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REVIEW

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Cell-Mediated Cytotoxicity Assays

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ABSTRACT

Cell-mediated cytotoxicity measurements can be divided in two methods, depending on whether radioactive material is used or not. Natural Killer cell-mediated cytotoxicity is routinely measured with a short-term assay by labeling the target cells with radioactive chromium (51Cr). The advantages of this method are (1) highly sensitivity, (2) easy execution, (3) low spontaneous release, and (4) nontoxic markers. The disadvantages include short half-life of the label and handling and disposal of radioactive supplies. Many attempts have been made to adapt this cytotoxicity assay to abolish radioactivity while maintaining its high sensitivity. Consequently, various nonradioactive methods have been developed. One of these methods utilizing the release of enzymes (LDH) as a result of cytolysis or membrane dyes (PKH-26) is usually less sensitive than radioactive assays. MTT-based colorimetric/enzymatic assays are highly sensitive and easy to use but unfortunately works only with adherent tumor cell targets. Fluorescent dyes such as carboxy-fluorescein diacetate can easily accumulate in the cytoplasm of effector or target cells. After cytotoxicity, the release of the dyes into the supernatant or their retention in target cells is calculated. However, spontaneous release of these dyes can be quite high, causing false positive results leading to a decreased sensitivity and restricting their use in short-term assays. More recently, flow cytometric methods using fluorescent monoclonal antibodies such as anti-CD56 for effector and anti-CD33 for target cells have been defined.

Keywords: Flow cytometry, Natural Killer cell, cell-mediated cytotoxicity, cytotoxicity

INTRODUCTION

In this review, we first define cell-mediated cytotoxicity. Cytotoxicity describes the realization of target cell death by mechanisms causing apoptosis/necrosis through mediators of the effector cell and/or membrane receptors (1). There are two general methods for the measurement of target cell death (cytolysis) described in the literature. One is based on radioactive material whereas the other is based on nonradioactive material. Two different main methods include the use of radioactive and nonradioactive enzymatic, membrane/fluorescent dyes and flow cytometry. Cell-mediated cytotoxicity measurement are mostly based on the presence of Natural Killer cells (NK) which play a role in the diagnosis of certain immunological and hemophagocytic disorders(2).

NK cells obtained from peripheral blood and NK cell l ines derived from tumor cells (KHYG-1, NK-92 and YT cell lines) are used as effector cells to evaluate cellular cytotoxicity. Unconjugated peripheral blood mononuclear cells are also used to measure peripheral blood NK cell cytotoxicity (3,4). Cells such as unconjugated CD8+-CTL (cytotoxic T lymphocyte) cells or CTL cell line AJY can also be used against different target cells (5). Effector cells are stimulated with specific cytokines such as IL-2 and/or IL-15, and transformed into lymphokine-activated killer (LAK) cells to evaluate cytotoxicity tests (6).

NK and CTL are the main cells of the natural and adaptive immune response. NK cells are predominant in defending against target cells that are infected with virus or that lose major histocompatibility complex class I (MHC-I) expression such as malignant cells. CTL cells function with at least three signals to generate response of the adaptive system. However, both cells function by containing cytolytic granules (7). Development of cytotoxicity due to cell-contact is a determining factor for CTL and NK cells. In vitro cytotoxicity studies have identified two major mechanisms of contact. The first is based on the activation of pathways leading to lysis in the target cell by discharging the lytic granules in the cell

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such as pore-forming perforin and pro-apoptotic serine proteases (granzymes). The second is that the effector cell stimulates apoptosis-inducing receptors in the target cell such as TNF family member, TNF-α, Fas ligand (FasL), or TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (7). Cell lines such as NK-sensitive tumor cells K562 (human chronic myeloid leukemia cell line) and YAC-1 (lymphoma), are used as target cell types in evaluating cellular cytotoxicity (8). NK-resistant/LAK-sensitive: Raji and Daudi (derived from Burkitt's lymphoma) cell lines are used. Other used leukemia cell lines are EM-2 (KML), EM-3 (KML), Jurkat, THP-1, HL60 (AML), U937 (9).

This review discusses the advantages and disadvantages of radioactive and non-radioactive methods for the measurement of cytotoxicity and will go in more detail regarding the non-radioactive methods.

RADIOACTIVE ASSAYS

Chromium (^{51}Cr) Release Assay: CRA

CRA was first described by Brunner et al. in 1968 (10) and is the the gold standard and most popular method. This assay is based on measurement of the release of radioactive ^{51}Cr - Na2 51CrO4 loaded into the target cell after lysis during *in vitro* evaluation of cell-mediated cytotoxicity. The advantages are that the test is easy to conduct and very reproducible (11). The radioactive or non-radioactive assays used to measure cytotoxicity are compared with CRA, which is the gold standard test, and the correlation between them is examined

Disadvantages / Limitations

^{51}Cr is radioisotope and a volatile element which requires license for storage and waste and needs control (12,13). Hence it is expensive and has a short half-life. Target cell with slow metabolism is slow or a low cytoplasm/nucleus ratio is hard to label. CRA delays in reflecting the damage/death in the cell and it measures at cell collection level rather than target single cell. It is time consuming to label the target cells and requires large number of target cells. Around 15% spontaneous release from the target cell can occur especially in long-term tests (5).

