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Laboratory Analysis of T-Cell Immunity

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1. ABSTRACT

Immune-based strategies for treating and preventing cancer are increasingly being tested and include cancer vaccination, adoptive T cell therapy, and cytokine therapy. An important component of testing and development of immune-based strategies is monitoring the immunologic response. The ability to monitor T cell immunity has been suboptimal. The measurement of tumor-specific immunity will aid in defining which strategies should be moved forward in clinical trials and which should be eliminated or evaluated further in the preclinical realm. Immunologic monitoring is necessary for determining if an approach has immunologic efficacy and ultimately whether immunologic responses correlate with a clinical response. This article discusses several important elements of measuring T cell immunity such as validation principles, laboratory issues, current approaches, and new paradigms and concepts for future testing. Informative immunologic monitoring of T cells will be one of the driving forces in advancing the field of tumor immunology.

2. INTRODUCTION

Our understanding of the immune response to tumors has improved dramatically over the past two decades. As a result, immune-based strategies for treating and preventing cancer are being tested frequently including cancer vaccination, adoptive T cell therapy, and cytokine therapy. Despite the rapid rate at which we are currently testing these treatments, the ability to monitor the immunologic consequences of such interventions has been suboptimal. The reproducible and quantitative evaluation of tumor-specific immunity will help define which strategies should be moved forward in clinical trials and which should be eliminated or evaluated further in the preclinical realm. Immunologic monitoring is necessary for determining whether or not the approach has immunologic efficacy and ultimately whether immunologic responses correlate with a clinical response. The study of immunologic monitoring is a growing field that needs to move forward technologically with the development of productive applications of quantitative and functional assessment. This article provides a general review of the

Table 1. Assay validation parameters-USP [1] and ICH [2]

Parameter	Definition
Accuracy	The agreement between assay results and an accepted reference
Precision	The variability of assay among replicate determinations
Limit of detection	Lowest detectable amount of analyte (neither precise nor accurate)
Limit of quantification	Lowest precise and accurate amount of analyte detectable
Specificity	Ability of assay to differentiate analyte from other similar analytes
Linearity	Ability of assay to produce results that are directly proportional to concentration of analyte
Range	The range of results of the assay where linearity, accuracy, and precision are similar
Ruggedness	The reproducibility of the assay under normal but unavoidable variable conditions (e.g. different analysts, reagent lots, etc.)
Robustness	The ability of the assay to remain unaffected by deliberate but small avoidable changes in conditions

[1]United States Pharmacopeia, [2]International Conference on Harmonization

current state of immunologic monitoring including general validation principles, laboratory issues, current approaches, and new paradigms and concepts for future testing.

3. VALIDATING ASSAYS FOR USE AS SURROGATES TO PROTECTIVE OR THERAPEUTIC IMMUNITY

The validation of an immunologic assay generally can be thought of in two phases. The first phase would involve determining the performance characteristics of the assay and defining the statistical correlation between the results of the test and the presence or absence of immunity. If the assay is to be eventually evaluated as a surrogate for a clinical response, then a second phase is needed to define the correlation between immunity and clinical outcome. This latter validation step is usually borne out in phase III clinical trials testing efficacy. Although this second phase of validation will not be further discussed, it should be pointed out that an immunologic response that is used as a surrogate for a clinical response need not be a marker of the mechanism central to improved therapeutic efficacy but rather only a marker of the therapeutic response.

Phase I of validation involves identifying sources of errors and quantifying the magnitudes of the errors (1). During validation of an assay, one must define the error in the context of several essential parameters mandated by regulatory agencies (Table 1). Accuracy is the determination of how close the assay comes to measuring the true quantity (e.g. antigen-specific precursors) of an entity as compared to an accepted reference value. Although accuracy can be determined in a number of different ways, one accepted approach for immune monitoring is to examine how closely an assay measures an acceptable reference T cell population such as a T cell line or clone (2). Precision, another widely measured parameter, is an expression of the nearness in agreement of repeated measures. There are 3 different levels of precision: repeatability, intermediate precision, and reproducibility. Repeatability is the measure of agreement among replicates in the same assay (intra-assay). Intermediate precision (inter-assay) variability describes the variability attributed to the assay being done on different days, by different analysts, with different equipment and other laboratory-specific variables. Typically, assays measuring T cell responses have high inter-assay variability indicating batch analysis may be needed to improve precision. Reproducibility is the measure of variability among different laboratories which is important to consider but not required for product development.

Specificity is the ability of the assay to measure the cell, antibody, or other immune parameter of interest at the exclusion of others. This is a particularly important point to consider for assays aimed at detecting T cell responses in mixed populations. For example, in the standard ELIspot analysis, whole PBMC are added for the measurement of T cell precursors. While it is typically assumed that the responding populations are T cells it is difficult to be completely certain because PBMC are a heterogeneous mix of cells. One way to avoid potential lack of specificity is to use purified T cell populations, improving the confidence in the identity of the cells, but at the cost of additional labor and errors that accompanying additional steps. Evaluating the limits of detection and quantification is important when establishing the parameters of an acceptable positive immune response (3). The detection limit is that minimum concentration of a T cell, antibody, or other immunologic parameter that one can measure using the assay. Because of wide variability at lower concentrations, the detection limit may not be accurate. In contrast, the quantification limit is the lowest concentration that can be defined with significant accuracy and precision. The linearity of the assay is the ability to provide results that are directly proportional to the concentration of the analyte in the sample. The range is the upper and lower bounds in which the assay remains accurate, precise, and linear. For immunologic assays, the range is influenced by a number of factors including the concentration of the specific T cells as well as the total number of T cells. Lastly, the robustness of the assay must be assessed. Robustness measures the ability of the assay to maintain its capabilities and capacities by slight changes in the methodology.

