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Immunologic Monitoring of Cancer Vaccine Therapy: Results of a Workshop Sponsored by the Society for Biological Therapy

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Summary: The Society for Biological Therapy held a Workshop last fall devoted to immune monitoring for cancer immunotherapy trials. Participants included members of the academic and pharmaceutical communities as well as the National Cancer Institute and the Food and Drug Administration. Discussion focused on the relative merits and appropriate use of various immune monitoring tools. Six breakout groups dealt with assays of T-cell function, serologic and proliferation assays to assess B cell and T helper cell activity, and enzyme-linked immunospot assay, tetramer, cytokine flow cytometry, and reverse transcription polymerase chain reaction assays of T-cell immunity. General conclusions included: (1) future vaccine studies should be designed to determine whether T-cell dysfunction (tumor-specific and nonspecific) correlated with clinical outcome; (2) tetramer-based assays yield quantitative but not functional data (3) enzyme-linked immunospot assays have the lowest limit of detection (4) cytokine flow cytometry have a higher limit of detection than enzyme-linked immunospot assay, but offer the advantages of speed and the ability to identify subsets of reactive cells; (5) antibody tests are simple and accurate and should be incorporated to a greater extent in monitoring plans; (6) proliferation assays are imprecise and should not be emphasized in future studies; (7) the reverse transcription polymerase chain reaction assay is a promising research approach that is not ready for widespread application; and (8)there is a critical need to validate these assays as surrogates for vaccine potency and clinical effect. Current data and opinion support the use of a functional assay like the enzyme-linked immunospot assay or cytokine flow cytometry in combination with a quantitative assay like tetramers for immune monitoring. At present, assays appear to be most useful as measures of vaccine potency. Careful immune monitoring in association with larger scale clinical trials ultimately may enable the correlation of monitoring results with clinical benefit. **Key Words:** Cancer vaccines—Immunologic monitoring—Immunotherapy.

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IMMUNE MONITORING: A DEVELOPMENTAL AND REGULATORY PERSPECTIVE

On November 8, 2001, the Society for Biological Therapy sponsored a 1-day workshop on immunologic monitoring of cancer vaccine trials that was held at the Natcher Auditorium on the campus of the National Institutes of Health in Bethesda, MD, U.S.A. The purpose of the workshop, which was organized by Michael Atkins, M.D., Ulrich Keilholz, M.D., and Jeffrey Weber, M.D., Ph.D. was to have an in-depth discussion about the roles various immune assays play in the monitoring of cancer vaccine trials. The organizers wished to address what state of the art assays were available for immune monitoring, discuss their methodology in depth, determine the usefulness, advantages, and disadvantages of each of the assays, and assess if there was a consensus for preparing guidelines for the most useful immune monitoring tools for evaluation of cancer vaccine trials. The Workshop had 120 participants, including academic clinical investigators and bench scientists, members of the biopharmaceutical industry, and representatives of the National Cancer Institute and the Food and Drug Administration (See Appendix 1).

The Workshop was felt to be a timely endeavor. Since the discovery of the first T-cell defined tumor antigen in Thierry Boon's laboratory a decade ago, numerous early phase clinical trials have been conducted with antigen-specific vaccine approaches. A wide variety of immune assays have been used in these trials without standardization of either approach or technique. The establishment of common methodologies and agreed upon assays for the measurement of immune responses in patients with cancer receiving vaccine therapy was felt to be critical to the assessment of immunologic effectiveness of these various approaches and to enable comparison of the results of different trials. Various nonantigen-specific vaccines are currently being tested in phase III trials, and the time was felt to be rapidly approaching when antigen-specific vaccine approaches would be sufficiently refined to justify their being evaluated in a similar setting. The ability of laboratory assays to function as surrogate markers for clinical benefit or at least indicators of immune response was felt to be a critical factor in performing such trials and in cancer vaccine development in general.