JAM (Jurkat Apoptosis Measurement) Test

Matzinger et al. developed an alternative radioactive method to measure cytotoxicity based on measuring apoptosis in Jurkat cell lines (14). The JAM Test is a method that measures the DNA fragmentation with labeled triitium [3H] methyl-thymidine. Target cell death is quantified by measuring the release of labeled DNA particles. It is considered as a quantitative radioactive assay that measures apoptosis in the target cell. Radioactivity can be seen as a disadvantage. However, compared to CRA, it has advantages of being cheaper, easier, more sensitive and reliable (15).

NON-RADIOACTIVE ENZYMATIC ASSAYS

These assays are based on the fact that the target cell releases enzymes that are related to apoptosis or cell damage.

Lactate Dehydrogenase (LDH)Release Assay

The LDH Release assay was first identified by Korzenniewski and Callewaert in 1983 (16). It is based on the fact that LDH is released from lysed target tumor cells. The amount of LDH released into medium is proportional to the number of dead cells. Ready-to-use measurement kits are commercially available (17). A major disadvantage of this test is that intact cells can re-uptake the released LDH. Advantages are it correlates with CRA, is sensitive, and easy to conduct.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide) Colorimetric Assay

This assay is used in research laboratories to measure the sensitive/resistant of tumor cells to chemotherapeutic agents. The MTT assay is a colorimetric assay that relies on the mitochondrial enzyme dehydrogenase to convert tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazon which than can be quantified by spectrophotometric means (18). The production of formazan is directly proportional to the number of live cells. At the end, NK cytotoxicity is measured against K562 tumor cell in vitro. Besides being reliable and well correlated with CRA, it is easy to conduct, and sensitive; it is also possible to measure cytotoxicity in multiple small wells with different effector/target cell ratios. The disadvantage is that this test can only be performed with adherent cells (19).

Granzyme B ELISPOT Test

This test is based on the measurement of serine esterase level in cytotoxic cells of CD8+- CTL, and NK cell. Granzim B enzyme level increases with stimulation that occurs in the cytotoxic cell encountering with the

target cell. A special kit has also been developed for this method (20). It is reliable, correlated with CRA, and easy to conduct. On the other hand, it has a disadvantage of reflecting capacity of cytotoxic cell rather than an actual cytolysis.

NON-RADIOACTIVE FLUORESCENT MARKING ASSAYS

Due to inefficiencies or difficulties in radioactive and non-radioactive assays, new assays have been developed using non-radioactive fluorescent dye assays or target cell marking techniques with monoclonal antibodies and their utilization in flow cytometer.

General Properties of Flow Cytometric Assays

No standard or routine flow cytometer assay are available yet. However, a lot of clinical and research centers have developed their own methods and determined the validity of according to chromium release assay, which is considered as the golden standard.

Flow cytometer assays have the ability to perform single, double, and multi-colored methods (1). Singlecolor flow cytometer assays are based on light scattering characteristics of cell (Figure 1). Flow cytometer assay measure the target cell death (cytotoxicity) by staining the membrane or DNA of the target cell with different fluorescent dye or mAb.

Examples of Membrane Staining with Fluorescent Substances

Examples of cytotoxicity measurements based on membrane staining techniques with some fluorescent substances will be introduced here.

Fluorescent Bound to Lipophilic Membrane

F-18 (octadecylamine- fluorescein isothiocyanate), PKH-2, PKH-26, Europium (Eu3+), and MitoTracker Green (MTG) are fluorescent Lipophilic dyes that will bind to membranes are used to measure cytotoxicity. These are usually green and red fluorescent dyes that mark target and effector cells. Membrane fixation is needed for dyes such as PKH-26 and DiOC18 however, its effect on cytotoxicity during co-incubation is not clear (5,9,11,21-24). This technique is easy to use, sensitive, and reliable; but leakage of these dyes from cells is a significant disadvantage.

Carboxy-Fluorescein Diacetate (CFSE: carboxyfluorescein succinimidyl ester)

This assay is based on measurement of dye release by microflorimeter after membrane staining and cytolysis. A high rate of release leads to incorrect results in short-term experiments (25).

DNA Staining Assays with Fluorescent Substance

Aminoactinomycin (7-AAD), PI (propidium iodide), CAM (Calcein-AM: calcein acetoxymethyl ester), and TO-PRO-3 iodide etc. are substance that will stain DNA in target cell and reflect cell death. PI and PKH-26 are red fluorescent dyes whereas PKH-2, F-18, CAM, DIOC18 (D275) are green fluorescent dyes (21-27). First, 7-AAD and PI were used frequently in the beginning (25,26), but CAM has become more prominent recently. The disadvantage of this method is that leakage can occur. Advantages for this method are DNA staining assays with fluorescent substance are easy to apply, sensitive, and reliable. They are also successful especially in long-term (over 4 hours) cytotoxicity tests (27).