Validation is essential prior to use of a specific monitoring assay in phase III clinical trials. Unlike assays for solutes or other single molecules, some immunologic assays are extremely variable for reasons that are not entirely understood. In fact, as will be discussed in a subsequent section, T cell-based assays can be extremely variable with precision error rates ranging from 25-50%. Some sources of error are identified during validation and can therefore be controlled or eliminated. It should be noted, however, that high variability does not mean that an assay cannot be validated. The validation process aids in defining the source and magnitude of the errors.

Table 2. Comparison of cryopreservation studies

Freezing media	Cell count/vial	Viability	Storage condition	Outcome	Reference
20% Human AB serum in RPMI/10% DMSO	$10x10^6$	Average of 97%	-196°C	Preserved cytokine secretion	6
90% Fetal Calf serum/10% DMSO	$5x10^6$	1-91%, median of 76%	Liquid nitrogen freezer	Viability predicts proliferative response	4
90% Fetal Calf serum/10% DMSO	$10x10^6$	≥85%	Liquid nitrogen freezer	Concordance of LPA response	52
10% FCS or Human AB serum in RPMI/10% DMSO	$10x10^6$	Median of 90%	-135°C vapor phase liquid nitrogen	Viability preserved over 12 years	53

4. LABORATORY ISSUES IN IMPROVING ACCURACY AND PRECISION

A number of sources of error can contribute to the outcome of immunologic monitoring and ultimately whether an assay can be used to evaluate an immune response. Two important potential sources of error are in cryopreservation and shipping of T cells. Cryopreservation of PBMC is of clinical importance in immunotherapeutic studies and often is not addressed or evaluated when developing methods of immunologic monitoring. The maintenance of the functional capacity of cryopreserved PBMC is essential when the cells are analyzed for antigen specificity at a time point distant from collection. The ability to effectively evaluate the function of lymphocytes that have been frozen will allow assessment of samples derived from the same individual over time simultaneously. Batching and analyzing samples as a group would reduce within-patient variability and allow selective study of subjects with specific outcomes. Batch analysis also eliminates inter-assay variability which can be significant. Cryopreservation of lymphocytes permits the analysis of specimens retrospectively, allowing assessment of assay precision and accuracy.

Several studies have shown that cryopreservation has an effect on T cell function. Many of these analyses have used retention of the viability of lymphocytes as the primary outcome measure for adequate cyropreservation. Investigation has demonstrated that viability is an excellent predictor of function. Data presented by Weinberg and colleagues suggested that in order to achieve consistently high viability, cryopreservation must be performed in laboratories whose staff members have been proven proficient in the technique (4). Furthermore, to obtain reliable results of functional assays, viability thresholds should be set. Those cyropreserved cells that do not demonstrate threshold viability should not be analyzed. In that study, 29 samples of PBMC were analyzed by 22 laboratories, and cell viability ranged from 4 to 96% (median of 68%). Samples frozen at laboratories with experienced staff members had viability greater than or equal to 70% and those sites which had viability less than or equal to 2% had similar results upon repeat testing. In a separate analysis, 45 specimens were evaluated for antigen-specific lymphoproliferation. Positive responses to CMV and pokeweed mitogen were significantly associated with increased viability of PBMC in each sample. When a median of 70% for viability was used as a threshold for evaluation, 100% of the samples had proliferative responses to PWM and 28% to CMV. In additional studies, investigators also assessed the feasibility of measuring CMV-specific cell-mediated immunity using cryopreserved cells (4). Specimens that were stored up to 2 years could still retain ≥85% viability. Stimulation of thawed lymphocytes in proliferation indicated that fresh and frozen cells had 84% and 85% concordance in HIV-infected patients and uninfected controls, respectively. Reimann and colleagues compared lymphocyte proliferation at multiple clinical sites using cryopreserved PBMC (5). Data demonstrated that if the viability of the samples was ≥85% after thawing, less than 10% of those specimens which had previous proliferative responses to mitogens or microbial antigens lost their responsiveness. Similarly, cytokine secretion can be preserved. Wang reported that upon PHA stimulation of cryopreserved T cells that retained 90% viability, the level of activity was comparable to that of fresh cells in the secretion of various lymphokines such as IL-2, IL-6, TNF-α, IFN-γ, and GM-CSF (6). Thus, when developing laboratory standard operating procedures of cryopreservation, the use of viability retention is an excellent method for prioritizing procedures and developing quality assurance programs.

There are several factors that contribute to successful cryopreservation of lymphocytes including the method of freezing, the procedure for thawing the frozen cells, and the media used for cryopreservation. In general, there are two methods laboratories utilize to freeze down cells. In one method, PBMC are processed and placed in alcohol-filled containers and are cooled at a rate of 1°C/min from 0°C to -70°C. A second more expensive method is to slowly cool processed cells to 1°C/min to -30°C and then 5°C/min to -70°C in the controlled rate freezer. When these two freezing process were compared, Venkataraman and colleagues reported there was no significant difference between the mean proliferative responses to both PHA and PWM and the IL-2-secreting abilities of cells frozen in inexpensive containers compared to cells frozen in controlled-rate freezers (7). No matter what the rate of freezing, it is recommended to keep cryovials in a nitrogen vapor phase to maintain high cell viability during long-term storage. Thawing and washing procedures can also attribute to recovery and viability of frozen cells. To prevent ice re-crystallization and cell disruption during the process of thawing, cells should be thawed rapidly in a 37°C water bath, DMSO diluted in warm media, and the lymphocytes should be washed once prior to analysis. Laboratories use a variety of freezing medias (Table 2).

A critical issue for multi-center clinical trials is the effect shipping samples has on the function of the T

cells. Betensky and colleagues evaluated the effects of shipping and handling on the T cell response to mitogens and recall antigens (8). The response to LPA by fresh versus shipped cells and fresh versus bench-held cells (held overnight in laboratory at room temperature) were compared. The odds of a positive response, to Candida, tetanus toxoid, or streptokinase were significantly higher for fresh specimens than for shipped specimens. Similarly, the odds of a positive response were significantly higher for fresh specimens than for bench-held samples for Candida, tetanus toxoid, and PWM. The investigators also found no difference in positive responses observed between heparin and ACD-processed samples or between heparin and ACD-tubes for any of the groups. Regardless of anticoagulant or stimulating antigen, there was a loss of LPA activity whenever the assay setup was delayed either by shipment or by overnight storage of whole blood.

