, with varying E/T ratios in every experiment. Thus, data collection from each sample could be standardized to acquire absolute cell counts. This enabled us to calculate the number of targets left alive per unit volume and to yield reliable intersample comparison. At least 1,000 fluorospheres were counted in each tube.

FCM Acquisition and Running

FCM was performed using an EPICS-XL MCL (multicarousel) (Coulter, Miami, FL) equipped with an argon laser (15 mW) source operating at 488 nm. The emission of three fluorochromes was recorded through specific band pass filters: 525 nm for FITC (FL_1), 575 nm for PE (FL_2), 620 nm for fluorospheres (FL_3), and 675 nm for PI (FL_4). The instrument was set for four-color analysis. As the emission spectra of the three different dyes and fluorospheres utilized in this bioassay interfere with one another, appropriate electronic compensations were adjusted by running individual cell populations stained with each dye consecutively through the EPICS. Once the compensations had been set, a gating was done on forward scatter (FS) (ordinate) versus log-scale red fluorescence of CD2 or other mAbs (abscissa), to separate target cells from effector cells. The different cell populations were gated with the use of control samples. To measure target-cell death and apoptosis, CD2-negative (effector staining approach) and CD33- or CD19-positive events (target staining approach) were gated on, and analysis of log green fluorescence (AnnV) versus log red fluorescence (PI) was performed (Fig. 1a and b). FCM analysis lasted until a minimum of 1,000 fluorospheres had been acquired in a fixed acquisition time on each tube.

FCM-CMCA Data Analysis

An average of 10,000 total events and 3,000 target cells were collected per sample. Data were reevaluated on list mode analysis using Coulter System II™ Version 3 software for the EPICS XL-MCL. The gating for the target cells was based on the target-alone analysis and kept constant throughout all tubes to avoid exaggeration of the counts due to apoptotic body contamination. Cytotoxicity calculations were based on viable populations in target-alone and coculture tube analysis results. Viable target-cell percentage and corresponding fluorosphere-adjusted counts were determined, and calculations were based on the control-tube values. We expressed the cell-mediated cytotoxicity in two different ways:

Percentage-Based (Cross-Sectional) Cytotoxicity

In this approach, we used the percent of AnnV-negative/PI-negative events (viable cell population) for the target-cell gate in target-alone (control) and coculture analysis, to correct for spontaneous apoptosis with the following formula:

$$\text{Percent Cytotoxicity (PC)} = \frac{[\text{Control-viable cell percent}] - [\text{Co-incubation-viable cell percent}]}{[\text{Control-viable cell percent}]}$$

The result reflects the rate of cells undergoing apoptosis/death ($AnnV^+/PI^-$ and $AnnV^+/PI^+$ events) at the time of sampling. Using the viable population helped us to account for all forms of cell death that occurred.

Cumulative Cytotoxicity

Instead of using percentages, we utilized corresponding fluorosphere-adjusted counts in the same equation given above. The result reflects the total cell loss that occurred during the entire incubation period rather than the rate of cell apoptosis/death at the time of sampling.

$$\text{Cumulative Cytotoxicity (CC)} = \frac{[\text{Fluorosphere-adjusted counts of control viable cells}] - [\text{Fluorosphere-adjusted counts of coincubation viable cells}]}{[\text{Fluorosphere-adjusted counts of control viable cells}]}$$

^{51}Cr-release Assay

A standard assay was performed as previously described (13). Briefly, the assay is a standard 4-h ^{51}Cr release assay