CAM (Calcein-AM: calcein acetoxymethyl ester)

It is a DNA dye used for effector and target cell staining. Spontaneous release after incubation and difficulty of loading are noteworthy, even at lower rates than others (27).

Red / Green Fluorescent Dyes

PI and PKH-26 of membrane and DNA markers are red fluorescent dyes. PKH-2, F-18, CAM, DIOC18 (D275) are known as green fluorescent dyes (21-27).

Monoclonal Antibody Marking of Target and Effector Cell in Flow Cytometry

The fluorescent monoclonal antibody (mAb) effector bound to membrane is used in the selection and marking of target cell and conjugation (adhesion of the effector cell to the target). The monoclonal antibodies used to mark the effectors are anti-CD2, anti-CD5, anti-CD7, anti-CD16, and anti-CD56 (8,9). The monoclonal antibodies used to mark the target cells are anti-CD19, anti-CD33, and anti-CD58. AnnexinV (AnnV) is used to measure apoptosis in the target cell; and 7- AAD or PI for necrosis (28-30).

We will briefly introduce our assay below as an example of assay using fluorescent mAb marking in a flow cytometry.

Our Assay: Flow Cytometric Cell-Mediated Cytotoxicity Assay

This is a three color flow cytometric assay. Target and effector cells are differentiated by specific fluorochrome-conjugated mAb staining. The anti-CD33-PE fluorochrome dye is used for K-562 target cell staining and anti-CD19-PE is used for Daudi or Raji cells (8).

During the development of this assay, anti-CD2-PE, anti-CD5-PE, anti-CD7-PE mAb were used for effector staining during development of this assay. Anti-CD2-PE was the most successful mAb. Annexin V (AnnV) / propium iodid (PI) is introduced to medium after co-incubation to measure target cell death (Figure 2,3). Absolute apoptotic/necrotic cell death counts are calculated with the help of fluorescent beads: fluorosphere numbers. Calibration and absolute number (not %, cumulative death) are measured with fluorosphere (8).

The main advantage of our assay is the lack of dye leakage problem and cell membrane fixation. Target cell and conjugation populations are selected and death is calculated directly by our assay. It is very effective in short time (≤4 hours) as well as long-term (≤24 hours) cytotoxicity measurements. Hence, this assay also measures early apoptosis and necrotic death, and correlates well with CRA (≥90%) (8,15,35). This assay can still be applied by changing effector or target cell, that is, using different effectors (mast cells) and target tumor cells (Daudi, Raji, U937, HLA, Meg-01 etc.) (31-33). It is open for multiparametric applications (different metabolic changes in cells other than death) at the same time in future. Another advantage is the fact that it can evaluate cumulative measurement rather than instant cross-sectional (34,35).

Target and effector cells are differentiated by specific fluorochrome-conjugated mAb staining. This method allows also for mast cells to be used as effectors instead of NK and LAK cells (6,31). If leukemia group tumor cells are used as target cells, anti-CD33-PE mAb is the most suitable antibody (Figure 2,3).

Starting from very low (½:1) E (effector):T (target cell) ratios, our assay was observed to give accurate and correlated results with CRA up to 128:1 or 256:1. We have also shown that cytotoxicity increases in parallel to increasing E:T ratios (8).

This assay also measured the cytotoxicity more successfully than other assays in short-term and long-term incubations of ½ to 18- 24 hours (36).

