Breast Cancer Research and Treatment (2005) 92: 85–93
DOI 10.1007/s10549-005-0988-1

Report

Evaluation of the CD107 cytotoxicity assay for the detection of cytolytic CD8$^+$ cells recognizing HER2/*neu* vaccine peptides★

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Key words: CD107, cytotoxicity, HER2/*neu*, peptides, vaccines

Summary

The recently reported FACS-based CD107 assay has been used in human HIV and CMV antigen models as well as in the *ex vivo* analysis of tumor cytolytic T cells in a melanoma model by a single group. The purpose of our study was to validate this assay and to use it in previously untested viral and tumor antigen models. Specifically, we investigated the use of the novel CD107 cytotoxicity assay in the detection of influenza and HER2/*neu* tumor-specific cytolytic CD8$^+$ T cells. CD8$^+$ T cells from HLA-A2$^+$ healthy donors were stimulated with autologous dendritic cells pulsed with FluM or the HER2/*neu* peptides, E75 or GP2. These CD8$^+$ T cells were then tested in cytotoxicity assays at varying effector:target (E:T) ratios against T2 targets. Cytotoxicity was measured by detection of CD107a and b on the surface of CD8$^+$ T cells. An E:T of 1:5 was found to optimize the resulting percentage of CD8$^+$CD107$^+$ T cells. E75- and GP2-stimulated CD8$^+$ T cells were then tested in cytotoxicity assays with MCF-7 (HER2/*neu*$^+$HLA-A2$^+$) and AU565 (HER2/*neu*$^+$HLA-A2$^-$) tumor cells. Cytotoxicity was measured by both the CD107 assay and the ^{51}Cr release assay. Results of cytotoxicity were then correlated between these two assays. In representative experiments, the CD107 assay identified average specific increases for E75- and GP2-stimulated cells of 4.26 and 3.57%, respectively. These results correlated favorably with cytotoxicity as measured by the traditional ^{51}Cr assay. These findings confirm preliminary reports of the CD107 assay and suggest its usefulness for monitoring cancer trials.

Introduction

The HER2/*neu* protein is an immune recognized tumor-associated antigen [1–3]. Among the immunogenic peptides from the HER2/*neu* protein recognized by cytotoxic T lymphocytes (CTL) are the HLA-A2 epitopes, E75 and GP2 [4,5]. E75 is the most studied of the HER2/*neu*-derived peptides, and it has been used in several clinical trials as an anti-cancer vaccine [6–12]. Our group has recently reported initial results from our ongoing clinical trial investigating the use of the E75 peptide mixed with GM-CSF as a simple vaccine strategy to induced E75-specific CD8$^+$ T cells. This vaccine appears to convey clinical benefit in immunocompetent patients with breast cancer who were rendered disease-free through standard conventional therapies but who were at high risk for recurrence.[1] GP2 is another immunogenic peptide from the HER2/*neu* protein that we and other groups are investigating for use in a second peptide-based cancer vaccine clinical trial [13].

As is the case for any immunotherapeutic trial, immunologic monitoring of the vaccine-elicited immune response is an important component of our HER2/*neu* peptide-based cancer vaccine trial. Newly developed technologies incorporating recombinant protein molecules and multi-parameter flow cytometry have enabled the enumeration of antigen-specific CD8$^+$ T cells using MHC Class I tetramers or HLA-A2:Ig dimers [14,15]. These techniques are able to determine the presence of these cells but are unable to provide information regarding their capacity to mediate functional immune activity. Functionality can be assessed by assays requiring minimal *ex vivo* manipulations such as the

★Funded by the Clinical Breast Care Project, a Congressionally funded program of the Henry M. Jackson Foundation for the Advancement of Military Medicine (Rockville, Maryland). Supported by the United States Army Medical Research and Materiel Command, and the Department of Clinical Investigations at the Walter Reed Army Medical Center. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

[1]Peoples GE, Gurney JM, Ryan GB, et al.: Clinical trial results of a HER2/*neu* (E75) vaccine to prevent recurrence in high risk breast cancer patients (submitted for publication).

CFSE assay for proliferation, intracellular cytotoxicity assays, and ELISPOT assays for cytokine secretion. Other functional assays such as the standard chromium (^{51}Cr) release or the 3H-thymidine proliferation assays also provide valuable functional information but they involve extended *in vitro* stimulation and generation of cell cultures [16–19]. From these assays, the most relevant and commonly used method for immunological monitoring of immune-mediated cytotoxic activity against tumor cells is the ^{51}Cr release assay despite the prolonged manipulations required to perform it. One drawback of the ^{51}Cr release assay is that it does not directly examine the CD8$^+$ T cells that mediate killing, rather it examines the death of target cells. While the ultimate goal for CTL induction is tumor cell killing, the ^{51}Cr assay does not allow for the identification of the specific effector cells responsible for target cell killing. Therefore, one cannot directly attribute the cytolytic activity to be due to antigen-specific CD8$^+$ T cells or other cellular populations present in the cultures being tested. In addition, the ^{51}Cr release assay does not allow for the quantification of effector cells. This test is, therefore, limited in its ability to characterize the phenotype and functional capacity of the responding effector cells.

