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Journal of Immunological Methods 300 (2005) 100–114

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

FL-CTL assay: Fluorolysometric determination of cell-mediated cytotoxicity using green fluorescent protein and red fluorescent protein expressing target cells

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Received 9 November 2004; received in revised form 24 February 2005; accepted 28 February 2005
Available online 2 May 2005

Abstract

Cytotoxic T lymphocytes (CTLs) are crucial effectors against intracellular pathogens and cancer. Accurate and efficient assessment of CTL activity is important for basic and clinical studies. Widely used CTL assays, including the chromium release, JAM test and ELISPOT, involve either radioisotopes or lengthy procedures. Here, we developed a new fluorolysometric CTL assay based on cell-mediated cytolysis of fluorescent protein (GFP or DsRed) expressing cells quantified by one of the fluoro-based methods: flow cytometry, fluorescence microplate reader, or fluorescence microscopy. With flexible detection methods and lentiviral vector transduced stable lines of either GFP^+ or $DsRed^+$ cells as targets for antigen presentation and equal number of the other as internal reference for consistency and accuracy, this assay is easy to perform and to scale-up for simultaneous multi-sample analyses. Using two different antigen systems, we demonstrated that this assay is very sensitive to determine primary CTL activity of both in vitro and in vivo primed antigen-specific T cells. Thus, this FL-CTL assay is highly sensitive, reliable, reproducible, economical, convenience and supports broad applications compared to conventional CTL assays.

Keywords: Cytotoxic T lymphocytes; CTL assay; Antigen-specific CTL function; Fluorometric-based CTL; GFP; Fluorescent proteins

1. Introduction

Cytotoxic T lymphocytes (CTL) are important effectors in host immune responses to tumors, intracellular pathogens and transplant rejection. This cytotoxicity is based on cell-surface antigen recognition and mediated through either release of perforin and

Abbreviations: CTL, cytotoxic T lymphocytes; FL-CTL, fluorolysometric cytolysis assay; GFP, green fluorescent protein; FACS, fluorescent activated cell sorting; ELISPOT, enzyme-linked immunospot assay; HA, hemagglutinin; TCR, T cell receptor; Tg, transgenic; NT, non-transgenic.

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doi:10.1016/j.jim.2005.02.010

granzyme containing cytolytic granules or engagement of cell-surface death receptors (Barry and Bleackley, 2002; Lieberman, 2003). Efficient and accurate evaluation of CTL function with a highly sensitive and convenient assay is important not only in clinical assessment of immune dysfunction, but also for the development and evaluation of therapeutic efficacy of cancer immunotherapy, viral infection and immune suppressive regimens to minimize transplant rejection.

Currently, several assay systems are widely used to evaluate CTL activity based on direct measurement of target cell killing or other indirect parameters. The chromium (^{51}Cr) release assay, which directly measures cytolytic activity as ^{51}Cr radioactivity released from killed target cells, is the most widely used CTL assay since its development (Brunner et al., 1968). Likewise, the JAM test directly evaluates CTL killing by measuring reduction in radioactivity of ^{3}H-thymidine pre-incorporated in genomic DNA from remaining target cells not eliminated by cytotoxic T cells (Matzinger, 1991). Both assays measure the total radioactivity released from target cells and sometimes may have limited sensitivity for determining CTL activity of in vivo activated cells. Under physiological conditions, antigen-specific CTL effector frequency of most immune responses is so low that the CTL activity often cannot be reliably measured via these conventional methods without further in vitro stimulation. Therefore, considerable efforts have been made to improve the sensitivity of CTL assays and/or determine CTL function at the single-cell level (Carter and Swain, 1997; Ewen et al., 2003). Towards this end, the ELISPOT (enzyme-linked immunospot) assay and intracellular cytokine staining have been developed to enumerate interferon-γ (or other cytokine) producing cells as estimates of CTL effector frequency (Miyahira et al., 1995; Carter and Swain, 1997). However, these assays often involve lengthy procedures and more importantly these cytokine producing cells may not truthfully represent cells with immediate cytolytic function (CTL effector), as recently demonstrated (Snyder et al., 2003). Likewise, development in tetramer technology significantly improves our capability of detecting and enumerating antigen-specific cytotoxic T lymphocytes, which, however, does not provide function determination (Altman et al., 1996).

Lately, a few flow cytometry (FACS)-based systems have been explored to directly evaluate antigen-specific cytolysis using target cells labeled with fluorescent dye or protein, or incorporated with fluorogenic caspase substrates (Papadopoulos et al., 1994; Lecoeur et al., 2001; Hoppner et al., 2002; Kienzle et al., 2002; Betts et al., 2003; Chahroudi et al., 2003; Hermans et al., 2004). The CTL activity was determined as decrease in viable fluorophore labeled cells or increase in caspase substrate positive cells, respectively. While these assays have been proven to be efficient in detecting cytolytic killing at the single-cell level, their major limitation on accurate determination of CTL activity is that they heavily relay on accurate enumeration of total apoptotic target cells of various stages and forms, which can be complicated especially with the extended duration of CTL assays. Since FACS is more appropriate in determining relative percentages of specific populations, total acquisition and accurate enumeration of absolute numbers of viable or apoptotic cells by FACS, in the absence of an internal control (reference), would be cumbersome. In fact, this issue was addressed in some of the recent modifications via introduction of the same number of distinct fluorescent dye labeled particles or cells to the target cells, which ideally would be of similar properties to the target cells (Estcourt et al., 2002; Kienzle et al., 2002). Thus, an optimal means of standardizing FACS-based CTL assay with convenience and reproducibility, especially in industry and clinical scale-up setting, is to have two easily accessible and similar cell populations (only different in fluorescent spectra) for targets and references to eliminate the needs and variability of fluorophore labeling each time before use. Furthermore, FACS-based analysis may not be generally accessible by all the laboratories, and more importantly, this operation often limits the number of samples to be analyzed. It is, thus, of major advancement if a CTL assay, based on the same principle, can be easily determined through other methods capable of simultaneous multi-sample analyses without additional and tedious preparation procedures.

In this study, we took advantage of efficient and long-term transgene delivery of lentiviral vectors to a wide variety of cells and established two P815 cell lines stably expressing green fluorescent protein (GFP) or red fluorescent protein (DsRed) distinguish-