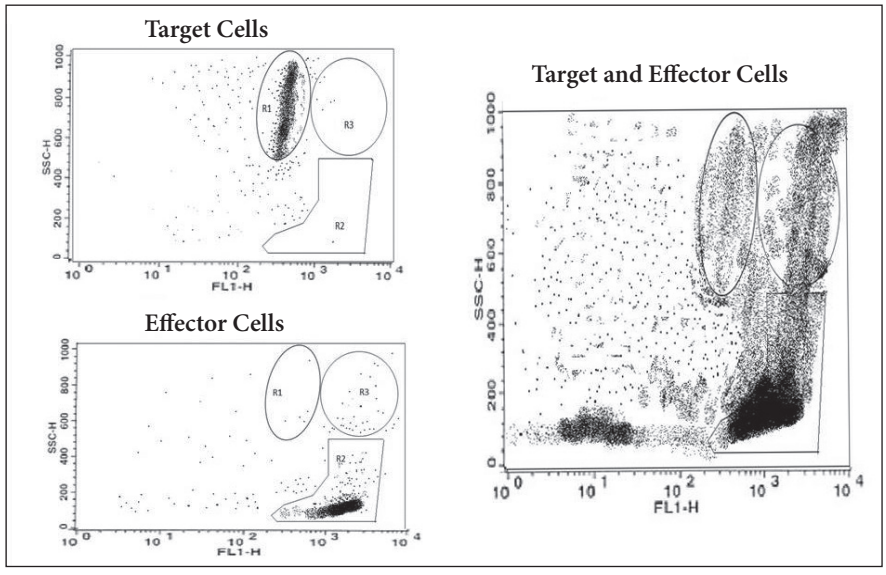

Figure 1. In the initial flow cytometric cell-mediated cytotoxicity studies, target and effector cells were distinguished by taking into account the light scattering characteristics of cells (such as SSC: granulation). The target cell K562 and the conjugate (effector bounds to target) population are located above the histogram and the effector peripheral blood lymphocyte is located below; clear distinction is observed. In the histogram; R1, R2, R3 fields are in the first, second and third regions. Representative distribution of target (R1) and effector (R2) cells (first alone then co-incubated), and conjugated (where the effector bounds to the target: R3 field) can be seen in histogram. In this method, cytotoxicity was calculated by measuring death in target cells after co-incubation and target cells in conjugate with fluorescent dyes such as PI, 7-AAD, AnnV in target cells in target cells and conjugate after co-incubation (adapted from reference 34).

In the cross-sectional 'percent cytotoxicity' calculation, the target cell death (cytotoxicity) is calculated. The rate of live cells in control is first found by subtracting the spontaneous death from the total number of cells in the control. It can be formulated as %cytotoxicity= [Control live cell (%)-Co-incubation live cell (%)]/ %Control live cell. In measurement of 'cumulative cytotoxicity', the above-mentioned ratios are calculated based on absolute numbers determined by fluorosphere. Most of the flow cytometer studies are insufficient to show cell concentration and absolute cell numbers. Fluorospheres (cell counting beads) have been developed to fill this gap (8,31).

Cytotoxicity Assay by Measuring Expression Change / Degranulation with Fluorescent Monoclonal Antibody in Effector Cell Membrane in Flow Cytometry

Effector cell in flow cytometry is also marked by lipophilic dye attaching to membrane or entering into the cell. Thus, the effector cell is also marked and separated from the target cell and death measurement can be made by gating to the target.

Effector cell is measured by the expression of degranulation molecules (LAMP1/ CD107a) and it is marked with anti-CD56 mAb. The effector NK, which responds to stimulation occurs after encountering with the target tumor cell, indicates the percentage of cells. However, there has been debate about whether this method actually reflects the cytolysis in the target cell correctly (37).

CD107a is considered to be a reliable marker of NK cell cytotoxicity, but may also be useful in detailed evaluation of NK cell functions. Although whole blood is used in the previously mentioned method, cell culture laboratories are not required as the cell line is not used. This test requires low amount of whole blood, minimal time and iis cost effective. Hence this test can be applied in routine clinic labs where flow cytometry is available (37).

Measurement of NK cell cytotoxicity is necessary when NK cell numbers and functions are impaired (classical or functional NK cell deficiencies). Genetic mutations that result in impaired or decreased NK cell and NK cell subgroup numbers are associated with more than 50 primary immunodeficiency diseases (38).

Lymphohistiocytosis type 2 disease, which is one of the immune regulation disorders can be diagnosed by adding intracellular perforin staining into our test. This method is also helpful in differentiation of type 3 and type 4 disease having defective granule release mechanism, Griscelli syndrome, and Chediak-Higashi syndrome from acquired lymphohistiocytosis disease (37).