Betts et al. recently described the CD107 assay as an alternative cytotoxicity assay that is able to address some of the shortcomings of the ^{51}Cr release assay [20]. The CD107 assay allows for precise phenotypic and functional characterization of responding CD8$^+$ T cells through flow cytometry using a marker that is only expressed during degranulation, the initial event that takes place during target cell lysis [20]. Degranulation of activated CD8$^+$ T cells occurs shortly after T cell receptor (TCR) stimulation. TCR stimulation initiates the transport of lytic granules in the effector CD8$^+$ T cell towards the synapse formed with the target [21]. These lytic granules contain a dense core composed of various cytotoxic mediators such as perforin and granzyme that are released once the granule fuses with the CD8$^+$ T cell's plasma membrane. The core within the granule is surrounded by a lipid bilayer containing lysosome associated membrane glycoproteins including CD107a and b. In their assay, Betts et al. described measuring the cumulative exposure of CD107a and b that become transiently mobilized to the T cell surface during the degranulation process that occurs upon contact with specific target cells [20]. A diagrammatic representation of these events is shown in Figure 1. The CD107 assay can provide assessment of the capacity, frequency and phenotype of CD8$^+$ T cells that kill in conditions similar to those used in a standard ^{51}Cr release assay [20]. Subsequent to its initial description, Betts et al. have reported on the use of the CD107a assay in the *ex vivo* analysis of tumor cytolytic T cells in a melanoma model and also for the study of T cell phenotype and functions in human HIV and CMV antigen-specific CD8$^+$ T cells [22,23].

In the present study, we have sought to corroborate the initial findings of Betts et al. and to expand this assay into previously untested viral and tumor antigen models. We have investigated the use of the novel CD107 cytotoxicity assay in the detection of influenza and HER2/*neu* tumor specific cytolytic CD8$^+$ T cells. In our approach to validate this assay, we specifically addressed three important aspects of an immunologic anti-tumor cytotoxicity assay. First, we tested various effector to target (E:T) ratios in order to optimize the activation and detection of the percentage of CD8$^+$CD107$^+$ cells. Second, we utilized the CD107

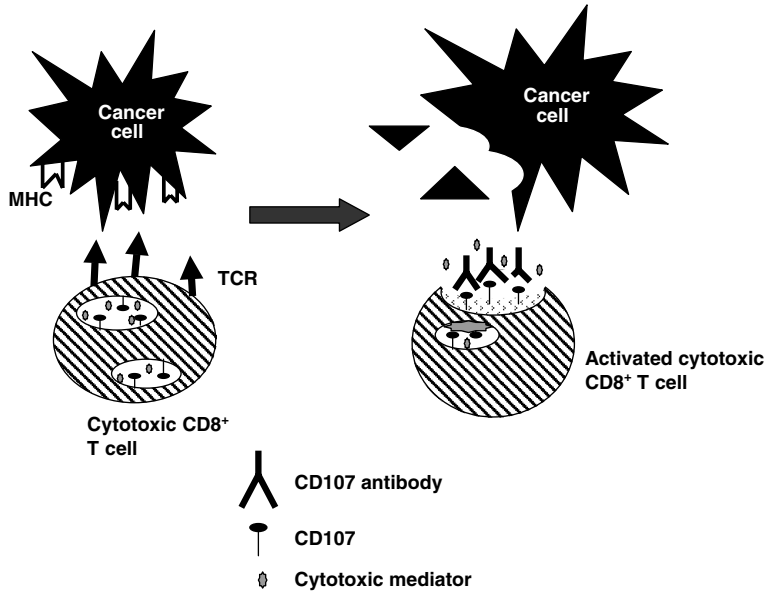

Figure 1. Stimulation of the T cell receptor on a CD8$^+$ T cell results in degranulation with release of lytic granules. Lysosome-associated membrane glycoproteins CD107a and b become transiently mobilized to the cell surface during this process. Identification and quantification of the CD107 molecules using a CD107 antibody and flow cytometry allows for precise phenotypic and functional characterization of activated CD8$^+$ T cells.

assay to detect the cytolytic activity of FluM- and HER2 peptide-stimulated $CD8^+$ T cells using peptide-pulsed T2 cells or HER2/*neu*$^+$ cancer tumor target cells. Finally, we correlated the results obtained from the CD107 assay with cytolytic activity measured by the traditional ^{51}Cr release assay.

Materials and methods

T2 cells and tumor cell lines

The HLA-$A2^+$ T-B lymphoblast hybrid cell line, T2, that is deficient in the transporter-associated protein (TAP), was obtained from ATCC (Manassas, VA). The HER2/*neu*-expressing breast cancer cell lines MCF-7 (HLA-$A2^+$) and AU565 (HLA-$A2^-$) were also obtained from ATCC. AU565 and T2 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Gemini Bioproducts, CA), 1% glutamine and 1% penicillin–streptomycin (GIBCO-BRL, VA). MCF-7 cells were cultured in DMEM supplemented with 10% FCS and penicillin–streptomycin, glutamine and insulin (GIBCO-BRL, VA).

Peptides and monoclonal antibodies

The E75 (HER2/*neu*, 369–377, KIFGSLAFL) and GP2 (HER2/*neu*, 654–662, IISAVVGIL) peptides were produced commercially in good manufacturing practices grade by Multiple Peptide Systems, Inc. (San Diego, CA). The purity of these peptides were verified by high performance liquid chromatography and mass spectrometry to >95% purity, and the amino acid content was determined by amino acid analysis. The influenza matrix peptide Flu-M (58–66, GILGFVFTL) was produced as research grade reagent at the BIC Peptide Synthesis Lab, Uniformed Services University of the Health Sciences (Bethesda, MD). The monoclonal antibodies, CD8-APC, CD107a-FITC, and CD107b-FITC were obtained from Pharmingen (San Diego, CA).