A recent detailed analysis suggests one potential mechanism for the loss of function with the shipping of cryopreserved or fresh samples to distant locations. Nyberg-Hoffmann and colleagues reported the effect of shipping adenoviral vectors on dry ice on the infectivity of the vectors (9). As the dry ice thawed during transport, the CO_2 level inside the shipping container increased, which then resulted in the seepage of CO_2 inside the cryovials. The increased CO_2 acted as a reducing agent lowering the pH of the storage buffer resulting in loss of viral infectious titer. To solve the problem, a heavy-duty sealable bag with controlled permeability to CO2 was used as a secondary container for cryovials shipped with dry ice. Whether this is an etiology for the loss of viability of T cells after shipping has yet to be investigated.

In summary, the *in vitro* assays currently used to measure immune function are technically complex, prone to variability, and usually performed using fresh specimens. The precision and accuracy of complex immunologic assays could be greatly improved if specimens obtained at multiple sites could be analyzed in a single, highly skilled laboratory. For example, inter-assay variability would be eliminated if multiple specimens obtained over time could be analyzed simultaneously in the same assay. The ability to standardize these cellular-based assays would be greatly improved if a reliable method of shipping, handling, and cryopreserving human lymphocytes could be developed.

5. COMMON IMMUNOLOGIC MONITORING TECHNIQUES

Several assays have been used over decades of immunologic monitoring but only a few of these methods have emerged as reproducible and sensitive enough so that they can be applied systematically throughout the course of a human clinical trial. In general, the techniques that have emerged have focused on only a few immunologic parameters. These parameters are proliferation rates, cytokine release, and cytotoxic activity. All of these parameters represent functional attributes of T cells. Assays that measure these T cell functions can be qualitative and quantitative.

5.1. T cell proliferation

The T cell proliferation assay is among the most well-tested and used assays in immunology. The proliferation assay is a measure of a functional attribute of antigen-specific T cells. Specifically, proliferation assays are used to detect clonal expansion in response to antigen stimulation in contrast to proliferation of T cell attributable to survival, homeostatic proliferation, or bystander proliferation. Proliferation is measured by a number of different methods but the predominant assay employs the use of the DNA precursor thymidine labeled with ^{3}H-hydrogen (tritium) or ^{14}C-carbon. Other novel methods of measuring proliferation have become available in recent times and are discussed in a subsequent section. The results of the proliferation assay are expressed either as a stimulation index (SI), number of responding wells, or as an estimate of the numbers of antigen-specific precursors. The desired outcome measure dictates the extent of the number of replicates, and hence the number of cells required for each experimental condition. The SI refers to the ratio of the mean of the antigen-containing wells to the control (no antigen) wells. The accuracy and precision of the SI is improved with increasing replicates but an SI can usually be calculated down 3 replicates per experimental condition if the magnitude of the response is large. However, the SI provides only minimal information regarding the immune response and only shows that the population of cells (e.g. PBMC) responds with higher proliferation in response to antigens present in the well. If the precursor frequency of antigen-specific T cells in the bulk population is low then the data are not normally distributed. The Poisson model has been used extensively to evaluate proliferation in clinical trials. Using this method, with a sizable number of replicates (e.g. n=16-24), a cutoff can be established which is equal to the mean plus 3 standard deviations of the control (no antigen) wells. Uptake of thymidine into DNA that is in excess of this value then is considered significantly different from the proliferation measured in the control wells that do not contain antigen. It is then possible to compare the numbers of positive wells in the experimental group (i.e. with antigen) to the number of positive wells in the control group. This method was successfully used to map the major T helper epitopes of tetanus toxin (10). A recent study describes a further modification of this assay in the context of a phase I clinical trial evaluating the immunogenicity of a peptide vaccine targeting HER-2/neu (11, 12). This modified limiting dilution analysis approach was used to determine T cell immunity for 38 patients that completed a full regimen of peptide immunizations. The proliferation assay can also be adapted into regular limiting dilution assay (LDA) to directly estimate precursor frequencies although, as will be discussed, newer less laborious methods are available by which to carry out frequency analyses (13, 14). In recent times the use of the proliferation assay has declined in favor of newer assays that either provide additional information (e.g. cytokine flow cytometry) or are less cumbersome (e.g. ELIspot). A significant problem with the proliferation assay is that it is typically done with unfractionated cells and therefore does not provide information about the responding cell population. An additional drawback of the thymidine

incorporation assay is that extended incubation times of 5-8 days are needed thus permitting the interjection of *in vitro* artifact. Additionally, full and modified LDA require enormous amounts of cells which can be prohibitive in some cases where only small volume blood draws are available. Lastly, the proliferation assay is highly variable and therefore reproducibility is not always evident. The correlation of proliferation with clinical outcomes, such as regression or time to relapse is currently unknown.