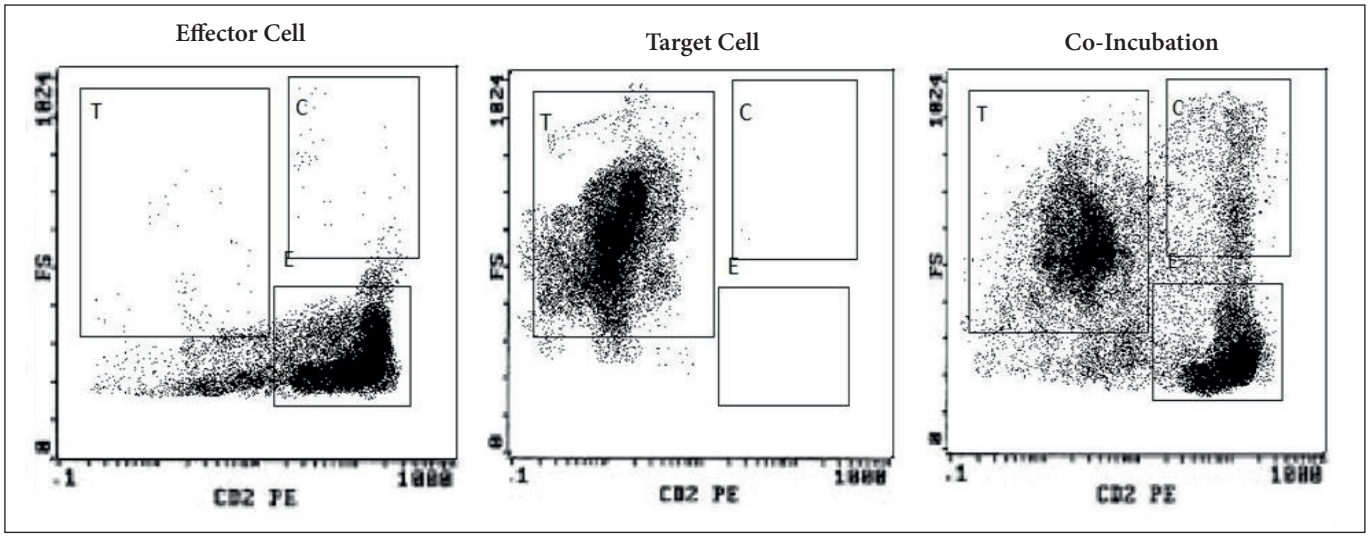

Figure 2. According to antibody bound and light scattering characteristic of fluorescent monoclonal anti-CD2 PE specific to target cell used in flow cytometry (FS: size); distinction of effector (E field), target cell (T field), and cells showing conjugation after the co-incubation (effector is bound to target) (C field) can be seen in histogram. The effector cells appear below, the target and conjugate cells appear above (adapted from reference 9).

Enzymatic (fluorogenic caspase substrate) Assay Measuring Apoptosis with Fluorescent Dye in Target Cell in Flow Cytometry

As mentioned above; membrane dye AnnV, which is bound to phosphatidylserine displacing outwardly in target cell membrane leading to apoptosis, is commonly used to measure apoptosis. Furthermore, an enzymatic method for measuring apoptosis in target cell has also been described. By this method; measurement of the apoptotic process beginning in CTO (cell tracker orange)-stained target cell after co-incubation with effector cell in C57BL/6 mice, is indicated by fluorescent staining (fluorogenic caspase substrate) indicating activation in the caspase enzyme. As is known, caspase is the first enzymatic (early apoptosis) component of pathway that induces apoptosis. It is claimed that this method measures apoptosis faster, is more sensitive, safer, and at a single cell level rather than population level (39). There are some disadvantages of this assay such as being triggered by various stimuli and questions about whether apoptosis starting with caspase will always result in death.

General Disadvantages of Flow Cytometric Assays

Fluorescent dyes may alter the sensitivity of target cell to cytotoxicity. Also most flow cytometric assays are based on the increase in membrane permeability of the target cell. This could cause artifacts due to leakage during incubation. Furthermore, fluorescent signal of some dyes are at low density, it may cause difficulty in detecting cytotoxicity (Table I) (3-5,11,15,22,23).

BRIEF COMPARISON OF CELL-MEDIATED CYTOTOXICITY ASSAYS

Although radioactive methods are still considered as the golden standard, advanced flow cytometric assays will become more utilized in the near future (Table I).

