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Technical note

A novel fluorescent sensitive assay for detection of differential T cell mediated lysis of multiple adherent target cells

Antony P. Black *, Louise Jones, Michael Ardern-Jones, Graham S. Ogg

Cutaneous Immunology Group, MRC Human Immunology Unit, University of Oxford, Weatherall Institute of Molecular Medicine, Oxford OX3 9DS, UK

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Abstract

There are few studies that have investigated T cell mediated lysis of adherent cells. We have developed a novel, rapid and sensitive fluorescent dye-swap assay that allows efficient detection of adherent target cell lysis. The assay allows simultaneous use of multiple differentially sensitised targets and facilitates concomitant surface or intracellular effector cell phenotypic analysis.

Keywords: Killing; Cytotoxicity; T cell; Adherent

1. Introduction

Cytotoxic T cells (CTL) are a major component of the adaptive immune system. Their ability to selectively lyse target cells presenting foreign antigen protects against intracellular pathogens such as bacteria, protozoa and viruses in addition to having a role in tumour and allogenic graft rejection (Zinkernagel, 1996). A number of assays have been used to assess the cytolytic activity of CTL, including release of chromium from labelled targets (Brunner et al., 1968), detection of caspase 3 induction (Liu et al., 2002) and a flow cytometry based assay for measuring loss of fluorescently labelled targets (Sheehy et al., 2001). These valuable approaches use suspensions of target cells, which if the targets are an adherent cell type, require trypsinisation or calcium chelation prior to use. Whilst in many settings such treatment may not alter the presenting capacity of adherent targets cells, we wished to use a technique for which we could exclude such potential artefact. However, there have been surprisingly few investigations that have used adherent cells as CTL targets. Several studies have used adherent target cell detachment as a marker for interaction with T cells, but target cell lysis was dissociated from target cell detachment (Russell et al., 1988; Abrams and Russell, 1991; Wang et al., 2004) suggesting that whilst the two processes are linked they are nevertheless distinct events. We therefore wished to develop a reproducible sensitive quantitative assay of adherent cell death by T cells, with the capacity to investigate differential killing of multiple targets at the same time.

2. Methods

2.1. Effector cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque

* Corresponding author. Tel.: +44 1865 222334; fax: +44 1865 222502.
E-mail address: ablack@hammer.imm.ox.ac.uk (A.P. Black).

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density gradient centrifugation. All samples were taken from consenting healthy adults under ethical approval from the Oxfordshire Ethics Committee. Cells were suspended in RPMI 1640 plus 10% human AB serum for generation of specific CD8+ T cell lines. The PBMC were incubated with 100 μM EBV BMLF1 280-8 GLCTLVAML peptide which was synthesized using standard Fmoc chemistry. Peptide purity was established by HPLC and the individual peptides were dissolved at 10 mg/ml in DMSO and stored at −20 °C. PBMC were then incubated for 10 days with IL-2 added on day 3 and 7 at a concentration of 100 U/ml. All cell lines were routinely maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin plus 10% human serum at 37 °C, in 5% CO_2.

2.2. HLA peptide tetrameric complexes

Complexes were synthesized as previously described (Altman et al., 1996; Ogg et al., 1998). Purified HLA heavy chain was modified by deletion of the transmembrane/cytosolic domain and C-terminal addition of a sequence containing the BirA enzymatic biotinylation site. Heavy chain, β2 microglobulin and peptide were refolded by dilution. The 45-kDa refolded product was isolated by gel filtration, and then biotinylated by BirA (Avidity, CO) in the presence of biotin (Sigma), ATP (Sigma) and Mg^{2+} (Sigma). Streptavidin–phycoerythrin conjugate (Sigma) was added in a 1:4 molar ratio and the tetrameric product was concentrated to 1 mg/ml.

2.3. Flow cytometry

Two colour flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson) with CellQuest software (Becton Dickinson). 10^6 PBMC were centrifuged at 300 g for 5 min and resuspended in a volume of 50 μl. Tetrameric complex was added and incubated at 37 °C for 20 min. Directly conjugated anti-CD69 FITC (BD Pharmingen) was added according to the manufacturers' instructions, and the samples incubated for 60 min at 4 °C. After two washes in cold phosphate-buffered saline the samples were fixed in 2% formaldehyde.

2.4. Target cells

NK is a human papilloma virus-16 immortalised keratinocyte line (gift from Dr. E. O'Toole) maintained in DMEM (Cambrex) supplemented with 10% fetal calf serum and 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma; Gillingham, UK). The line was HLA-typed by PCR-SSP phototyping (Bunce et al., 1995).

3. Results and discussion

3.1. Fluorescent labelling of keratinocytes

Keratinocytes were treated with 300 U/ml IFN-γ (Roche) overnight. IFN-γ treatment was necessary to sensitise keratinocytes to T cell mediated cytotoxicity (Symington and Santos, 1991). The keratinocytes were then trypsinised and incubated with 5 μM CFSE (carboxy-fluorescein succinimidyl ester; Invitrogen) or CellTracker Orange CMTMR ((5-(and 6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine; Invitrogen) diluted in PBS 0.05% bovine serum albumin for 10 min at 37 °C. Both CFSE and CMTMR are vital dyes which diffuse through the cytoplasm and allow cells to be detected by flow cytometry or fluorescent microscopy. The reaction was quenched with RPMI 10% foetal calf serum for 20 min. Labelled keratinocytes were mixed in equal ratios at a concentration of 10^6 cells/ml. Keratinocytes were allowed to adhere to glass 8-well culture slides (BD falcon) or plastic flat-bottomed 96 well plates (Corning Incorporated, Corning, USA) for 1 h prior to addition of effector cells.

Fig. 1A shows an example of fluorescently labelled keratinocytes illustrating stable interaction between differential populations of cells within the same slide left overnight. Having established that adherent human keratinocytes are stably labelled overnight with the differential fluorescent dyes we were able to proceed to investigate whether lysis could be detected.

3.2. Differential lysis of adherent targets

By using different populations of target cells simultaneously, we were able to sensitise each target differentially and then compare lysis. We incubated either the CFSE or CMTMR labelled keratinocytes with peptide (EBV BMLF1 280-8 GLCTLVAML) prior to adherence of a total of 10^5 keratinocytes on each slide or 10^4 keratinocytes on each well of the 96-well plates. Effector cells were then added in a final volume of either 100 μl or 300 μl of medium for 96-well plates and culture slides, respectively. Following 4 hour or overnight incubation, three separate fields of the slides and plates were chosen at random for image capture using Openlab software (Improvision). Quantitative fluorescent image analysis was performed using Image J software (http://rsb.info.nih.gov/ij). Images were split into red (CMTMR) and