5.2. Cytolytic T cell assay

The cytolytic T cell (CTL) assay is another assay that measures a specific effector function, cytolysis, typically attributable to the CD8+ T cells. This assay measures the ability of CD8 T cells to lyse antigen-expressing target cells in an MHC class I-restricted fashion. The standard chromium (^{51}Cr) release assay (CRA) that measures the cytolytic activity has long been the gold standard by which to monitor CD8 T cell responses. In this assay, target cells (e.g. tumor cells) are labeled with ^{51}Cr. In most cases, healthy target cells can retain the majority of the radiolabel over the course of the assay. Effector T cells are added to the target cells, usually at varying concentrations, and killing of the target cells is detected by release of the ^{51}Cr into the medium. Like proliferation, CRA assay can take many forms including simple setups aimed at detecting the presence of antigen-specific T cells to more complex designs such as LDA to measure antigen-specific T cell frequencies. Cebon and colleagues reported on a unique modification that permits evaluation of cytotoxic T cells in a semi-quantitative fashion without a full LDA analysis (15). Cells are plated with unpulsed or antigen-pulsed targets in a 24-well paired replicate format. Rather than scoring the percent lysis as is commonly done, the data is read out as the numbers of positive wells that are above a predefined cutoff value. In that study patients were immunized with both an HLA-A2 Melan-A peptide and an HLA-A2 influenza matrix peptide. A positive well was defined as one in which the percentage of T2 cell lysis was more than twice that of its paired control well and more than the mean and one standard deviation of the values of all of the control wells. Induction of a CTL response was defined as a doubling in the number of positive wells over baseline or an increase of at least 4 wells if baseline was 4 or less. The investigators observed increased levels of CTL activity to the Melan-A peptide in 3 of 28 individuals and to the influenza peptide in 9 of 28 individuals as well as some clinical responses. There was no correlation between the generation of immunity and a clinical response. The CRA lacks sensitivity, and CTL often require one or more cycles of *in vitro* expansion in order to be detected. Although the CRA provides useful information regarding the immune response it has been largely replaced by other assays which are either more informative, less laborious, or both. However, the CTL assay is still used on a smaller scale to verify the results of larger data sets such as those collected with ELIspot analysis (12).

5.3. Enzyme Linked Immunosorbent Spot (ELISpot) Assay

The ELIspot assay was originally described in 1983 as a method to detect low level frequencies of antigen-specific B lymphocytes (16). The assay, however, was not adapted for the detection of T cells until about 15 years later, primarily due to the lack of reagents and supplies. In recent years, it has become one of the workhorse assays of modern immunologic monitoring strategies. In principle, the assay is a modified capture ELISA assay that allows enumeration of individual antigen-secreting cells. In this assay, T cells are stimulated on a bed of cytokine-specific antibodies that have been coated directly onto nitrocellulose-based wells. Once stimulation has occurred, a fraction of cytokine that has been released binds to the coating antibody. The cells are then removed and a second detection antibody which has been conjugated to a detection reagent is used to detect the bound cytokine. The bound cytokine is at its highest concentration around the cell from which it was secreted. A number of commercial reagent systems have been developed in recent times for use in ELIspot assays. Since the assay directly and visually measures antigen-specific T cells, LDA techniques do not apply. Originally, the predominant use of ELIspot was to measure the CD8 T cell immune response to HLA class I peptides, but in more recent times it has been applied to tumor cells, tumor cell lysates, HLA class II peptides, and protein antigens. The majority of ELIspot assays are conducted to measure for IFN-γ-secreting cells, but newer antibody pairs are being defined that permit measuring other cytokines such as TNF-α, IL-4, and IL-5.

The popularity of the ELIspot assay has led to many publications evaluating its suitability for monitoring immune responses to either infectious disease or cancer vaccines. Many of these publications have addressed one or more of the various issues associated with suitability and validation. Scheibenbogen and colleagues studied the assay to look for reliability among different sites and to compare the assay with LDA (CRA). In this study six samples from healthy normal volunteer donors were assessed for influenza-reactive CD8 T cells at 4 different centers using ELIspot analysis and CRA. The comparison found essentially concordant results among all 4 centers with respect to the outcome of ELIspot analysis suggesting the possibility that conditions for the assay can be standardized. Furthermore, the assay showed remarkable correlation and agreement with the LDA suggesting that the ELIspot is an alternative assay to classical techniques. A significant advantage of the ELIspot, when compared to other assays such as CFC, is that the limits of detection are typically low ranging from 1:300,000 to 1:100,000. However at the lower frequencies, an *in vitro* stimulation step is required which may introduce artifact. Two major formats for the ELIspot are used to evaluate precursor frequencies, the 3-day and 10-day formats. The 3-day format is useful if the precursor frequencies are high such as with the case of viral antigens (17). In this assay, the effector cells are stimulated with antigen and APC for 24 hours followed by an overnight incubation with the secondary detection antibody. On the 3rd day the cytokine bound to the nitrocellulose plate is detected. In contrast, the longer 10-day ELIspot developed by McCutcheon and colleagues requires an intermediate *in vitro* sensitization step (18). The 3-day format has been used successfully to

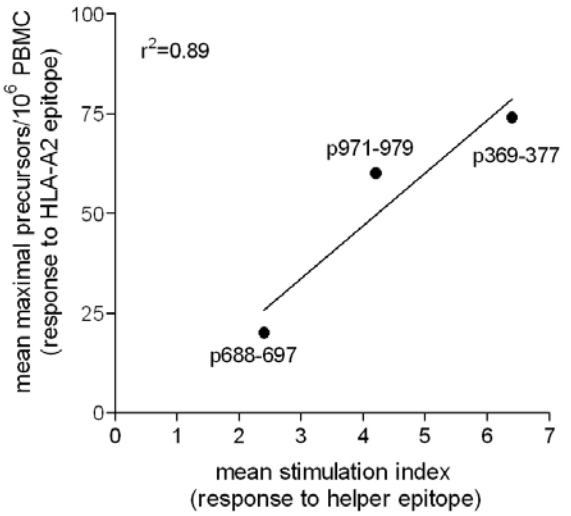

Figure 1. The magnitude of CD8 T cell immunity as assessed by IFN-γ ELIspot correlates with proliferative response to helper epitopes encompassing the CD8 T cell 9-mer epitopes. Shown is the linear correlation of the overall magnitude of CD8 T cell precursor frequency associated with helper peptide T cell response after vaccination. The y-axis is the mean of the maximal peptide-specific T cell precursor frequencies to the HLA-A2 peptides detected in 16 patients by IFN-γ ELIspot. The x-axis, on both graphs, is the mean of the maximal stimulation indices to the Th peptides calculated from 16 patients using T cell proliferation assays. The r2 value was determined with linear regression analysis and is the correlation coefficient. The HLA-A2 sequence designations of HER-2/neu shown in the graph are fully contained within the helper epitopes, p369-384, p688-703, and p971-984.