Blood samples and HLA-A2 typing

Peripheral blood mononuclear cells (PBMC) were prepared by density-gradient centrifugation of leukophoresis blood products provided by healthy donors and commercially available from BRT Laboratories (Baltimore, MD). The cells were washed in HBSS and resuspended in complete culture medium (CM) consisting of Iscove's Modified Dulbecco's medium containing 10% human AB serum (Gemini Bio-Products, Woodland, CA) supplemented with 1X penicillin/L-glutamine/streptomycin, 1X sodium pyruvate, 1X nonessential amino acids and 50 μM of 2-mercaptoethanol (Life Technologies, Rockville, MD). Aliquots of freshly isolated PBMC were cryo-preserved in 90% FCS and 10% DMSO in liquid nitrogen for future experiments. The HLA-A2 status of the blood samples was confirmed by indirect staining with 10 μl of anti-HLA-A2 monoclonal antibodies (mAbs), BB7.2 and MA2.1 (1:10 dilution of culture supernatant) (ATCC) at 4 °C for 30 minutes followed by 30 min of incubation with FITC-conjugated goat anti-mouse antibodies (Caltag Laboratories, Burlingame, CA) and analyzed on a FACSCalibur Analzyer (Becton Dickinson).

In vitro *stimulation of PBMC cultures for cytotoxicity assay*

The PBMC preparations re-suspended in CM were used for the preparation of dendritic cells (DC) and as a source of $CD8^+$ T lymphocytes for *in vitro* stimulation with the peptides. Highly pure populations of $CD8^+$ T cells were isolated by magnetic beads separation technique using CD4-, CD14-, and CD19-coated Dynal beads. Highly enriched populations of CD14 monocytes were prepared by magnetic bead depletion of PBMC that had been incubated with CD2-, CD19-, and CD8-coated beads from Dynal. The CD14 cell population was cultured in Macrophage Serum Free Medium (GIBCO-BRL, MD) with 100 μg/ml of GM-CSF and 50 μg/ml of IL-4/ml (R&D) to obtain monocyte-derived DC populations. Recombinant human TNF-α (R&D Systems) was added at 30 ng/ml on day three to induce maturation. DC were then harvested at 6 days, incubated with E75, GP2, Flu-M, or no peptide for 2 h and then used to stimulate the purified preparations of $CD8^+$ T cells. Cultures were set up in CM with 10 ng/ml of IL-7 and this was followed by the addition of 25 ng/ml of IL-2 on the second day. All cultures were maintained in a humidified incubator at 37 °C with 5% CO_2.

^{51}*Chromium release cytotoxicity assay*

Peptide-specific cytotoxicity in the $CD8^+$ T cell cultures stimulated with peptide-pulsed DC was determined by standard 4-h chromium release assay. Briefly, targets were labeled with 100–150 μCi of sodium chromate (Perkin-Elmer, Boston, MA) for 1 h at 37 °C then washed twice and plated at 2500 cells/well in 100 μl in 96-well U-bottom plates (Becton Dickinson). Effectors were added at an effector: target (E:T) ratio of 10:1–20:1 in 100 μl/well. After 4 h of incubation, 100 μl of culture supernatant was collected and radionuclide release measured on a Microbeta Trilux counter (Perkin-Elmer). All determinants were done in triplicate. Results are expressed as percent specific lysis as determined by: (experimental mean cpm-spontaneous mean cpm)/(maximum mean cpm-spontaneous mean cpm) $\times$ 100. The target cells used in the cytotoxicity assays were peptide-pulsed T2 cells or MCF-7 (HER2/*neu*$^+$HLA-$A2^+$) and AU565 cells (HER2/*neu*$^+$HLA-$A2^-$).

CD107 cytotoxicity assay

The CD107 assay with the T2 and the breast cancer cells was set up based upon a similar approach described by

Betts et al. with a few modifications [20]. The breast cancer cells, MCF-7 and AU565, were plated at various cell numbers in a 24-well plate and incubated at 37 °C overnight prior to their use in the assay on the next day. This was done in order to allow the tumor cells to become adherent on the bottom of the wells. (Experiments performed by plating the cells on the same day as the running of the assay did not significantly change the results obtained.) On the day of the assay, the culture supernatant was removed from the wells and the effector cells (DC-stimulated $CD8^+$ T cells in CM) were added to the wells at various E:T ratios. Control wells containing either only T cells or tumor targets were also set up with each assay. Ten microliters each of CD107a-FITC and CD107b-FITC was added to each well at the same time as addition of the T cells. The plate was then centrifuged for three minutes at 1000 rpm in order to facilitate immediate contact between the T cells and the tumor targets at the bottom of the wells. The plate was then incubated at 37 °C and after the first hour of incubation 1 μl/well of Brefeldin A (BD-Fastimmune Golgi Plug) was added to each of the wells and the plate was returned to the incubator for an additional 4 h. At the end of the incubation period the cells were harvested into separate tubes and washed once with Pharmingen Stain Buffer (PSB, BD Pharmingen, CA) after which they were re-suspended in 1 ml PSB and stained with anti-human CD8-PE antibodies for 30 min. The cells were then washed again and re-suspended in PSB and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The forward and side scatter profiles obtained using the control cultures containing only the tumor cells or T cells enabled us to gate on the lymphocyte populations present in the mixed cultures of T cells and tumor cells. The CD107 assay with the T2 cells were set up in an identical manner and experimental design with the only exception being that the T2 cells were prepared by pulsing with the respective peptides and using these cells on the same day the assay was performed.

