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Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays

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Abstract

Cytotoxicity is one of the major defence mechanisms against both virus-infected and tumor cells. Radioactive ^{51}chromium (^{51}Cr) release assay is a "gold standard" for assessment of natural killer (NK) cytolytic activity *in vitro*. Several disadvantages of this assay led us to design alternative tools based on flow cytometry analysis. Four different fluorescent dyes, calcein acetoxymethyl ester (CAM), carboxyfluorescein succinimidyl ester (CFSE), Vybrant DiO (DiO) and MitoTracker Green (MTG) were tested for labeling of NK target K-562 cells. Target staining stability, spontaneous release of fluorochromes and subsequent accumulation in bystander unstained cells were measured using fluorimetry and flow cytometry. Healthy donor peripheral blood mononuclear cells and affinity column purified NK cells were used as effectors coincubated with target K-562 cells at different E:T ratios for 3 h and 90 min, respectively. Fluorescent probe 7-amino-actinomycin D was used for live and dead cell discrimination. Bland–Altman statistical method was applied to measure true agreement for all CAM–^{51}Cr, CFSE–^{51}Cr, DiO–^{51}Cr and MTG–^{51}Cr pairs analyzed.

Based on the data, none of the four proposed methods can be stated equivalent to the standard ^{51}Cr release assay. Considering linear relationships between data obtained with four fluorochromes and ^{51}Cr release assay as well as linear regression analysis with $R^2 = 0.9393$ value for CAM–^{51}Cr pair, we found the CAM assay to be the most closely related to the ^{51}Cr assay.

Keywords: Calcein-AM; CFSE; Cr release; Cytotoxicity; Mitotracker Green; Natural killer; Vybrant DiO

Introduction

Natural killer (NK) cells are the primary effector cells of the innate immune system through their ability to eliminate pathogen-infected and tumor cells (Whiteside and Herberman 1994). They sense pathological changes in

Abbreviations: ^{51}Cr assay, ^{51}Cr release assay; CAM, acetoxymethyl ester of calcein; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; CFSE, carboxyfluorescein succinimidyl ester; DiO, Vybrant DiO; FC assay, flow cytometric assay; MTG, MitoTracker Green FM; NK, natural killer.

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tissues via balanced cognate activities of the NK inhibitory receptors and NK activating receptors, which recognize decreased expression of major histocompatibility complex (MHC) class I molecules and increased expression of non-classical MHC molecules (MICA and MICB) on affected cells, respectively. The receptor stimulation induces activation of NK cells and leads to direct elimination of target cells through NK cell-mediated cytotoxicity (Lanier 2005). Therefore, monitoring of NK functional alteration emerges as important and may be useful in prognosis, or follow-up during treatment of patients with a variety of diagnoses (Mazodier et al. 2005; Santin et al. 2000; von Zons et al. 1997; Wiltschke et al. 1994).

Cytotoxicity follows a number of steps such as the recognition of the foreign antigen or other molecules expressed on the surface of target cells, the creation of conjugates between effector and target cells and the activation of effector killer cells. NK cells mediate non-major-histocompatibility-complex-restricted lysis and antibody-dependent cytotoxicity, which can be mediated by two major classes of contact-dependent mechanisms such as killing by a secretory/necrotic cytotoxic mechanism, associated with a perforin/granzyme-mediated pathway, or by an apoptotic mechanism based on receptor–ligand interactions (Lanier 2005).

The standard method for determination of NK cytotoxic activity *in vitro* is the ^{51}chromium (^{51}Cr) release assay (Brunner et al. 1968). Though this method has the benefits of being reproducible and relatively easy to perform, it has several limitations including short half-life of chromium, poor loading and high spontaneous release by some cell types and the measurement of cytolysis at the population versus single-cell level. The most serious disadvantage is potential environmental and health hazard associated with the use of radioactive isotope (Jakubek et al. 1983; Slezak and Horan 1989).

Besides assays analyzing the release of endogenous enzymes (e.g., lactate dehydrogenase, alkaline phosphatase), other methods using non-radioactive compounds (e.g., dimethyl-thiazol-diphenyl bromide tetrazolium bromide, Alamar blue) measure directly the proportion of viable cells by evaluating variations in metabolic state (Hussain et al. 1993; Korzeniewski and Callewaert 1983; Nociari et al. 1998; Szekeres et al. 1981). In addition, several groups have developed multicolor flow cytometry-based assays to study cell-mediated cytotoxicity (Fischer and Mackensen 2003; Kantakamalakul et al. 2003; Kasatori et al. 2005; Langhans et al. 2005; Lecoeur et al. 2001; Lee-MacAry et al. 2001; Sheehy et al. 2001). However, the leakage of the dyes used for cell discrimination causes labeling of nearby cells during the assay and thus prevents proper discrimination of target and effector cell populations. Thus, the choice of a stable primary fluorochrome is highly important.

Although many flow cytometry-based alternatives have been designed, they were not correlated among themselves. For this reason, we correlated the most suitable fluorescent probes acetoxymethyl ester of calcein (CAM) (Neri et al. 2001; Roden et al. 1999), carboxyfluorescein succinimidyl ester (CFSE) (Jedema et al. 2004; Westerhuis et al. 2005), Vybrant DiO (DiO) (Hoppner et al. 2002; Piriou et al. 2000), and MitoTracker Green FM (MTG) (Vizler et al. 2002) among other fluorescent fluorochromes, which could be used for labeling target cells, with the still widely used ^{51}Cr release assay considered as the reference assay (Brunner et al. 1968). The aim was to find a stable alternative flow cytometric method for monitoring NK activity that could be incorporated into the evaluation of immune parameters in patients and possible correlate NK cell activity with disease outcome and progression.

Material and methods

Effector and target cells preparation

Effector PBMNCs were isolated from buffy coats of healthy donors (National Transfusion Service, Bratislava) by Pancol density gradient centrifugation (1.077 g/ml, PAN-Biotech, Germany). The mononuclear cells from the interface were collected, washed twice with PBS and once in RPMI, and then resuspended in 10 ml of complete culture medium (CM), consisting of RPMI 1640 medium, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and supplemented with 10% fetal calf serum (FCS). Purified NK cells were obtained from PBMNCs by positive selection using CD56 mAb-conjugated magnetic beads according to the manufacturer's instructions (Miltenyi, Biotec). Target K-562 cells were maintained in RPMI 1640 cell CM supplemented with 10% FCS and 2 mM glutamine. The cells were split every 3–4 days. For assay purposes, they were centrifuged once, washed with PBS and the pellet resuspended in FCS free RPMI medium. To determine the absolute number of viable and dead cells equal volumes (100 μl) of both Flow-Count fluorospheres (Coulter) and cells were mixed. Cell concentrations of the samples were calculated according to $\text{cells}/\mu\text{l} = (\text{counts}_{\text{cells}} \times \text{volume}_{\text{beads}} \times \text{concentration}_{\text{beads}})/(\text{counts}_{\text{beads}} \times \text{volume}_{\text{cells}})$ where "$\text{concentration}_{\text{beads}}$" indicates the concentration of the beads suspension as given by the manufacturer and "volume" indicates volumes pipetted per sample. Target and effector cells viability was determined by 7-amino-actinomycin D (7-AAD; Molecular Probes, USA) viability assay; viability of $>95\%$ was required to proceed.

Labeling of target cells

Four different fluorescent dyes were used for labeling of K-562 cells before effector and target coincubation. All fluorochromes were purchased from Molecular