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Research paper

Statistical determination of threshold for cellular division in the CFSE-labeling assay

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Abstract

The combination of flow cytometry and carboxyl fluorescent succinimidyl ester (CFSE) labeling techniques has been widely used in the study of cellular proliferation, including measurement of the percentage of proliferated cells and the number of cell divisions undergone by proliferated cells. However, the smallest numbers that represent true cell division rather than experimental variation are not known. To define a threshold that separates true proliferation from experimental variation, we performed a large number of replicate CFSE labeling experiments using polyclonal stimulation, obtained the percentages of proliferated cells using ModFit software, and then analyzed these data using several statistical methods. Our results indicate that the threshold of proliferation lies between 0.071% (95% confidence) and 0.114% (99% confidence) of total CFSE-labeled cells under our laboratory conditions. We offer our methods presented here for other investigators to calculate a threshold in their own CFSE-labeling experiments.

Keywords: Threshold; Cell proliferation; Cell division; Flow Cytometry (FACS); CFSE

1. Introduction

For quantitative assessment of immune responses, the carboxyl fluorescent succinimidyl ester (CFSE) labeling assay has been used in recent studies to track cellular divisions in mice and humans (Gett and Hodgkin, 2000; Champagne et al., 2001; Lyons et al., 2001; Migueles et al., 2002; Wherry et al., 2003; Lichterfeld et al., 2004). However, each research group analyzes their data differently, thus making precise comparison of published results difficult. In the current study, we present some data and analytic interpretation of our data in the hope for standardizing data analysis process.

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In the CFSE assay, cells are labeled with CFSE in conjunction with other antibodies to various cell surface or intracellular markers. A control sample of cells is set aside without any stimulation, while the remaining samples are stimulated for proliferation. As CFSE labeled cells divide, the fluorescent CFSE is equally partitioned between daughter cells, and thus the fluorescence intensity halves. Once the CFSE labeled cells are run through a flow cytometer (FACS), computer collects data in standard FACS format (FCS files), in which the fluorescent intensity of CFSE and labeling antibodies are recorded for each cell (Seamer et al., 1997; Givan et al., 1999). With the combination of CFSE labeling and FACS techniques, divided cells can be easily enumerated, and the phenotypes of proliferated cells can be identified and characterized by cell surface or intracellular markers. Furthermore, the CFSE intensity can be quantified, and the histogram of the CFSE intensity provides profile for cell divisions. CFSE labeling assay has several advantages over the traditional ^{3}H-thymidine incorporation assay: (1) It provides information on the phenotypes of cells; (2) It is less labor intensive; and (3) It is safer, without involvement of radioactive materials (Wells et al., 1997; Hasbold et al., 1999; Givan et al., 1999; Gett and Hodgkin, 2000).

The importance of quantitative investigation of T cell proliferation using CFSE labeling and FACS techniques has been highlighted in several recent studies. In HIV-infected subjects whose viremia levels are controlled in the absence of antiviral therapy, HIV-specific CD8+ T cells have normal capacity to proliferate and synthesize perforin. In contrast, CD8+ T cells proliferate poorly, and do not synthesize perforin in patients whose HIV viremia is not controlled (Champagne et al., 2001; Migueles et al., 2002; Appay et al., 2002). Consistent with the human studies, in the murine lymphocytic choriomeningitis virus (LCMV) infection model, central memory T cells (T_{CM}) are more capable than effector memory T cells (T_{EM}) in controlling LCMV replication in vivo, and they have a greater capacity to proliferate upon peptide stimulation in vitro (Wherry et al., 2003, 2004). Other recent studies in HIV-infected subjects have suggested that defective CD4 helper T cell responses may be partly responsible for the diminished proliferation of HIV-specific CD8+ T cells (Younes et al., 2003; Papagno et al., 2004; Lichterfeld et al., 2004). Collectively, these data indicate that proliferation potential may be one of the most important functional properties of antigen-specific T cells, and a surrogate of antiviral activity.

Several computer programs, including FlowJo (Tree Start, Inc.), ModFit LT (Verity Software House, Inc), and CellQuest (Becton Dickinson) are currently available for analyzing cellular proliferation in CFSE labeling experiments. These programs generate histograms of fluorescence intensity, and apply deconvolution algorithms (Givan et al., 1999) to determine the proportion of proliferating cells at each cell division. It is straightforward to determine the proportion of proliferating cells based on the difference between control and stimulated cells; however, it is practically difficult to determine the number of cell divisions if the proportions of dividing cells are small. One approach would be performing a statistical test to compare replicates of stimulated cell cultures with that of unstimulated cell cultures as we will show later in this paper. The other approach would be determining the smallest percentage that constitutes a true cell division rather than experimental variation. To define a threshold separating a small proportion of cells that had CFSE dilution as a result of proliferation from that due to experimental variation, we have performed repeated experiments to study cell proliferation using the CFSE labeling technique. We then demonstrate how to determine the threshold value using statistical methods, including hypothesis testing and model fitting procedures.

2. Materials and methods

2.1. Source of cells

Heparinized blood samples were obtained from two anonymous healthy human volunteers (New York Blood Center, New York). Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll/Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation and were frozen at −150 °C until use. Human subject study regulations and guidelines were strictly followed in all studies.

2.2. CFSE labeling assay

PBMCs were quickly thawed in a 37 °C water bath, washed twice with RPMI 1640 (GIBCO) medium containing 10% fetal calf serum, penicillin (100U/ml) and streptomycin (100U/ml), and resuspended into RPMI-1640 medium at a concentration of 1×10^{7} cells/ml. Carboxyl fluorescein succinimidyl ester (CFSE) (Molecular Probes, Inc., Eugene, Oregon) was added to the cell suspension at the final concentration of 12 μM and incubated for 10 min at room temperature in the dark. Labeling was