Results

Optimization of the E:T ratio used in the CD107 assay

The first step in evaluating the CD107 cytotoxicity assay for its potential use in immunologic evaluation of blood samples from patients enrolled in a peptide-based vaccine trial was to determine the E:T ratio that would be optimal for stimulation and detection of the number of $CD8^+CD107^+$ T cells being generated in cell cultures. In order to determine this ratio, we began with $CD8^+$ T cells from HLA-$A2^+$ healthy donors that were stimulated with autologous DC pulsed with FluM peptide, a standard viral antigen used extensively in immunological studies of infectious diseases. After being pulsed with the peptide, the cells were expanded then incubated with T2 target cells at varying E:T ratios. Flow cytometric analysis was performed to determine the percentage of $CD8^+$ T cells that expressed surface CD107a or b during the incubation. Figure 2 shows histograms from an initial experiment and data from subsequent experiments with Flu-M stimulated cultures from additional HLA-$A2^+$ healthy donors as well as E75- and GP2-stimulated cultures from HLA-$A2^+$ donors versus T2 targets at varying E:T ratios. These results reflect the detection of specific $CD8^+CD107^+$ cells where background numbers of $CD107^+$ cells generated against un-pulsed T2 target cells have been subtracted out. An E:T of 1:5 provided the greatest percentage of $CD8^+CD107^+$ T cells. Ratios of 1:2 also resulted in an adequate percentage of $CD8^+CD107^+$ cells identified suggesting that any ratio providing more targets then effectors is adequate to run this assay. In separate experiments, the ratio was taken to 1:10 at which point there was a decrement in CD107 detection (data not shown).

Similar results have been observed by Betts et al. (personal communication). These sets of results with peptide-pulsed T2 cells confirm the utility of the CD107 assay as a feasible cytotoxicity assay with T2 target cells in both viral and tumor antigen systems.

Detection of cytolytic activity of E75- and GP2-specific $CD8^+$ T cells in peptide-stimulated cultures against tumor targets

We next investigated the use of the CD107 assay for the detection of cytolytic activity of E75- and GP2-specific $CD8^+$ T cells against HER/*neu*-expressing tumor targets. E75- or GP2-stimulated cells were incubated with MCF-7 (HER2/neu^+HLA-$A2^+$) or AU656 (HER2/neu^+HLA-$A2^-$). To confirm that an E:T whereby targets exceeded effectors was optimal when assessing cytotoxicity against tumor targets as opposed to T2 targets, initial experiments were performed using E:T ratios of 1:1 and 1:5. At a 1:1 E:T ratio, E75-stimulated cells from two different PBMC had average specific increases (defined as % $CD8^+CD107^+$ cells with AU565 subtracted from % $CD8^+CD107^+$ cells with MCF-7) of 1.25%. GP2-stimulated cells from two PBMC had average specific increases of 1.12%. At an E:T of 1:5, the average specific increases for E75- and GP2-stimulated cells were 4.26 and 3.57%, respectively (Figure 3a).

Based on these initial findings, additional experiments were designed to validate the utility of the CD107 assay as a tumor-specific cytotoxicity assay. $CD8^+$ T cells from two additional HLA-$A2^+$ healthy donors were stimulated with autologous DC pulsed with E75 or GP2 peptides and then expanded. The cells from these stimulated cultures were incubated at the optimal E:T of 1:5 with MCF-7 and AU565 cells. Specific increases of $CD8^+CD107^+$ cells shown in Figure 3b were determined using FACS analysis. Results of these experiments confirm that we are able to identify populations of tumor-cytolytic T cells from peripheral blood samples using this flow cytometric quantification of the surface mobilization of CD107.

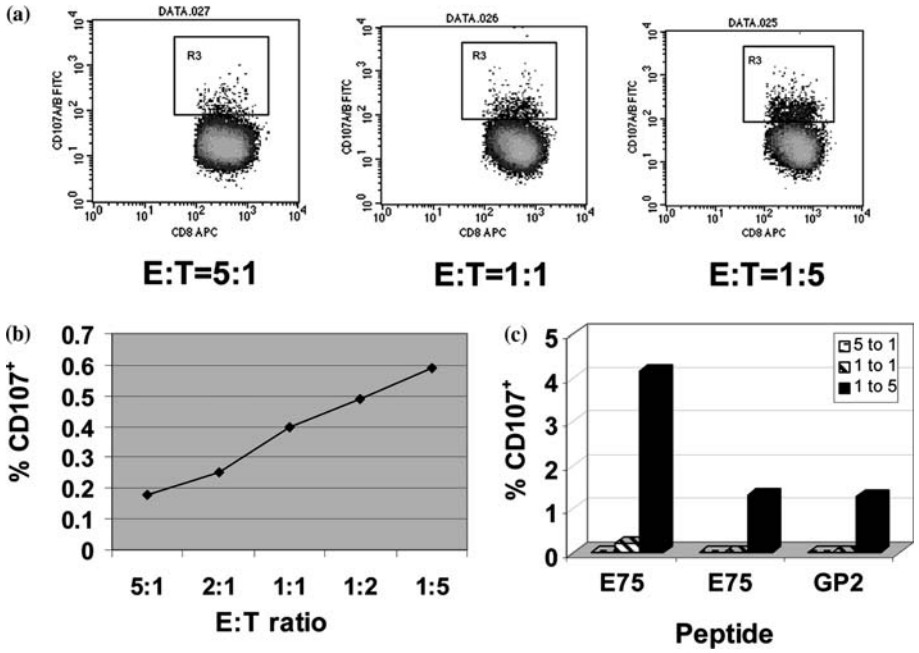

Figure 2. Percentage of $CD8^+$ T cells expressing surface CD107a or b. (a) Histograms showing FluM-stimulated effectors versus T2 targets at the indicated E:T ratios. An E:T of 1:5 resulted in the highest percentage of resulting $CD107^+CD8^+$ T cells. This histogram is representative of results obtained using $CD8^+$ T cells from 4 HLA-$A2^+$ healthy donors stimulated with autologous dendritic cells pulsed with FluM. (b) Line diagram depicting results of additional experiments evaluating the optimal E:T ratio. These experiments again involved $CD8^+$ T cells from HLA-$A2^+$ healthy donors stimulated with FluM peptide versus T2 targets. Additional ratios including 2:1 and 1:2 were evaluated. (c) Percentage of specific $CD107^+$ T cells at varying ratios using $CD8^+$ T cells from HLA-$A2^+$ donors stimulated with E75 (PBMC 1 and 2) and GP2 (PBMC 3), two immunogenic peptides derived from the HER2/*neu* protein.