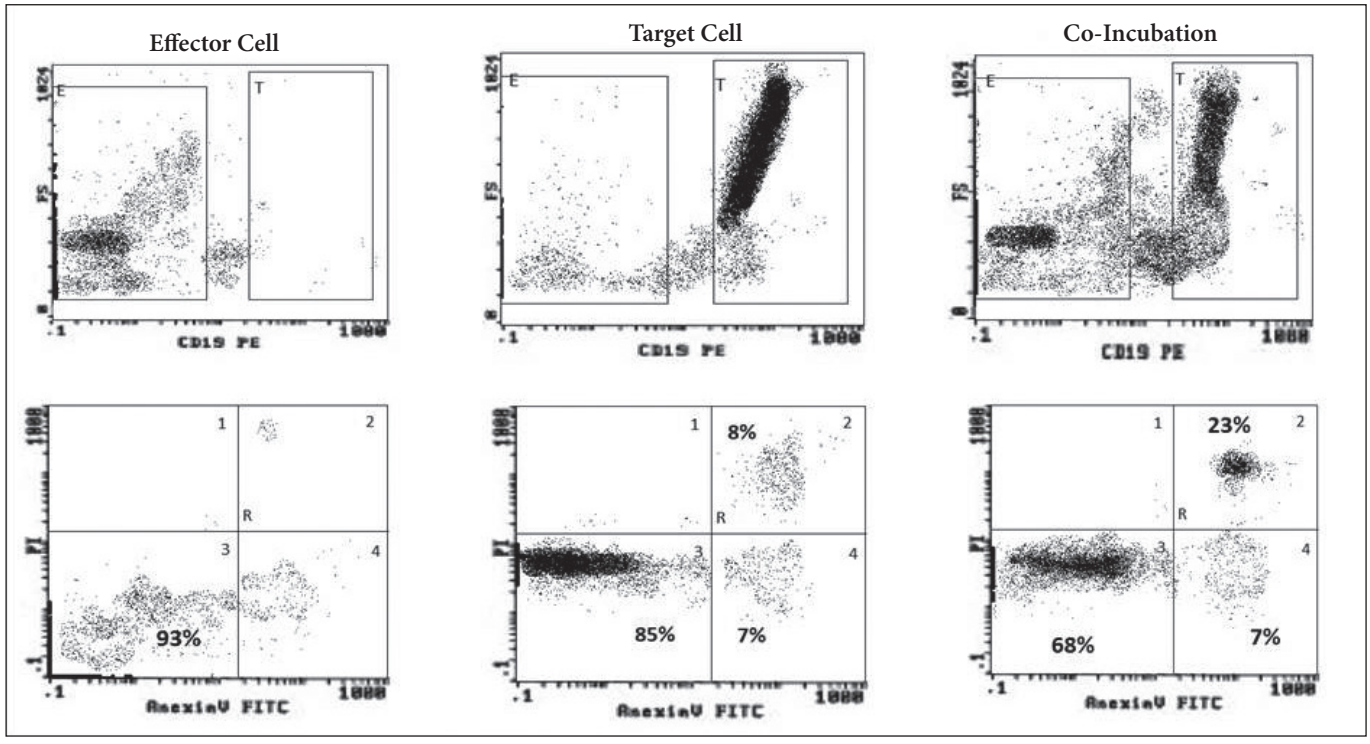

Figure 3. According to antibody bound and light scattering characteristic of fluorescent monoclonal anti-CD19 PE specific to target cell used in flow cytometry (FS: size); distinction of effector (E field), target cell (T field), and conjugation (effector is bound to target) cells can be seen in above histograms. Conjugating cells are collected in the target cell population (the first stage of co-incubation) and at the bottom with the progression of co-incubation. The first graphs below is gated to effector (E field), the second to target (T) population, and the third again to T population. In the histograms below; effector, target, and conjugated target cell death can be evaluated with annexin V/PI dyes. In the histograms below, R1 refers to necrotic (AnnV- / PI+), R2 refers to late apoptotic (AnnV+/ PI+), R4 refers to early apoptotic (AnnV+ / PI-), and R3 refers to live cells (AnnV- / PI-). It is inferred that liveliness in the target cell after co-incubation decreased from 93% to 68%. (Adapted from reference 9).

Table I. Comparison of cell (NK) - mediated cytotoxicity assays.

Assays	Advantages	Disadvantages	References
I. Radioactive			
CRA	Golden standard	Radioisotope	5,12,13
	Simple test	Expensive and short half-life	
	Repeatable results	Spontaneous release	
	Measures necrosis	Requires large and many cells	
JAM	Measures apoptosis	Radioactive	14,15
	Cheap		
	Easy, sensitive, and reliable		
II. Non-radioactive Enzymatic assays			
LDH, MTT, Granzyme B	Correlated, easy, sensitive	Reuptake (LDH) Adherent cell (MTT) Indirect measurement (Granzyme)	16-20
III. Non-radioactive Fluorescent Dyes			21-27
DiOC18, PKH, CAM	Easy, sensitive, and reliable	Membrane fixation	21-27
		Leakage	
Fluorogenic caspase	Faster, more sensitive and reliable	Does it reflect cell death?	38
	Measurement at single cell level	The effect of different stimuli?	
FCM- mAb	Single cell level	Antibody specificity	28-30
	Conjugation	Dead cell marking difficulty	
	Easy and direct measurement	Low fluorescent signal	
	Multi-parametric		
FCM- CMCA (mAb)	Apoptosis-Necrosis measurement	Dead cell marking difficulty	6,8,9,31-33
	Long-short term		
	Well correlated		
	Cumulative cytotoxicity		
Degranulation (mAb)	Easy	Indirect measurement (real death?)	37
		The effect of different stimuli?	

NK: Natural killer, **CRA**: Chromium Release Assay, **mAb:** Monoclonal antibody, **FCM:** Flow cytometric, **CMCA**: Cell-mediated cytotoxicity assay

Fluorescent mAbs markers are mostly used for target and effector cells because staining with these mAb occurs after-incubation prohibiting potential false positive results on cytotoxicity due to membrane fixation (3-5,8,9,11,15,22,23). Table 1 presents comparison of all assays within/to each other.

FUTURE EXPECTATIONS: MULTI-COLOR FLOW CYTOMETRIC ASSAY, MULTIPARAMETRIC ASSAYS AND IMAGING

Today's flow cytometric assays have the opportunity to clearly distinct between effector and target cells by using different mAb. For example effector cells can be labeled with mAB for CD3-CD16/56+ and target cell with fluorescent dye such as CTO with light scattering characteristics such as FCS/SSC leading to cytotoxicity measurements in target cell with 7-AAD. Using large number of dyes simultaneously and own characteristics of cells, cells can be differentiated and evaluated (25,35).