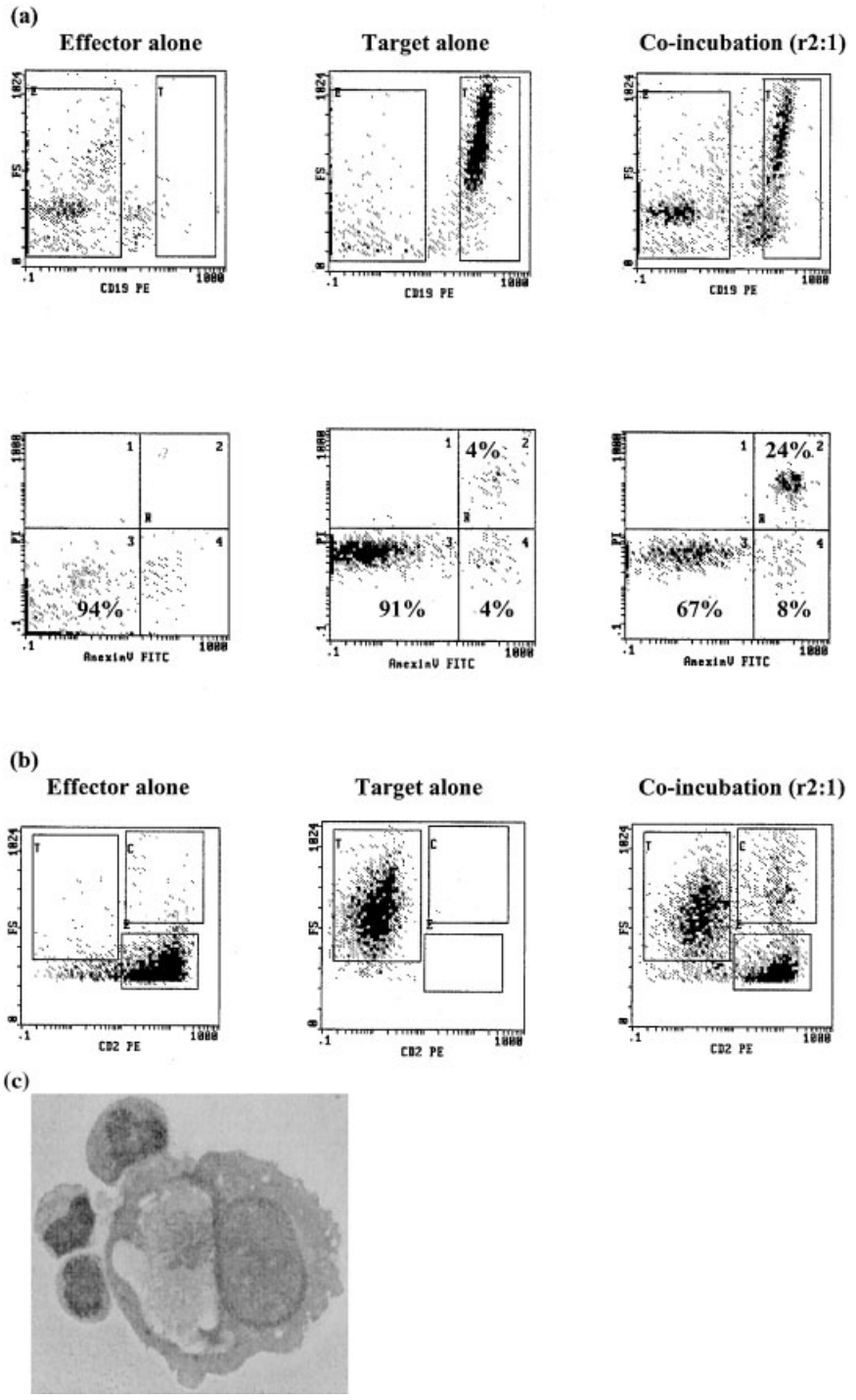

FIG. 1. **a**: Identification of effector and target cells in coincubations using target-ell labeling is shown in histograms obtained from an experiment where LAK cells were coincubated with CD19-positive Daudi cells. Evaluation of the cell populations gated by AnnV/PI staining is shown in the rectilinear boxes in the bottom row of histograms. In this representative sample, Daudi cell viability decreased from 91% to 67% with coincubation (E/T ratio: r2:1). The early apoptotic population increased from 4% to 8% as well as the late apoptotic population, from 4% to 24%. (E: effector area, T: target area, R2: late apoptotic/dead [$AnnV^+/PI^+$] population, R3: viable [$AnnV^-/PI^-$] population, R4: early apoptotic [$AnnV^+/PI^-$] population.) **b:** The location of the conjugate population using effector cell labeling is shown in a coincubation histogram (E: effector, CD2 positive events; T: target, CD2 negative events; C: conjugate area, CD2 positive events those with larger sizes [higher FS values]). **c**: Conjugate formation between K-562 and LAK cells with W/G staining.

using target cells that have been prelabeled with 100 μCi ^{51}Cr (Perkin Elmer, Boston, MA) for 1 h. Prior to use in the assay, the target cells were washed three times with medium to remove excess ^{51}Cr. Several concentrations of PBL or LAK cells were added to a fixed number of target cells (5,000) in a round-bottom microtiter plate to a total volume of 0.2 ml. The plate was then centrifuged for 5 min at 1,000 rpm, after which the plate is incubated at 37°C for 4 h. Following the 4-h incubation, 0.1 ml of the supernatant was carefully harvested and counted on a scintillation counter (Packard, Downers Grove, IL). The percentage of cytotoxicity was calculated using the following equation: (E – S)/(M – S) × 100, where E is the experimental counts per minute, S is the spontaneous counts per minute and M is the maximum counts per minute. Maximum release was determined from wells