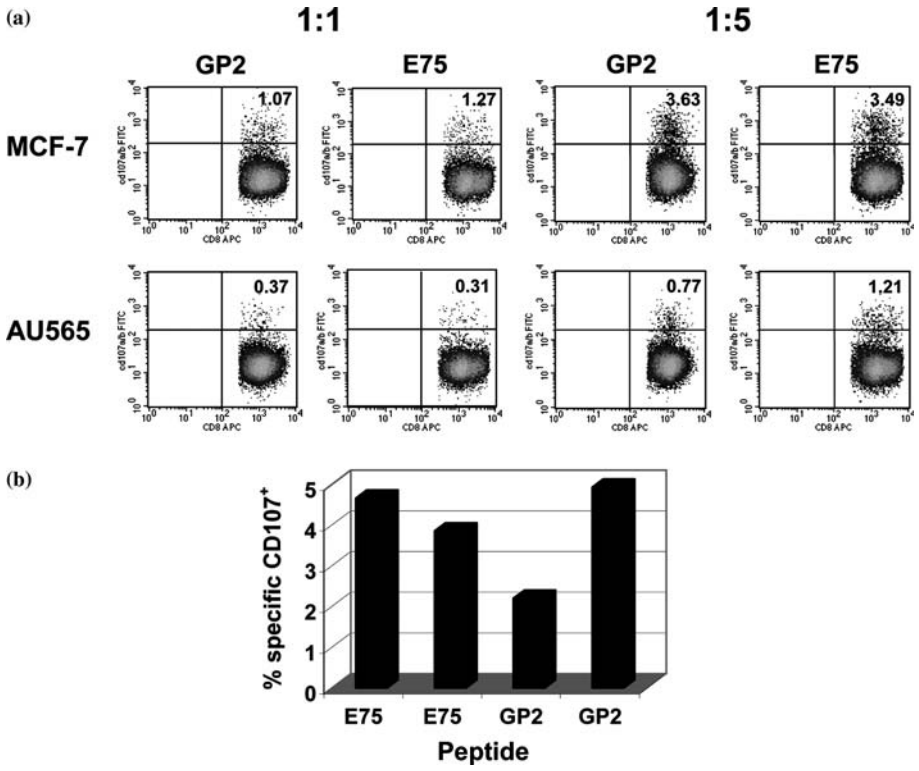

Figure 3. (a) Representative histograms showing E75- or GP2-stimulated effectors at the indicated E:T ratio against the HER2/neu^+, HLA-$A2^+$ control breast cancer cell line MCF-7 and the HER2/neu^+, HLA-$A2^-$ control cell line AU565. (b) Detection of specific cytolytic activity (defined as % $CD8^+CD107^+$ cells with AU565 subtracted from % $CD8^+CD107^+$ cells with MCF-7) from E75- or GP2-stimulated $CD8^+$ T cells in peptide-stimulated cultures against tumor targets.

Correlation of cytotoxicity detected by CD107 assay with traditional ^{51}Cr release assay

Having shown that the CD107 assay could be used to detect the cytolytic activity of HER2/*neu* peptide-specific $CD8^+$ T cells in peptide-stimulated cultures against HER2/*neu*-expressing tumor targets, our final objective was to correlate the CD107 assay with the ^{51}Cr release assay that we are currently using to measure cytolytic activity in our peptide-based vaccine trials. $CD8^+$ T cells from an HLA-$A2^+$ healthy donor stimulated with FluM, E75 and GP2 peptide were incubated with T2 target cells at a 1:5 ratio for the CD107 assay and at our standard 20:1 ratio for the ^{51}Cr release assay. Figure 4a shows a positive pattern of response demonstrated between cytolytic activity detected by the two assays.

Additional experiments performed which showed poor target killing with GP2-stimulated $CD8^+$ T cells versus T2 targets confirmed that there was a negative correlation as well, whereby when no target cell killing was detected by the CD107 assay, the standard ^{51}Cr release assay also showed no or negligible target cell killing (data not shown).

Similar experiments were performed using E75- and GP2-stimulated cells versus tumor targets in order to correlate the validity of results obtained from these two assays. Results of these experiments (Figure 4b) confirm that a good overall correlative pattern of response is seen between the CD107 assay and the standard ^{51}Cr release assay.

Discussion

In this study we have corroborated the preliminary reports by Betts et al. suggesting the utility of the CD107 assay as an alternative cytotoxicity assay [20]. Furthermore, we have expanded the scope of the assay to viral and tumor antigen systems not previously tested. Specifically, we have shown that this assay can be used to detect cytolytic activity of $CD8^+$ T cells recognizing the HER2/*neu* vaccine peptides E75 and GP2 which are currently being investigated in ongoing peptide based vaccine trials. Additionally, we have optimized the assay by determining the best E:T which had not been specified in previous reports. We also validated the CD107 assay against the longstanding standard cytotoxicity assay used for the monitoring of clinical cancer vaccine trials.