Besides conduction of multiparametric evaluation of cells by using multi-purpose staining in flow cytometric assays, conjugated cells in addition to effector and target cells can be imaged clearly at single cell level (40,41). Moreover, assays measuring cell-mediated cytotoxicity in flow cytometry are being used and developed (accelerated) for other T-cells (γδ T cell) than NK or CTL (42,43).

Although clinical application area of these assays is not common today; the flow cytometric assays, in which multicolor and multivariate measurements can be

performed at the same time, will gain importance in fields such as cancer immunotherapy (such as new clinical trials and measurement of immune response to cancer vaccines) or immune system diseases with impaired cytotoxicity in the near future.

CONCLUSION

Although radioactive assays measuring cytotoxicity do not lose their golden standard characteristics, flow cytometric assays using target and effector cell marker mAbs are prominent today.

REFERENCES

1. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: Differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med 1998;188(12):2375-80.
2. Sieni E, Cetica V, Mastrodicasa E, Pende D, Moretta L, Griffiths G, et al. Familial hemophagocytic lymphohistiocytosis: A model for understanding the human machinery of cellular cytotoxicity. Cell Mol Life Sci 2012;69(1):29-40.
3. Suck G, Branch DR, Smyth MJ, Miller RG, Vergidis J, Fahim S, et al. KHYG-1, a model for the study of enhanced natural killer cell cytotoxicity. Exp Hematol 2005;33(10):1160-71.
4. Valiathan R, Lewis JE, Melillo AB, Leonard S, Ali KH, Asthana D. Evaluation of a flow cytometry-based assay for natural killer cell activity in clinical settings. Scand J Immunol 2012;75(4):455-62.
5. Goldberg JE, Sherwood SW, Clayberger C. A novel method for measuring CTL and NK cell-mediated cytotoxicity using annexin V and two-color flow cytometry. J Immunol Methods 1999; 224(1-2):1-9.
6. Ozdemir O, Savaşan S. Combinational IL-2/IL-15 induction does not further enhance IL-15-induced lymphokine-activated killer cell cytotoxicity against human leukemia/lymphoma cells. Clin Immunol 2005;115(3):240-9.
7. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. Expert Rev Vaccines 2010;9(6):601-16.
8. Kasatori N, Ishikawa F, Ueyama M, Urayama T. A differential assay of NK-cell-mediated cytotoxicity in K562 cells revealing three sequential membrane impairment steps using three-color flow-cytometry. J Immunol Methods 2005;307(1-2):41-53.
9. Ozdemir O, Ravindranath Y, Savaşan S. 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. Cytometry A 2003;56(1):53-60.
10. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; Inhibition by isoantibody and by drugs. Immunology 1968;14(2):181-96.
11. King MA. Detection of dead cells and measurement of cell killing by flow cytometry. J Immunol Methods 2000;243:155-66.
12. Roder JC, Haliotis T, Klein M, Korec S, Jett JR, Ortaldo J, et al. A new immunodeficiency disorder in humans involving NK cells. Nature 1980;284(5756):553-5.
13. Morales A, Ottenhof PC. Clinical application of a whole blood assay for human natural killer (NK) cell activity. Cancer 1983;52(4):667-70.
14. Matzinger P. The JAM test: A simple assay for DNA fragmentation and cell death. J Immunol Methods 1991;145:185-92.
15. Langhans B, Ahrendt M, Nattermann J, Sauerbruch T, Spengler U. Comparative study of NK cell-mediated cytotoxicity using radioactive and flow cytometric cytotoxicity assays. J Immunol Methods 2005;306(1-2):161-8.
16. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983;64(3):313-20.
17. Weidmann E, Brieger J, Jahn B, Hoelzer D, Bergmann L, Mitrou PS. Lactate dehydrogenase-release assay: A reliable, nonradioactive technique for analysis of cytotoxic lymphocyte-mediated lytic activity against blasts from acute myelocytic leukemia. Ann Hematol 1995;70(3):153-8.
18. van de Loosdrecht AA, Nennie E, Ossenkoppele GJ, Beelen RH, Langenhuijsen MM. Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay. A methodological study. J Immunol Methods 1991;141(1):15-22.
19. van de Loosdrecht AA, Beelen RH, Ossenkoppele GJ, Broekhoven MG, Langenhuijsen MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J Immunol Methods 1994;174(1-2):311-20.
20. Ewen C, Kane KP, Shostak I, Griebel PJ, Bertram EM, Watts TH, et al. A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for Granzyme B. J Immunol Methods 2003;276(1-2):89-101.
21. Ozdemir O. Evaluation of human mast cell-mediated cytotoxicity by DIOC18 target cell labeling in flow cytometry. J Immunol Methods 2007;319(1-2):98-103.
22. Fischer K, Mackensen A. The flow cytometric PKH-26 assay for the determination of T-cell mediated cytotoxic activity. Methods 2003;31(2):135-42.
23. Hopkinson K, Williams EA, Fairburn B, Forster S, Flower DJ, Saxton JM, et al. A MitoTracker Green-based flow cytometric assay for natural killer cell activity: Variability, the influence of platelets and a comparison of analytical approaches. Exp Hematol 2007;35(3):350-7.