monitor immunologic responses to viral vaccines. For example, Smith and colleagues used it to monitor the increase in varicella-zoster virus-specific immunity in elderly individuals following booster immunizations. The precursor frequencies of varicella zoster-specific T cells are typically measured in the range of 1:20,000 to 1:2,000 (17). They also observed that enumeration of precursors by humans was similar to computer video imaging albeit with a higher intra-assay variation. They followed 165 subjects who had been immunized 8-10 years prior with either a single dose of inactivated virus (n=39), a single dose of attenuated live virus (n=104), or a double dose of attenuated live virus (n=22) and who received a booster with a single dose of a live attenuated virus (19). The ELIspot demonstrated that patients who had originally received a double dose of live attenuated vaccine had a stronger response (~2.2 fold increase) compared to individuals who received a single dose of inactivated or live attenuated (~1.6 fold and ~1.7 fold increase, respectively).

Knutson and colleagues used the 10-day format to monitor low level CD8 T cell precursor frequencies that were specific for HLA-A2 motifs contained with the tumor antigen HER-2/neu. In that study, patients with advanced stage breast and ovarian cancer received a vaccine that consisted of 3 helper peptides 14-18 amino acids in length, all of which encompassed HLA-A2 binding 9-mer peptides (12). Prior to immunization less than 10% of patients had preexistent immunity to either the full length helper peptides or the HLA-A2 peptides. After immunization the majority developed responses to the helper peptides as well as to the HER2 protein as assessed by proliferation analysis. The 10-day IFN-γ ELIspot revealed that CTL precursors specific for the encompassed HLA-A2 peptides were detectable in greater than 60% of patients following vaccination. The assay was taken through validation steps prior to use in the clinical trial with IFN-γ-coated beads which revealed that the assay had a detection limit of 1:100,000 and a detection efficiency of about 93%. Preliminary assays evaluating for CTL precursors specific for the HLA-A2 influenza matrix peptide using a range of PBMC concentrations showed that the assay was linear over a PBMC range of 1.0-3.5 x 10^5 cells. In a subsequent analysis, the results of the IFN-γ ELIspot were compared with the responses to the helper peptides as measured by proliferation assay (Figure 1). It was observed that the magnitude of the CTL responses was highly associated with the magnitude of the helper epitope proliferation responses. For example, the higher p369-377 responses correlated with higher responses to the longer Th epitope that fully encompassed it (r^2=0.89). These findings not only demonstrate agreement among different assays but also give credence to the idea that the results of the ELIspot assay may be a trustworthy representation of the actual immune response.

Despite the fact that the ELIspot analysis is a feasible and useful assay for the measurement of precursor frequencies, like most assays that measure T cell function there is a higher inherent variability than with other assays that do not directly measure a functional response (e.g. ELISA, RIA). Lathey studied the intra-assay, inter-assay, and biological variability of ELIspot (3). She analyzed the background (ie. no antigen, low) spots, response to Candida antigen (intermediate), and PHA (high). If the spot counts were below 20 the intra-assay coefficient of variation was >30. The variability however decreases with increasing numbers of spots until a plateau of 200 spots are achieved at which time the CV is 7-8. The mean CV at baseline in the absence of antigen is high at 45-50%. In response to stimulation with either Candida or PHA the CV drops to 30-40% and 0-20%, respectively. Lathey also observed that the inter-assay variability CVs were approximately double of the intra-assay variability CVs suggesting the assays be done in batch rather than sequential. Lastly, and aside from experimental variability, is the biologic variability which can be as high as the intra-assay variability. Biological variability is the measure of fluctuations in immunity that occur naturally. One approach to assess natural waxing and waning of the immune response is to follow the immune response of several viral antigens in individuals over time. Biologic variability could be an important factor in establishing the cutoff of what is considered a response to vaccination in individuals that already have pre-existent immunity to an antigen. While ELIspot is a desirable assay from a number

of different standpoints, it is clear that like other T cell-based assays, extreme variability is a limitation.

The inability of the assay to reproducibly detect response at lower precursor frequencies has led to the development of strategies to improve the signal without extending the time of *in vitro* stimulation. Jennes and colleagues described an approach that amplifies the responses by the inclusion of IL-7 and IL-15 to provide for a modification that the authors' termed the Amplispot assay (20). The addition of the cytokines to the 3-day format improved detection of antigen-specific CD4 T cells up to 2.4 fold and antigen-specific CD8 T cells up to 7.5 fold.

5.4. Cytokine Flow Cytometry (CFC)

CFC is another cytokine-based assay that has evolved into a method that can be applied directly to the monitoring of human clinical trials of immune-based therapies. The overall approach of the assay is to stimulate the T cells with antigen leading to the production of cytokines which are then trapped in the cell with the use of chemicals that block intracellular transit and secretion. The cytokines are stained with fluorochrome-conjugated specific antibodies following permeabilization and fixation of the cells. Several cytokine-specific antibodies are now commercially available that are conjugated to a wide variety of fluorochromes. Co-staining is performed with antibodies that detect cell surface markers or other surface molecules that demonstrate phenotype (e.g. CD4, CD8, and CD45). The cells are analyzed using flow cytometry. With the technological improvements in flow cytometers allowing for the simultaneous detection of multiple colors, exquisite phenotypic detail of the cytokine producing cells can be obtained.