Immunologic response monitoring is an important component of a peptide-based cancer vaccine immunotherapy trial. Currently, to determine the response to an administered vaccine, both phenotypic and functional assays are performed in order to examine the presence and functionality of induced, antigen-specific $CD8^+$ T cells. In our ongoing trial investigating the HER2/*neu*-derived peptide E75, we are using the HLA-A2:Ig dimer assay to quantify the number of E75-specific $CD8^+$ T cells that are induced by our vaccine [14]. We use the ELISA and ELISPOT assays to investigate cytokine secretion, and we perform the ^{51}Cr release assay to determine cytotoxic activity in *ex vivo* stimulated cell cultures from the PBL of the vaccinated patients. The ^{51}Cr release assay has several drawbacks, however, most notably that it does not allow for the determination of the actual cell population responsible for the killing activity. The assay only measures target cell death and not the actual cytolytic process; therefore, it cannot differentiate cell death due to antigen-specific $CD8^+$ T cells that may have been induced by vaccination from cell death due to other effector cell populations. It also

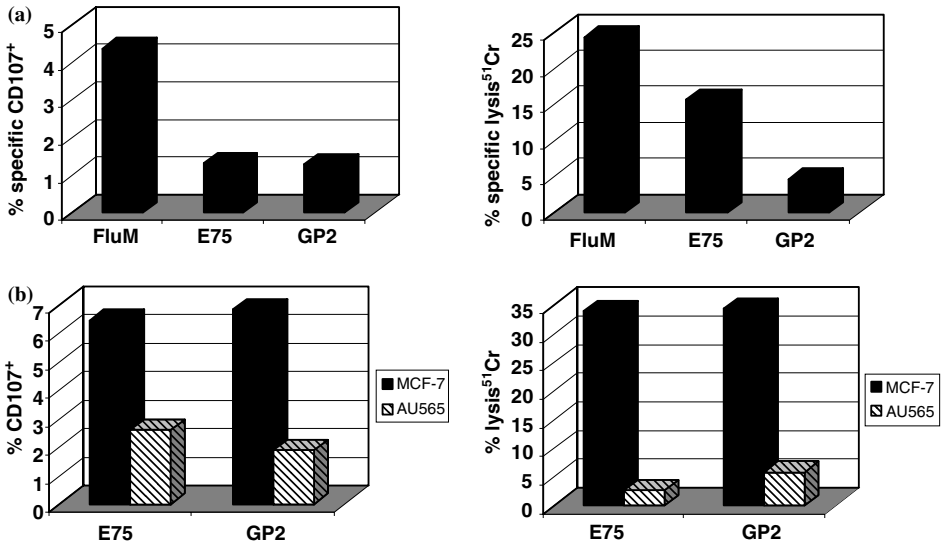

Figure 4. Cytolytic activity of E75- and GP2-specific $CD8^+$ T cells as detected by the ^{51}Cr release assay. (a) Correlation of lytic activity of E75- or GP2-stimulated $CD8^+$ T cells versus T2 targets as measured by the CD107 and ^{51}Cr assays. Values indicate specific lysis (T2 without peptide subtracted from T2 with peptide). (b) Correlation of lytic activity of E75- or GP2-stimulated $CD8^+$ T cells versus HER2/*neu*-expressing tumor targets. Killing versus the HLA-$A2^+$ tumor target MCF-7 as well as the HLA-$A2^-$ tumor target AU565 are shown. Specific lysis would be determined by subtracting AU565 value from MCF-7 value. Both figures depict a positive correlation between the two cytotoxicity assays.

does not allow for a determination of the percentage of T cells actually responsible for the effector function. Additionally, the ^{51}Cr release assay is cumbersome and potentially insensitive in that it is difficult to standardize for consistencies in the various components and steps involved. The requirement for radioactive material to perform the assay is another drawback. In an attempt to better monitor the immune response being induced in our patients receiving an E75 peptide vaccine, we have investigated the CD107 assay that allows for precise phenotypic and functional characterization of the cytotoxic activity of the responding $CD8^+$ T cells. Interestingly, this assay has the potential to allow for the possibility of detecting peptide-specific CTL in the act of killing tumor cells through the use of double staining methods with peptide-specific HLA-dimer molecules and CD107 antibodies. Additional studies to explore this strategy are currently being conducted in our laboratory for eventual use with the blood samples obtained from the patients enrolled in our clinical trial.

The effector functions of $CD8^+$ T cells include the production of soluble factors such as cytokines or chemokines and target cell killing. Target cell killing can be accomplished via one of two pathways, including the granule-independent pathway, which mainly involves ligand–ligand induced cell death and the granule-dependent pathway. The granule-dependent pathway relies on preformed lytic granules that are intracellular membrane bound secretory lysosomes that contain a dense core composed of various proteins including perforin and granzyme [21]. This dense core is surrounded by a lipid bilayer containing the lysosome-associated membrane glycoproteins CD107a and b. Upon interaction with the appropriate peptide-MHC complex presented by the target cell, transport of an activated $CD8^+$ T cell's lytic granules towards the synapse with the target begins [24]. Once the lytic granules reach the $CD8^+$ T cell's plasma membrane, they fuse resulting in the release of the cytotoxic mediators perforin and granzyme into the immunological synapse. Of interest, monitoring of the presence or absence of granzyme or perforin using the ICC assay gives no indication as to the ability of the T cell to degranulate and cause target cell death [20]. Additionally, perforin or granzyme detection by ELISPOT identifies the release of these mediators but does not provide information about the cell source.

The fusion of the granule with the $CD8^+$ T cell's plasma membrane also results in the exposure of CD107a and b. These two glycoproteins are not normally expressed on the surface of a T cell and their function has not been elucidated. Their presence in the cytotoxic granular membrane has been proposed to protect against the leakage of contents from the granule by coating the interior of the membrane [21]. For the purposes of immunologic monitoring, the CD107a and b glycoproteins provide a marker that can be quantified to indicate if degranulation has occurred. The CD107 assay was first described by Betts et al., who utilized CD107a and b along with $CD8^+$ antibodies and multiparameter flow cytometry to measure degranulation in primary responding antigen-specific $CD8^+$ T cells [20]. Using a CMV-specific $CD8^+$ T cell population known to degranulate in response to an HLA-A2 restricted CMV-derived peptide, these authors further showed that antigen-specific $CD8^+$ T cells that degranulate are the same cells mediating cytotoxic activity [20].