24. von Zons P, Crowley-Nowick P, Friberg D, Bell M, Koldovsky U, Whiteside TL. Comparison of europium and chromium release assays: Cytotoxicity in healthy individuals and patients with cervical carcinoma. Clin Diagn Lab Immunol 1997;4(2):202-7.
25. Lecoeur H, Février M, Garcia S, Rivière Y, Gougeon ML. A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. J Immunol Methods 2001;253(1-2):177-87.
26. Lee-MacAry AE, Ross EL, Davies D, Laylor R, Honeychurch J, Glennie MJ, et al. Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. J Immunol Methods 2001;252(1-2):83-92.
27. Jang YY, Cho D, Kim SK, Shin DJ, Park MH, Lee JJ, et al. An improved flow cytometry-based natural killer cytotoxicity assay involving calcein AM staining of effector cells. Ann Clin Lab Sci 2012;42(1):42-9.
28. Thakur A, Zaman A, Hummel J, Jones K, Hortelano G. Single-colour flow cytometric assay to determine NK cell-mediated cytotoxicity and viability against non-adherent human tumor cells. Biotechnol Lett 2012;34(3):447-53.
29. Cholujová D, Jakubíková J, Kubes M, Arendacká B, Sapák M, Ihnatko R, et al. Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays. Immunobiology 2008;213(8):629-40.
30. Zimmermann SY, Esser R, Rohrbach E, Klingebiel T, Koehl U. A novel four-colour flow cytometric assay to determine natural killer cell or T-cell-mediated cellular cytotoxicity against leukaemic cells in peripheral or bone marrow specimens containing greater than 20% of normal cells. J Immunol Methods 2005;296(1-2):63-76.
31. Ozdemir O. Flow cytometric mast cell-mediated cytotoxicity assay: A three-color flow cytometric approach using monoclonal antibody staining with annexin V/propidium iodide co-labeling to assess human mast cell-mediated cytotoxicity by fluorosphere-adjusted counts. J Immunol Methods 2011;365(1-2):166-73.
32. Savaşan S, Buck S, Ozdemir O, Hamre M, Asselin B, Pullen J, et al. Evaluation of cytotoxicity by flow cytometric drug sensitivity assay in childhood T-cell acute lymphoblastic leukemia. Leuk Lymphoma 2005;46(6):833-40.
33. Ozdemir O. Flow cytometric cell-mediated cytotoxicity assay. J Immunol Methods 2007;318(1-2):158-9; author reply 160-1.
34. Godoy-Ramirez K, Franck K, Gaines H. A novel method for the simultaneous assessment of natural killer cell conjugate formation and cytotoxicity at the single-cell level by multi-parameter flow cytometry. J Immunol Methods 2000;239(1-2): 35-44.
35. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: Comparisons to a 4h 51Cr-release assay. J Immunol Methods 2007;325(1-2):51-66.
36. Johann S, Blümel G, Lipp M, Förster R. A versatile flow cytometry-based assay for the determination of short- and long-term natural killer cell activity. J Immunol Methods 1995;185(2):209-16.
37. Shabrish S, Gupta M, Madkaikar M. A modified NK cell degranulation assay applicable for routine evaluation of NK cell function. J Immunol Res 2016;2016:3769590.
38. Mace EM. Requirements for human natural killer cell development informed by primary immunodeficiency. Curr Opin Allergy Clin Immunol 2016;16(6):541-8.
39. Chahroudi A, Silvestri G, Feinberg MB. Measuring T cell-mediated cytotoxicity using fluorogenic caspase substrates. Methods 2003;31(2):120-6.
40. La Muraglia GM, O'Neil MJ, Madariaga ML, Michel SG, Mordecai KS, Allan JS, et al. A novel approach to measuring cell-mediated lympholysis using quantitative flow and imaging cytometry. J Immunol Methods 2015;427: 85-93.
41. Somanchi SS, McCulley KJ, Somanchi A, Chan LL, Lee DA. A novel method for assessment of natural killer cell cytotoxicity using image cytometry. PLoS One 2015;10(10):e0141074.
42. Jin Q, Jiang L, Chen Q, Li X, Xu Y, Sun X, Zhao Z, Wei L. Rapid flow cytometry-based assay for the evaluation of γδ T cell-mediated cytotoxicity. Mol Med Rep 2018;17(3): 3555-62.
43. Kandarian F, Sunga GM, Arango-Saenz D, Rossetti M. A flow cytometry-based cytotoxicity assay for the assessment of human NK cell activity. J Vis Exp 2017;(126).