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Journal of Immunological Methods 304 (2005) 43–59

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

A sensitive flow cytometry-based cytotoxic T-lymphocyte assay through detection of cleaved caspase 3 in target cells

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Received 24 January 2005; received in revised form 3 June 2005; accepted 8 June 2005
Available online 1 July 2005

Abstract

We describe a highly sensitive flow cytometry-based CTL assay using the cleavage of caspase 3 in target cells as a readout. The assay involved labeling of cells with a cell tracker dye and staining permeabilized cells with an antibody recognizing cleaved caspase 3. The assay proved to be robust and reliable in measuring antigen-specific CTL activity in a number of human and murine systems, including MLR, human peptide-specific T-cell responses induced in vitro, and CTL responses following immunization of mice with viral and peptide vaccines. The assay was found to yield comparable results as ^{51}Cr-release, but with markedly higher sensitivity. When compared to detection of antigen-specific T cells via HLA tetramer/pentamer-based methods of T-cell staining in HIV gag peptide-specific human T cell lines the caspase 3 cleavage readout assay exhibited a comparable level of sensitivity with detection of CTL function at antigen-specific T-cell frequencies of 1:15,000 or lower. A similar level of sensitivity was obtained when murine CTL assays were performed with MLR in which effector cells were highly diluted with naïve syngeneic spleen cells. Our results indicate that the caspase 3 cleavage assay may be a powerful tool to measure antigen-specific CTL responses in human vaccine trials and in pre-clinical animal models of CTL function at both high and low effector cell frequencies.

Keywords: Cytotoxicity assay; CTL; Caspase 3; Granzyme B; Apoptosis; Immune monitoring

Abbreviations: CTL, cytotoxic T-lymphocyte; CMA, Concanamycin A; DDAO-SE, CellTrace™ Far Red DDAO-succinimidyl ester; ELISPOT, enzyme-linked immunospot; FACS, fluorescence-activated cell sorting; MLR, mixed lymphocyte response; 7-AAD, 7-aminoactinomycin D.

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doi:10.1016/j.jim.2005.06.005

1. Introduction

The measurement of ^{51}Cr-release from lysed target cells has been the gold standard to measure the cytotoxic function of activated CD8$^+$ T cells and NK cells for over 3 decades (Boyle, 1968; Thorn et al., 1974). However, overall the ^{51}Cr-release assay is rather insensitive and is also plagued with a number of safety and environmental limitations. In addition, the membrane leakage events measured by ^{51}Cr-release and other "release" methods may only reflect the action of perforin, especially at high effector to target ratios and not the true apoptotic nature of target cell death in vivo involving granzymes (Bashford et al., 1988; Shiver and Henkart, 1991). Newer methods at enumerating antigen-specific CD8$^+$ T-cells, such as intracellular cytokine staining, the ELISPOT assay, and staining for T-cells using HLA/MHC class I tetramers have been introduced as substitutes, but none of these approaches measures the ultimate cytolytic function of CTL and NK cells as ^{51}chromium-release. Another approach for the measurement of CTL activity is using flow cytometry (FACS). A number of FACS-based and fluorimeter-based assays have been developed that mostly involve the detection of target cell lysis using the release of pre-loaded dyes or uptake of external dyes much like ^{51}Cr-release (Mattis et al., 1997; Roden et al., 1999; Sheehy et al., 2001; Fischer et al., 2002; Kienzle et al., 2002; Betts et al., 2003; Hermans et al., 2004). Recently, the application of FACS to monitor products of apoptosis generated in CTL target cells, such as monitoring of Annexin V binding and the detection of caspase activity and caspase cleavage, have been introduced as an alternative to the membranolytic-based detection methods (Fischer et al., 2002; Jerome et al., 2003a,b).

The induction of apoptosis before the onset of membrane leakage is one of the hallmarks of the target cell killing. The role of Granzyme B, and more recently Granzyme A, in cleaving a number of proteins such as caspases and other downstream mediators of apoptosis in target cells has been well established (Pham and Ley, 1997; Lieberman, 2003; Trapani and Sutton, 2003). Caspases are key enzymes regulating the apoptotic program leading to DNA fragmentation in all mammalian cells. Although experiments using caspase inhibitors and cytoplasts have shown that caspase 3 and other caspases are not always needed for target cell death (Henkart et al., 1997; Lieberman, 2003), the cleavage of caspase 3 as a direct consequence of the insertion of perforin and granzymes into CTL target cells can nevertheless be used as a marker for antigen-specific CTL activity. Thus, the detection of caspase 3 cleavage and other early products of apoptosis (e.g., phosphorylated histones) in target cells using FACS may serve as useful alternative assay to ^{51}Chromium (Cr)-release and methods measuring target cell membrane lysis. The caspase 3 cleavage assay has been proposed as an alternative CTL assay system recently (Jerome et al., 2003a,b). However, a comprehensive study on the versatility of the assay in different CTL/target cell combinations, its applicability to different human and murine systems, the sensitivity of the assay, and how it performs relative to other cell-mediated immune response assays such as IFN-γ ELISPOT, HLA/MHC tetramer/pentamer staining, and other FACS-based methods has not been done.

In the work presented here we optimized a FACS-based CTL assay using caspase 3 cleavage as a read-out and tested its applicability to a wide variety of both human and murine systems immune responses. We also determined its performance in monitoring CD8$^+$ T cell responses in comparison to ^{51}Cr-release, IFN-γ ELISPOT, and HLA tetramer/pentamer staining in human and murine systems. In addition, we studied the sensitivity limits of the assay in monitoring rare antigen-specific CD8$^+$ effector cells in highly diluted T-cell populations. Overall, the assay proved to be highly sensitive, reproducible, and applicable to a variety of CTL effector cell situations with a variety of different target cell types. The assay showed the same degree of specificity and precision as ELISPOT and ^{51}Cr-release. Moreover, its sensitivity to enumerate antigen-specific T-cell activity in highly diluted samples of effector cells was comparable to HLA tetramer/pentamer-based methods.

2. Materials and methods

2.1. Reagents and cell culture media

Anti-cleaved caspase 3 (reactive against both human and mouse forms) was phycoerythrin (PE)-labeled and purchased from BD Biosciences (Mississauga, ON).