including target cells and 10% sodium dodecyl sulfate (SDS) in the medium. Spontaneous release was measured from wells containing a fixed number of target cells in the medium.

Cell Staining for Microscopy With Wright/Giemsa

At the end of the coincubation period, the sample was gently mixed and 10 μl was used to prepare slides at room temperature without cytospinning. The slides were then stained with Wright/Giemsa (W/G). A conjugate formation between K-562 and LAK cells with W/G staining is demonstrated in Figure 1c.

Statistical Analysis

The correlation between PC and CC measured by flow cytometer and the CRA results was studied using Pearson's correlation analysis with the Statistical Package for Social Sciences (SPSS-11.5 for Windows). Significance was determined as < 0.05.

RESULTS

Two leukemia/lymphoma cell lines, K-562 and Daudi, were used as the main targets in this study to compare our method with the standard CRA. K-562 is a NK-sensitive cell line and has been used as a standard target cell in cytotoxicity assays. However, Daudi is known to be NK-activity—resistant, but LAK-sensitive. Because we have shown that LAK activity reaches a peak level at three-day incubation of PBL in culture with a combination of IL-2 and IL-15, we utilized fresh PBL and LAK cells obtained following three-day culture as effectors (14). This cytokine combination also produced more effective LAK activity compared to other combinations such as PHA+IL-2, anti-CD3+IL-2 and IL-15. We also evaluated the LAK cytotoxicity of these three protocols by CRA in certain experiments and saw a very good correlation between CRA and our method.

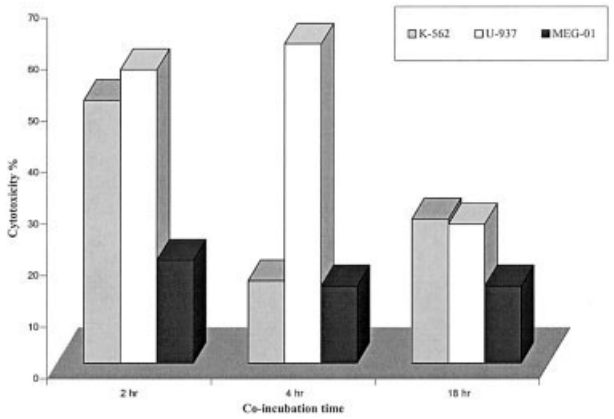

FIG. 2. Kinetics of LAK cytotoxicity by PC in three different cell lines at 2:1 ratio at different coincubation times by effector cell labeling. The mean PC reaches a maximum level at 2–4 h of coincubation in all myeloid leukemia cell lines tested in three different sets of experiments.