In the overall evaluation of the CD107 assay, there are several theoretical and technical features of the assay that should be elaborated upon. To begin with, CD107a and b are likely differentially regulated within the cell types capable of producing these proteins. They are encoded on different chromosomes and are expressed at different copy numbers within the cell [25]. It has not been determined if these two proteins are differentially expressed in cytotoxic granules within individual $CD8^+$ T cells. For this reason, we believe it is important to use antibodies to both CD107a and b to ensure the greatest sensitivity for the assay.

Timing of CD107a and b expression on the cell surface is a second important technical aspect of the assay. Degranulation of activated $CD8^+$ T cells occurs rapidly after TCR stimulation as a result of the polarized mobilization of microtubules that transport the lytic granules toward the immunological synapse formed between the cytotoxic T cell and the target [24]. Significant expression of cell surface CD107a and b is observed as early as 30 min after stimulation of $CD8^+$ T cells and reaches maximum by four hours. Cell surface expression of CD107a and b on T cells is transient and any CD107 externalized to the cell surface is rapidly retrieved via the endocytic pathway. Therefore, as the $CD8^+$ T cells degranulate they become positive for cell surface CD107 for only a brief period of time. In light of this, there are two important strategies to employ when performing this assay. First, the antibodies conjugated to CD107a and b are added prior to the 4-h incubation. The antibodies are therefore present during the entire duration of the stimulation so that any transient surface expression of CD107 would lead to antibody binding and subsequent fluorescent labeling of that cell. Second, Brefelden A, a secretion inhibitor, is added one hour into the incubation. Brefelden A works to minimize involution of CD107a and b thereby maintaining it on the cell surface where it can be detected for analysis. Taken together, these two steps have resulted in our findings with FluM and HER2/*neu* peptides to be in agreement with those of Betts et al. who used the CD107 assay in a different viral and tumor model [22,23].

In our experiments designed to determine the E:T ratio that was optimal for the performance of this assay, we found that a ratio whereby targets exceeded effectors provided the best conditions. Specifically, an E:T ratio of 1:5 resulted in optimal stimulation and generation of $CD8^+$ $CD107^+$ T cells. This is true for both viral antigen-stimulated (FluM) and the HER2/*neu*-derived peptides, E75- and GP2-stimulated $CD8^+$ T cells

against T2 targets as well as tumor targets. Interestingly, this pattern of E:T ratio is reversed when compared with the standard ^{51}Cr assay where an excess of effectors has always been required for the optimal detection of cytotoxic activity. This may reflect the need for individual effector cells to come into contact with target cells in order to be activated. A ratio of 1:5 ensures that each effector is contacted by a target thereby providing the opportunity for it to be activated and degranulate with the resultant exposure of CD107a and b. In addition, the CD107 assay may require many fewer effector cells for the detection of cytotoxic activity because it identifies the specific cell responsible for the target cell killing, an event that can be identified individually by flow cytometry analysis. As previously mentioned, the CD107 assay specifically detects CD8$^+$ T cells that have cytolytic activity. In contrast, the ^{51}Cr assay simply detects target cell death and is unable to differentiate between CD8$^+$ T cells, cytotoxic CD4$^+$ T cells, and natural killer cells that may be responsible for eliciting the lytic activity. It may be that a certain threshold lysis of the target cell membrane has to be achieved in order for the release of measurable ^{51}Cr to occur and that this can only be achieved as a combined effect brought about by a large number of cytotoxic CD8$^+$ T cells. Therefore, due to the method of detection, the CD107 assay is more efficient and the number of effector cells required to perform the assay is much less.

In order to be acceptable in assessing the functional cytotoxic activity of antigen-specific CD8$^+$ T cells that are induced by vaccination in our HER2/*neu*-derived peptide vaccine study, the results of the CD107 assay must be comparable to the established ^{51}Cr release assay. In this study, we have shown a favorable correlation between these two assays that measure different ends of the cytolytic process. The CD107 assay measures the effectors responsible for eliciting cytolytic activity by identifying their degranulation while the ^{51}Cr assay measures cell death of the targets. The assays, therefore, have two different end points as readouts for cytotoxic activity. The positive correlation is important in that it is in agreement with the findings of Betts et al. that detection of CD107a and b identifies cells that degranulate and that degranulation is requisite for target cell killing [20].

We have shown in this study that the CD107 cytotoxicity assay can be used for the detection of peptide vaccine-stimulated HER2/*neu* tumor-specific cytolytic CD8$^+$ T cells. The CD107 assay utilizes flow cytometry to detect a marker that is only expressed during degranulation, an event that occurs early during target cell killing. Identification of this marker along with the simultaneous staining of other T cell antigens allows for both phenotypic and functional characterization of responding CD8$^+$ T cells. Therefore, this assay provides a link between the direct and indirect methods of CD8$^+$ T cell effector analysis employed to monitor the immunologic response in cancer patients receiving immunotherapy. We are currently utilizing this assay to evaluate its potential to be used for monitoring the cytotoxic activity induced in breast cancer patients receiving the HER2/*neu* peptide vaccine in our ongoing clinical trial.

Acknowledgements

This work was supported by funds from the Department of Defense to the Henry M. Jackson Foundation for the Advancement of Military Medicine (Rockville, MD) for the Clinical Breast Care Project. We thank Ms. Diane Papay and Ms. Stacy O'Neill of the Clinical Breast Care Project who provided excellent patient care and administration of the clinical trial. We also thank the staff of the Clinical Breast Care Project Immunology and Research Center for their clinical and administrative assistance.