A significant advantage of CFC is that it has been adapted for measurement of cytokine producing cells from whole blood without prior processing with agents such as ficoll (21). Unlike ELIspot, the limits of quantification and detection are problematic for CFC for a number of reasons. These limits tend to be at greater than 1 antigen-specific T cells in 10,000 PBMC or other cell types (e.g. CD3, CD4 T cells). Although CFC is advantageous because it has been adapted to measuring responses after only short periods (e.g. 6-8 hrs) of *in vitro* manipulation, this could pose a significant limitation on its ability to accurately measure all of the antigen-specific precursors since it would be expected that there would be a broad variability in the amount of time it takes to generate a recall response to antigens. Some studies indicate that different cytokines are elevated at different times during the recall response (22). Furthermore, there are noted differences in the time required to activate a CD4 T cell as compared to a CD8 T cell (23). Little is known about the temporal requirements for re-activation of memory T cells but it is likely that the time required for activation of the bulk of the antigen-specific T cells is heterogeneous. In a study by Nomura and colleagues it was found that activation of IFN-γ or TNF-α producing CD4 T cell specific for CMV antigen required at least 10 hours of antigen exposure for the maximal response (24). Incubation times of more or less decreased the response. The duration of exposure to toxic uncoupling agents such as Brefeldin or Monensin is one limitation to extended *in vitro* stimulation. Nomura observed that longer exposure times to Brefeldin enhanced the results up to about 10 hours after which decreases were observed. Similar to ELIspot, efforts have been made to enhance the response to antigen. Waldrop and colleagues showed enhanced activation of antigen-specific T cells by inclusion of monoclonal antibodies to the CD28 and CD49b costimulatory molecules (25). Unlike ELIspot, however, the intra-assay and inter-assay variability may be reduced. Normura and colleagues reported that the intra-assay CVs for IFN-γ and TNF-α were 8.4 and 4.1, respectively (24). However, the inter-assay variability was somewhat higher for both cytokines at 23.7% and 18.4%, respectively, indicating that the most appropriate way to evaluate with CFC is by batch analysis, rather than sequentially on different days.

Nonspecific background staining, which is likely attributable to many potential sources, is a major shortcoming of CFC keeping its limit of detection/quantification relatively high at >1:10,000 (antigen-specific T cells per total cell count). One source of error that has been identified is cytokine production by platelets and monocytes. Nomura and colleagues developed an exclusion gating strategy to minimize the signal contributed by monocytes and platelets. Staining with either CD33- or CD62P-specific 4th-color antibodies allowed them to filter out activated monocytes and platelets, respectively (21).

Recent clinical trials have demonstrated the feasibility of using CFC as a monitoring tool. Smith and colleagues used the technique to evaluate the immune responses in patients immunized with a modified HLA-A2, gp100 peptide, g209-2M (26). In that study, 29 patients were immunized with the modified peptide. While the bulk of the immunologic monitoring was done with peptide-specific tetramers, CFC was used in 9 patients to compare the immune responses to native HLA-A2 gp100 peptide (g209-217) and g209-2M. The assay revealed that the response to native peptide was generally lower, at about 75% of that observed to the vaccine epitope. In addition, the investigators also demonstrated that the results of the CFC were comparable to the tetramer assay in 3 of 4 patients examined. Although CFC has been used for several years, improvements are continually being made to cytometry equipment, reagents, and software, all of which have led to improved capabilities and the emergence of multiparameter CFC as an excellent tool for immunologic monitoring.

5.5. Tetramers

Tetramers represent the most direct approach to the identification and visualization of antigen-specific T cells. Tetramers are composed of four MHC class I molecules, each bound to a specific peptide of interest. The MHC molecules are held together by biotinylating each monomer followed by binding to flourochrome-conjugated avidin. As a tetramer, the MHC class I molecules bind with greater affinity to the T cell receptor TCR than they would as monomers (27). Recently, MHC class II

tetramers have been developed to identify CD4 T cells (28). The limit of detection of the assay has been reported to be greater than 1 CD8+ T cell per 10,000 freshly prepared peripheral blood mononuclear cells which is consistent with limitations of flow cytometry based methods such as CFC (27). Cells are typically co-stained with fluorochrome-conjugated anti-CD8 T cells in order to enumerate only those cells that co-express both CD8 and the antigen-specific TCR. When used alone with CD8 staining, tetramers only provide information about the TCR of the CD8 T cell but nothing related to the overall phenotype or function of the cell. Because tetramer staining of cells does not require activation of the T cells, the variability is similar to regular flow-cytometry with intra- and inter-assay variability typically less than 10%. There has been interest in combining this approach with an activation step to ensure that the identified T cells are functional. A major problem with this strategy is that activated T cells tend to down regulate surface expression of the TCR following activation. An alternative approach would be to run parallel samples, one stained with tetramers and the other taken through a CFC assay, ELIspot, or CTL assay. Studies comparing tetramer assays to these other assays have, however, revealed that the tetramer assays consistently tend to show higher precursor frequencies (29-32). Although the reason for these discrepancies is unclear there are some potential mechanisms that may be implicated in the observation. First, as previously mentioned, if the cytokine-based assays to be used as comparators are not optimized to detect all of the responding cells, discrepancies may be observed. Alternatively, it could be possible that not all of the tetramer binding antigen-specific T cells are functional as has been reported (33). Engstrand and colleagues have previously shown that while immunosuppressed patients possess tetramer positive pp65-specific T cells, a fraction of these cells are functionally impaired. Whatever the mechanism for the discrepancy, one caveat to tetramer analysis is that it may overestimate functional immunity.

Drawbacks to tetramer analysis is that its application is limited by knowledge of biologically relevant MHC epitopes contained within tumor antigens, the sources for tetramers are extremely limited, and the analysis is expensive. Tetramer analysis as a tool for immunologic monitoring is typically confined to clinical trials involving immunization with the peptides to which the tetramers are targeted. The bulk of the human clinical trials in which tetramers were used focused on melanoma where many biologically relevant tumor antigen-derived peptides have been identified including Mart-1, gp-100, and tyrosinase (34-40). Weber and colleagues recently published the results of a clinical trial involving 48 patients receiving 2 HLA-A2 peptides, $gp100_{209-217}$ and $tyrosinase_{368-376}$ admixed with either IFA, or with both IFA and GM-CSF (41). The study followed 39 of the patients with peptide-specific tetramers and set a cutoff of 0.01% CD8 T cells as a response to immunization. Prior to immunization, all patients tested did not have levels of above the cutoff. Following immunization, 33 patients had tetramer- positive cells of greater than 0.01%. For the patients that received the vaccine with IFA alone, the tetramer-positive cells increased from below the limits of detection to 0.028% of total CD8+ T cells. For patients receiving the vaccine with IFA and GM-CSF the post-immunization value was 0.083% of total CD8 T cells.