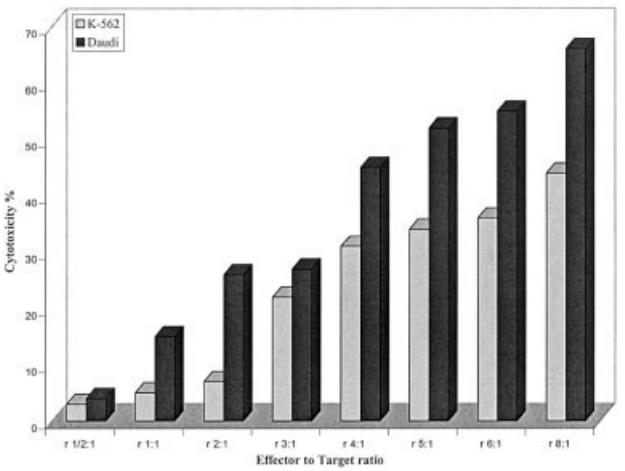

FIG. 3. LAK cell cytotoxicity in representative samples of K-562 and Daudi cell lines by target labeling at eight different ratios (r). Increasing cytotoxicity with higher E/T ratios up to 8:1 by PC calculation was observed in these studies. A superior LAK cell killing was observed in Daudi cells than K-562 cells, reflective of the known enhanced sensitivity of Daudi cells to LAK cells.

Percentage-based cytotoxicity reached peak levels at the end of 2–4 h of coincubation and started declining after 4 h, depending on the target cell population (5,8,15). In comparison studies with CRA, samples were coincubated for 4 h, whereas in some experiments samples were coincubated for 2 h (Fig. 2).

After studying several different E/T ratios in both cell lines, we used a 2:1 ratio for many comparisons due to the superior separation of the target and effector cell populations at this ratio. There was ratio-dependent cell kill as shown in Figure 3. It has been reported that E/T ratios up to 10:1 and 20:1 have been successfully used in flow cytometric cell-mediated cytotoxicity assays (4).

Fresh PBL/NK Cytotoxicity Evaluation by FCM-CMCA Using Effector Cell Labeling

Fresh PBL/NK cytotoxicity experiments using effector cell labeling with CD2 were evaluated in a total of five different experiments with a minimum of five different E/T ratios each time, using K-562 and Daudi cells, and results were compared with CRA. Consistent with the literature, lower target-cell killing was observed compared to the results obtained with LAK cells as effectors. In fresh PBL studies, early apoptotic cells ($AnnV^+/PI^-$) were more pronounced than the late apoptotic/dead ($AnnV^+/PI^+$) events. There was a positive correlation between PC and CRA results in K-562- and Daudi-cell killing ($r = 0.94$; $P = 0.009$ and $r = 0.95$; $P = 0.007$, respectively) (Fig. 4). Since our calculation approach takes early apoptotic cells into account, the PC and CC appeared to be higher than the CRA results. In other words, early apoptotic cells may still have their membrane integrity, preventing ^{51}Cr release consistent with the literature (5,16). Similarly, CRA results correlated with FCM-CMCA when CC was calculated for both cell lines ($r = 0.93$; $P = 0.012$ and $r = 0.81$; $P =$