References

1. Ioannides CG, Fisk B, Fan D, Biddison WE, Wharton JT, O'Brian CA: Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER2/neu proto-oncogene. Cell Immunol 151: 225–234, 1993
2. Yoshino I, Goedegebuure PS, Peoples GE, Parikh AS, DiMaio JM, Lyerly HK, Gazdar AF, Eberlein TJ: HER2/neu-derived peptide(s) are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res 54: 3387–3390, 1994
3. Disis ML, Smith JW, Murphy AE, Chen W, Cheever MA: *In vitro* generation of human cytolic T-cells specific for peptide derived from the HER-2/neu protooncogene protein. Cancer Res 54: 1071–1076, 1994
4. Peoples GE, Goedegeburre PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci USA 92: 432–436, 1995
5. Fisk B, Blevins TL, Wharton JT, Ioannides CG: Identification of an immunodominant peptide of the HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J Exp Med 181: 2709–2717, 1995
6. Zaks TZ, Rosenberg SA: Immunization with a peptide epitope (pp. 369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. Cancer Res 58: 4902–4908, 1998
7. Knutson KL, Schiffman K, Cheever MA, Disis ML: Immunization of cancer patients with HER-2/neu, HLA-A2 peptide, pp. 369–377, results in short-lived peptide-specific immunity. Clin Cancer Res 8: 1014–1018, 2002
8. Murray JL, Gillogly ME, Przepiorka D, Brewer H, Ibrahim NK, Booser DJ, Hortobagyi GN, Kudelka AP, Grabstein KH, Cheever MA, Ioannides CG: Toxicity, immunogenicity, and induction of E75-specific tumorlytic CTLs by HER-2 peptide E74 (369–377) combined with granulocyte macrophage colony-stimulating factor in HLA-A2+ patients with metastatic breast and ovarian cancer. Clin Cancer Res 8: 3407–3418, 2002
9. Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W: Induction of cytotoxic T-lymphocyte responses *in vivo* after vaccinations with peptide-pulsed dendritic cells. Blood 96: 3102–3108, 2000
10. Kono K, Takahashi A, Sugai H, Fujii H, Choudhury AR, Kiessling R, Matsumoto Y: Dendritic cells pulsed with HER-2/neu-derived peptides can induce specific T-cell responses in patients with gastric cancer. Clin Cancer Res 8: 3394–3400, 2002
11. Disis ML, Grabstein KH, Sleath PR, Cheever MA: Generation of immunity to the HER-2/neu oncogenic protein in patients with

breast and ovarian cancer using a peptide-based vaccine. Clin Cancer Res 5: 1289–1297, 1999
12. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K: Generation of T-cell immunity to the HER-2/neu peptide-based vaccines. J Clin Oncol 20: 2624–2632, 2002
13. Dees EC, McKinnon KP, Kuhns JJ, Chwastiak KA, Sparks S, Myers M, Collins EJ, Frelinger JA, Deventer HVan, Collichio F, Carey LA, Brecher ME, Graham M, Earp HS, Serody JS: Dendritic cells can be rapidly expanded *ex vivo* and safely administered in patients with metastatic breast cancer. Cancer Immunol Immunother 53: 777–785, 2004
14. Woll MM, Fisher CM, Ryan GB, Gurney JM, Storrer CE, Ioannides CG, Shriver CD, Moul JW, McLeod DG, Ponniah S, Peoples GE: Direct measurement of peptide specific $CD8^+$ T cells using HLA-A2: Ig dimer for monitoring the *in vivo* immune response to a Her2/neu vaccine in breast and prostate cancer patients. J Clin Imm 24: 449–461, 2004
15. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes. Science 274: 94–96, 1996
16. Kern F, Surel IP, Brock C, Freistedt B, Radtke H, Scheffold A, Blasczyk R, Reinke P, Schneider-Mergener J, Radbruch A, Walden P, Volk HD: T-cell epitope mapping by flow cytometry. Nat Med 4: 975–978, 1998
17. Sheehy ME, McDermott AB, Furlan SN, Klenerman P, Nixon DF: A novel technique for fluorometric assessment of T lymphocyte antigen specific lysis. J Immunol Methods 249: 99–110, 2001
18. Brenchley JM, Douek DC, Ambrozak DR, Chatterji M, Betts MR, Davis LS, Koup RA: Expansion of activated human naïve T-cells precedes effector function. Clin Exp Immunol 130: 432–440, 2002
19. Liu L, Chahroudi A, Silvestri G, Wernett ME, Kaiser WJ, Safrit JT, Komoriya A, Altman JD, Packard BZ, Feinberg MB: Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nat Med 8: 185–189, 2002
20. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA: Sensitive and viable identification of antigen-specific $CD8^+$ T cells by a flow cytometric assay for degranulation. J Immunol Methods 281: 65–78, 2003
21. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, Slot JW, Geuze HJ: Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 173: 1099–1109, 1991
22. Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roderer M, Lee PP: *Ex vivo* identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 9: 1377–1382, 2003
23. Betts MR, Price DA, Brenchley JM, Loré K, Guenaga FJ, Smed-Sorenson A, Ambrozak DR, Migueles ST, Connors M, Roederer M, Douek DC, Koup RA: The functional profile of primary human antiviral $CD8^+$ T cell effector activity is dictated by cognate peptide concentration. J Immunol 172: 6407–6417, 2004
24. Barry M, Bleackley RC: Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol 2: 401–409, 2002
25. Fukuda M: Lysosomal membrane glycoproteins: structure, biosynthesis and intracellular trafficking. J Biol Chem 266: 2127–2133, 2001

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