6. EMERGING TECHNIQUES AND CONCEPTS

Quantitative methods of T cell analysis are becoming more robust and reproducible. Flow cytometric-based assays, such as cytokine flow cytometry and MHC tetramer analysis, use a minimal amount of patient material but are, in general, less sensitive in detecting responses. Methods that require an *in vitro* stimulation, such as ELIspot, are more sensitive with a lower limit of detection, but require significant patient material for analysis. The next generation of assays currently under development focuses on increasing the sensitivity of detection while maximizing the amount of information obtained concerning the character of the immune response. In addition, newer techniques are being developed that more closely simulate the terminal function (e.g. proliferation and lysis) of tumor antigen-specific effector cells.

The analysis of the cellular immune response by real time PCR (RT-PCR) can be quantitative and sensitive. Indeed, the method can be used not only to assess changes in peripheral blood mononuclear cells (PBMC) after active immunization but also changes in the tumor itself. Primarily, RT-PCR has been used to determine a comprehensive cytokine profile in stimulated PBMC after immunization. The benefits of RT-PCR in immunologic monitoring are that the method is sensitive and can detect approximately 1/20,000-1/50,000 antigen-specific T cells. Furthermore the assay does not require *in vitro* expansion and yields a great deal of information about the phenotype of the response (42). Most notably, RT-PCR can be performed on very minimal amounts of material. In fact, using RT-PCR to analyze the cellular immune response occurring after vaccination against a tumor antigen in mice can be monitored serially in murine blood without euthanizing the animal (43). The disadvantage of RT-PCR is that, while the assay can give comprehensive information concerning gene expression, the method does not provide any indication of actual protein expression. Furthermore, RT-PCR can not discriminate between various cell subsets (42).

Methods have been developed to detect secreted cytokines in small samples of peripheral blood (44). Beads coupled with antibodies specific for a variety of cytokines can be used to capture secreted proteins found in blood after the activation of a specific immune response. Techniques have been developed to allow the simultaneous detection of 15 immune related cytokines in a single blood sample. This type of analysis has demonstrated performance characteristics well within guidelines for a clinical assay (44). The use of antibody-coated beads will allow adaptation to flow cytometric analysis where specific evaluation of cellular subsets can be performed readily. Development of highly reproducible assays that can determine multiple immune response related parameters will allow fine-detail characterization of the tumor antigen-specific immune response generated after vaccination.

Terminal functions of tumor antigen-specific effector cells are, in general, proliferation due to antigen recognition and death of a target cell. The development of biomarkers to quantitatively assess clonal proliferation after antigen stimulation and the generation of antigen- specific immunologic memory is a goal of immunologic monitoring. One of the problems with the incorporation of radiolabeled thymidine is that it is often not possible to determine the optimal incubation time to measure proliferation. Techniques have been developed, such as the measurement of serial halving of the fluorescent intensity of the vital dye carboxyfluorescein diacetate succinimidyl ester, or CFSE, that reflect a lifespan of proliferation in a highly quantitative fashion. CFSE diffuses through the cell membrane, and the protein has a very low turnover rate. One can assess the rate of proliferation by measuring the serial halving of the number of CFSE staining cells. Recent studies have demonstrated that this method of analysis can detect 8-10 cycles of cell division by flow cytometry (45). Not only can CFSE analysis assess the proliferative potential of the immune response, the technique can also determine the kinetics of that response.

Effective immunization results in the development of immunological memory which is antigen specific, often lifelong, and results in a heightened response to the pathogen upon re-infection. The generation of memory T cells is a multi-step process that starts with antigenic stimulation of naïve T cells during a primary response. Upon activation there is rapid division of these antigen-reactive T cells. The expanded T cell population then differentiates into effector cells which are G_1 stage lymphocytes. This process takes 4-5 days during which there are changes in their expression of cell surface adhesion molecules (46, 47). The differential expression of adhesion molecules allows the effector T cells to migrate to distinct areas where they come into contact with the targeted antigen. After the response has subsided, the majority of the effector cells are destroyed, and only a fraction of primed T cells persist as circulating memory cells that can provide protection and give, upon secondary challenge, a more rapid and effective response. After immunization, T cells undergo quantitative and qualitative changes which result in the development of memory T cells. First, there is an increase in the frequency of antigen-reactive T cells, and this increased frequency can be maintained for long periods of time. Secondly, unlike naïve T cells, memory T cells express different cell surface markers and behave in functionally different ways (46, 48). The characteristic surface phenotype of memory T cells includes upregulation of CD44 and integrins and downregulation of CD62L and high molecular weight CD45 isoforms (46). Based on their proliferation *in vivo* and the expression of activation markers, memory T cells comprise two distinct subsets, "central memory" T cells (T_{CM}) and "effector memory" T cells (T_{EM}). T_{CM} express the lymph node homing receptors CD62L and CCR7 and lack immediate effector function. However, upon a secondary challenge, they can stimulate DCs and also differentiate into effector T cells. T_{EM} do not express CD62L or CCR7 but rather express receptors for migration to non-lymphoid peripheral tissues to mediate inflammatory reactions or cytotoxicity. The identification of phenotypic changes that occur in a T cell as it makes a transition to a memory cell has allowed the development of methods that can more specifically quantitate and phenotype memory cells, i.e. flow cytometric methods. Memory/effector subsets of CD4+ T cells are delineated by differential expression of CD45RO isoforms which can be easily characterized with flow cytometry. In addition, other surface markers, specifically CD62L and CCR7, can be evaluated to differentiate between effector memory T cells and central memory T cells.