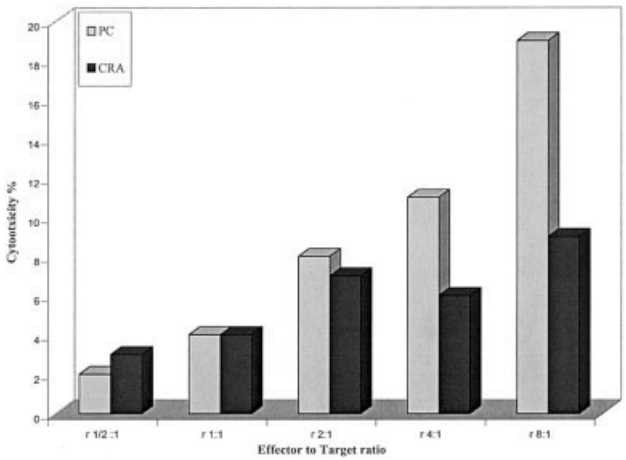

FIG. 4. Fresh PBL/NK cytotoxicity evaluation by the FCM-CMCA method (PC) and CRA in K-562 cells are compared. The values represent mean killing of K-562 cells in three different sets of effector labeling experiments and CRA. Since early apoptotic cells were included in PC calculations, PC values were higher than CRA results.

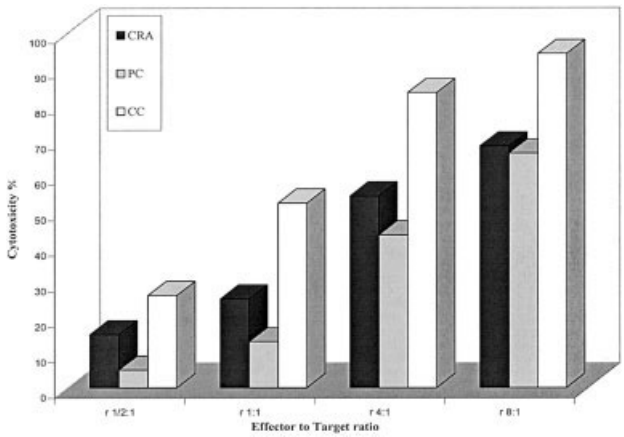

FIG. 5. LAK cell cytotoxicity on Daudi by different FCM-CMCA calculation methods (PC and CC) and CRA. The results reflect mean values of three different sets of experiments. Target cells were labeled with CD19 mAb.

0.04, respectively). As expected from the above results, there was a statistically significant correlation between PC and CC calculations for both cell lines (r = 0.96; P = 0.004 and r = 0.92; P = 0.01, respectively).

LAK Cytotoxicity Evaluation by FCM-CMCA Using Target Cell Labeling

LAK cytotoxicity was evaluated in six different experiments with a minimum of five different E/T ratios each time. CD33 labeling was used for K-562 cells and CD19 for Daudi cells. PC and CRA results correlated very well for both cell lines (r = 0.97; P = 0.007 and r = 0.99; P = 0.007, respectively). There was also a positive correlation between CC obtained from FCM-CMCA and CRA results for both cell lines (r = 0.98; P = 0.003 and r = 0.98; P = 0.02, respectively) (Fig. 5). Again, PC correlated significantly with CC in both cell lines (r = 0.90; P = 0.040 and r = 0.96; P = 0.044, respectively).

One of the advantages of our approach is to be able to further characterize the fate of target cells in coincubation. Since PI^+ cells represent either dead or late apoptotic cells, CRA results are expected to correlate better with cytotoxicity analysis taking only these cells into account, excluding $AnnV^+/PI^-$ events. As shown in Figure 6, CRA results have correlated with PC calculations based on viable target cell population ($AnnV^-/PI^-$) as described under the methods section, as well as PC-PI calculations based solely on dead/late apoptotic (PI^+) events (n = 4; r = 1; P = 0.002, and n = 4; r = 1; P = 0.003, respectively).

When we used our experimental data together (fresh PBL and LAK) in comparing FCM-CMCA with CRA results, there was a significant correlation for both PC (n = 19; r = 0.95; $P < 0.001$) and CC (n = 19; r = 0.77; $P <$ 0.001).

Comparison of Simultaneous Target and Effector Cell Labeling

In a total of seven different experiments, we compared the reproducibility of the result using target-cell and effector-cell labeling with specific mAbs simultaneously. K-562 and Daudi cells were incubated with either fresh PBL or LAK cells. CD2 was used for effector-cell labeling, and CD33 or CD19 were used for target cell labeling in different coincubations, as stated above. Both PC and CC correlated for these two different mAb-staining strategies performed simultaneously in K-562 cells (r = 0.86; $P <$ 0.001, and r = 0.92; $P < 0.001$). A similar correlation was observed for Daudi cells (r = 0. 87; P = 0.029, and r = 0.89; $P < 0.001$).

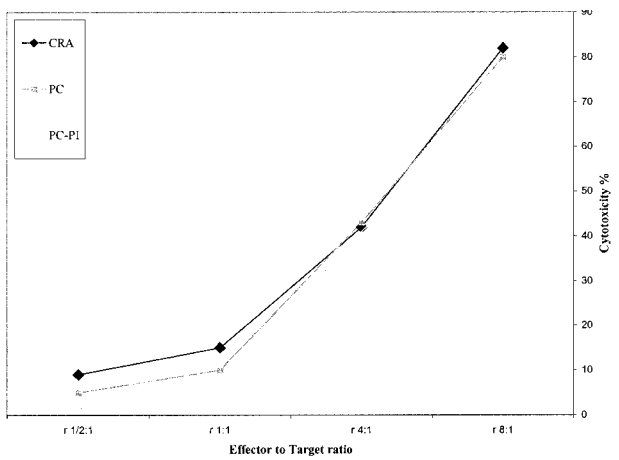

FIG. 6. Comparison between CRA and FCM-CMCA analysis using different PC calculation methods. LAK cell killing was evaluated using target labeling in Daudi cells in this representative sample. There was a positive correlation between CRA and PC calculated in two different ways, based on either viability (PC) or late apoptotic/dead population (PC-PI) percentages. The observed correlation may partially be secondary to the relatively low percentages of early apoptotic cells at the time of analysis.

Conjugate Evaluation

This assay can also be used to study the formation of conjugates between effectors and targets (8). Since cell-to-cell interaction is required for target-cell kill in the cellular immune reaction, conjugate formation is a necessary step in this process. However, the formation of conjugates may occur very early during the course of incubation, and detection may be difficult if samples are analyzed after a certain time period (Fig. 1b and c). Conjugate formation has been reported to reach a maximum after 40–60 min of coincubation when LAK cells are used as effectors (9). It has also been shown that after 1 h of culture with high E/T cell ratios, about one-third of the dead targets were still conjugated to effectors. We have observed different patterns of conjugate formation depending on the target cell type and E/T ratio, as well as the incubation time. This information can also be used in kinetic analysis of cell-mediated cytotoxicity.

DISCUSSION

This is the first study to our knowledge to combine effector- or target-cell staining using fluorescence-conjugated mAb with AnnV/PI colabeling to elucidate the fate of target cells by FCM-CMCA. We also integrated the use of fluorospheres to account for the entire target-cell loss throughout coincubation, rather than determining the cross-sectional assessment of the dynamic cell elimination process at the time of sampling using PC calculations. This approach appears to be reproducible, easy, and reliable with very significant correlations obtained when compared to CRA results.

The determination of target-cell death/apoptosis by AnnV/PI or AnnV/7-AAD staining has been used with PKH-26 and PKH-67 labeling, respectively (7,16). However, some investigators expressed concern about such dyes leaking and labeling other cells in the environment during coincubation (8). In a limited number of experiments in which we utilized such dyes, we also faced the same difficulties. Moreover, in studies where target-cell labeling with PKH or other dyes was used, cell elimination mediated by the effector cells involving cell membrane disruption or permeabilization through cytolytic pathways such as perforin/granzyme B, resulted in dye leakage into the medium and artifactual staining of other populations in the coincubation (15). The use of mAbs does not pose such a disadvantage due to their specificity for either effector or target cells.