The lytic potential of an antigen-specific T cell has long been acknowledged as the functional measure of viral eradication. Likewise, the generation of cancer-specific CTL has been touted as the major goal of a tumor antigen-specific vaccine. Quantitative assays are being developed that measure specific lytic and apoptotic functions ascribed to antigen-specific CTL. The release of chromium from labeled target cells after they have been destroyed by cytolytic T cells has been the gold standard for the assessment of CTL activity. Unfortunately, chromium release (^{51}Cr) assays are fraught with technical problems that make them difficult to adapt to analysis of multiple specimens and even more difficult to standardize. First, ^{51}Cr assays require incubating target cells with radiolabel. Chromium is not only toxic to cells limiting the time of incubation with CTL, but it also inconsistently labels human tumor cells. In addition, a quantitative ^{51}Cr release assay requires the analysis of multiple concentrations of effector cells and targets in a limiting dilution format. Consequently, patients must be able to undergo a leukapheresis to allow a detailed analysis of precursor frequency. Investigators have circumvented the need for ^{51}Cr by developing non-toxic methods of cell labeling. Snyder and colleagues describe a "Lysispot" assay as a measure of direct target cell killing (49). In the Lysispot, target cells are transduced to express a foreign marker, in this case *Escherichia coli* β-galactosidase or β-gal. β-gal is a non-secreted foreign protein, and high avidity monoclonal antibodies are widely available, specific for β-gal, facilitating assay development. Simply, β-gal is introduced into a target cell via a viral vector such as herpes simplex. Maximal amounts of β-gal are produced in the target cell within 3 hours, thus the lytic assay can be performed in a minimal period of time. If CTL specific for the target are present, the target cell will be lysed, β-gal will be released and imbedded on a nitrocellulose membrane impregnated with anti-β-gal antibodies. Complementary antibodies specific for β-gal can be used to develop the membrane, and then spots are counted that correspond to individual lyse target cells. Results demonstrate that the Lysispot compares favorably to both ELIspot and chromium release assays. Furthermore the Lysispot assay is adaptable to both human and murine experimental systems (49).

Further variations on the analysis of lytic activity actually focus on the measurement of enzymes and substrates involved in initiating the lytic and apoptotic cascade in cell death. The measurement of granzyme B production and release by CTL has been adapted to a

highly quantitative format (50). The development of the assay is based on the basic biologic function of antigen-specific CTL that release granzyme B and perforin when they recognize antigen in the context of MHC. Similar to the Lysispot described above, nitrocellulose membranes are impregnated with antibodies specific for granzyme B. When the enzyme is released by activated T cells in the presence of their specific target, secreted granzyme is bound to an antibody and presumably can be detected by an additional granzyme B-specific antibody. Individual spots on plates represent an activated T cell in the process of lysing its target (50). Measurement of granzyme B release result in markedly decreased assay backgrounds as compared with the standard chromium release assay. In addition, the method was adaptable to direct *ex vivo* measurements of CTL precursors without a prolonged *in vitro* stimulation (50). Finally, a recently described approach enumerated CTL via the measurement of CTL-induced caspase activation in target cells which will result in their apoptosis (51). The use of fluorogenic caspase substrates allows not only the quantification of lytic CTL but also directs visualization of the effect. This approach may be more sensitive than measuring up stream effects of lytic initiation by CTL. In general, CTL kill target cells by one of two means, either directly lysing the cell via perforin or granzyme pathways, or inducing apoptosis via activating the fas/fas ligand pathway. An immediate consequence of either method of inducing cell death is activating the caspase cascade within the target cell. Thus, the approach described by Liu and colleagues may allow assessment of all effected target cells whatever their method of cell death. The measurement of caspase induction has been adapted to flow cytometric analysis and termed the FCC assay (fluorescent based cellular cytotoxicity). In murine models of LCMV the FCC assays were quite comparable to chromium release (r=0.8754) (51).

Novel methods of immunologic monitoring are being developed to be reproducible at low precursor frequencies and to provide a maximal amount of information concerning the nature of the immune response elicited after any particular intervention. Techniques are being developed which will delineate a response in an effector cell that would presumably correlate with a beneficial clinical outcome. Whether any of these assays will function as a validated biomarker for an effective immune response must be addressed prospectively in large randomized clinical trials of immune-based cancer therapy.

7. CONCLUSIONS

Over the last decade, significant molecular and cellular technologies have emerged permitting the testing of novel T cell-targeting immune-based approaches in the clinic to treat and prevent cancer. As a result, the need for reproducible and informative immunologic assays for assessing T cell function has increased. A number of assays have been developed for monitoring human clinical trials including proliferation, cytotoxicity, ELIspot, CFC, and tetramer assays. In recent times, emphasis has been placed on assays that provide fine detail of the immune response and are relatively easy to use in a clinical trial setting. Appropriate validation techniques will aid, and careful consideration of several laboratory variabilities may improve, the reproducibility of T cell assays. Alternatively, a number of newer assay concepts have been recently described which may ultimately replace currently used assays. Informative immunologic monitoring of T cells will certainly be one of the driving forces in advancing the field of tumor immunology.

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Abbreviations: ACD: Acid citrate dextrose, CFC: Cytokine flow cytometry, CFSE: Carboxyfluorescein diacetate, succinimidyl ester, CRA: Chromium release assay, CTL: Cytotoxic T lymphocytes, DMSO: Dimethylsulfoxide, DNA: Deoxyribonucleic acid, ELIspot: Enzyme linked immunosorbent spot , FCC: Fluorescent based cellular cytotoxicity, GM-CSF: Granulocyte macrophage-colony stimulating factor, IFN-g: Interferon gamma, IL-2: Interleukin-2, IL-6: Interleukin-6, LCMV: Lymphocytic choriomeningitis virus, LDA: Limiting dilution assay, PBMC: Peripheral blood mononuclear cells, PHA: Phytohemaglutinin, PWM: Pokeweed mitogen, TNF-alpha: Tumor necrosis factor alpha

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