An additional aspect of using dyes such as PKH-26, $DiO18_{(3)}$ is the need for cell-labeling prior to initiation of coincubation (16,17). Although this may not influence the effector- or target-cell function, this needs to be studied carefully in every new application of this approach. Again, this potential risk is not an issue for our approach due to the addition of mAb at the end of coincubation, just before AnnV and PI. Since we did not fixate the cells for mAb staining, surface binding of mAb did not appear to influence cell functioning or staining with other dyes.

Cell-mediated cytotoxicity is a dynamic process. As stated earlier, cell-to-cell contact occurs very early during the course of coincubation and cell-kill reaches a maximum within 2–4 h. This is one of the potential explanations of the low percentages of conjugated cells observed in our studies. Target- or effector-cell labeling with fluorescence-conjugated mAb enables identification of a well-defined population of conjugates. Due to our method's lack of the nonspecific labeling complication, several aspects of conjugate formation may be studied, including kinetics (Fig. 1b).

The ability to study cell-mediated cytotoxicity in longer coincubation times (18 h), in addition to shorter coincubation times (2 h), is another advantage of this method (15). This appears to be a drawback for CRA as well as for the methods utilizing PKH-26, PKH-67, or DiO18 due to increased release of ^{51}Cr and dye leakage, resulting in staining of the other population. This application is potentially important for studying certain apoptotic pathways that take longer to become operational, such as soluble and membrane-bound TNF-α-induced apoptosis. TNF-α is expressed by various cytolytic cells both in transmembrane and soluble forms. TNF-α has been shown to kill TNF-susceptible targets in 24-h assays (18).

An interesting observation was the correlation between CRA results and the PC calculated based on the viable population, which took early apoptotic cell percents into account as well as the dead/apoptotic (PI^+) cell percents (PC-PI). One might assume that CRA results would better-correlate with the latter approach (PC-PI), since ^{51}Cr is released from cells that have lost their membrane integrity; however, we were able to show that this was also the case for the former approach (PC). This correlation indicates that PI may be replaced with a different fluorescent marker that may help further study of the mechanism of target cell fate, since the results based on the AnnV staining pattern are sufficient to calculate PC.

The use of fluorospheres in determining the absolute number of cells in our method provides an opportunity to calculate the total cell loss (1,3). Although there was a very good correlation between PC and CC in short-incubation experiments, in our limited experience this would not seem to hold true for longer incubation times. Since the rate of apoptosis and oncosis is known to change at any given time during the coincubation, utilizing fluorosphere-adjusted counts in calculations reflects the accurate eventual result, expressed as cumulative cytotoxicity (CC) in this work. Nevertheless, we have observed quite high target-cell viability percents and low PC, despite high CC based on fluorosphere-adjusted counts in certain experiments. Use of fluorosphere-adjusted counts in calculations also proves to be more accurate in experiments that are associated with very high target-cell kill, such as high E/T ratio coincubations or in the presence of very cytotoxic effector cells.

The demonstration of a significant correlation between effector- and target-cell labeling with fluorescence-conjugated mAb emphasizes an important aspect of this approach, enabling its application to various effector- and

target-cell types with different cellular characteristics. Namely, effector-cell staining may be more practical or appropriate in a setting where target-cell fate needs to be studied—not just a determination of viability. We have successfully utilized this method in studying LAK elimination of acute myeloid leukemia cells from patients (19). Additionally, we were able to measure mast cell-mediated killing of myeloid leukemia cells (20). With this method, it is also possible to carry out experiments on more than one target cell population in the same tube.

This method can be utilized in investigating cell-mediated cytotoxicity in a variety of different laboratory settings for various purposes. With the advantage of multiparameter flow cytometry, several different variables could be integrated into the setting, which would enable study of the mechanisms of death/apoptosis in in vitro cell-mediated cytotoxicity.

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