The format of the Workshop included lectures on important issues in the field of cancer vaccines and six breakout sessions to discuss specific immune monitoring modalities (Appendix 1). A number of questions and issues were to be addressed by each breakout group. These included a brief review of the principles of the specific assay, a discussion of the methodology, how well developed and reliable the assay was, a summary of how the assay has been used to measure immune responses in vaccine trials, a discussion of whether clinical relevance has been established for the assay, a description of the clinical scenarios for which the assay would best be suited, and a comparison of the assay with other assays. The breakout-session leaders then reported on the consensus reached within their session regarding these issues. A panel discussion ensued in which the conclusions presented in the current document, particularly those in the summary, were developed. The Workshop included lectures on two issues felt to be of importance to cancer-vaccine development. Dr. Norman Letvin discussed lessons learned from HIV-vaccine development, a field that might allow cancer-vaccine researchers to learn from the lessons and mistakes of medical history. Dr. James Finke and Dr. Dmitry Gabrilovich addressed the critical issue of tumor-related immune suppression, covering aspects of T-cell and dendritic cells (DC) dysfunction. Although much effort in the field has focused on the generation of potent and long-lasting immune responses in patients with cancer, emerging data indicate that the success of any immune strategy in tumor bearers depends on the ability to overcome active and passive immune suppression. Summaries of Dr. Finke's and Dr. Gabrilovich's lectures are included in this document.

The six breakout sessions each discussed an immune assay that was felt to be important for cancer-vaccine development. Breakout session leaders then wrote conclusions of their session for publication in this document (Appendix 2 lists the authors of the specific sections of this document). The reports establish some common guidelines for the performance and interpretation of these assays and describe the relative merits of each particular assay. Assays fell into one of three groups: (1) an assay that has promise but needs further development; (2) an assay that is unlikely to be of great usefulness in monitoring cancer vaccine trials; and (3) an assay that, either alone or combined with others, has the greatest likelihood of providing important information in a cancer-vaccine trial. Relatively little information was available to the participants on the clinical value or relevance of any assay. Such data can only derive from large trials with long follow-up examinations, which are mostly lacking in this field. Thus, any conclusions reached on the clinical relevance of these assays will be tentative at best. However, significant information was available to discuss the scientific importance of each assay and its ability to provide valid information regarding specific immune activation.

As our understanding of the immunologic basis of vaccines used for the treatment of patients with malignancy becomes more refined, critical questions that need to be addressed include an assessment of those strategies, which are most efficacious and a consideration of the optimal patient populations to be enrolled in vaccine studies. Whereas most tumor vaccines have been, or are being evaluated in patients with advanced or end-stage malignancies, it is possible that patients with minimal residual disease would be most likely to demonstrate a robust antitumor immune response. In addition, the tumor-vaccine community will need to address those components of vaccine development that will be necessary to gain U.S. registration, namely tests of identity, purity, potency of the vaccine product, as well as the demonstration of safety and efficacy (1). The development of reproducible immunologic assays might result in the application of these techniques in product potency assays as well as the use of the assays as an immune "pharmacokinetic" measurement to help prioritize strategies for larger scale clinical trials.

Relevant issues that manufacturers of tumor vaccines face in formulating a final product for human administration are the: generation of a consistent product from inconsistent starting material; qualification of the source and characterization of the cell substrate used to produce the vaccine; evaluation of the effect of cryopreservation on yield, viability, and activity of the vaccine; timing and methods of sterility testing; and lot release specifications for identity, purity, and potency.

Potency carries a strict regulatory definition: "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" (CFR 21.600.3). Potency assays serve as measures of quality control and ensure that patients receive a standardized and reproducible vaccine dose on each treatment. Even though there are no U.S. Food and Drug Administration standards at this time, the Food and Drug Administration is formulating recommendations for each vaccine class. Surrogates for potency may be established using either an in vitro cell-based assay or an in vivo animal model "to affect a given result." In the context of drug development, it is important to develop reliable measures of potency before conducting pivotal (Phase 3) clinical trials to assure that all study patients receive similar vaccine doses throughout the trial and that a consistent product can be guaranteed for a wider patient population after licensing approval.

In assessing potency, immunoassays or tests that measure a predesignated immunologic parameter may be useful, but will require adherence to standard operating procedures, standardization, and validation to be deemed reliable. Assay validation will require an evaluation of assay accuracy, precision, limit of detection, limit of quantitation, specificity, linearity and range, ruggedness, robustness, and system suitability.

Clinicians have an expectation that with each laboratory test they employ, the result is backed by a rigorous analysis of a reference population to define baseline responses as well as validation of the reproducibility of the result. These data are rarely published or discussed in reporting results from cancer-vaccine trials; however, the steps needed for assay-validation are well defined. Quality control monitoring and assay validation is composed of several analytical measures. These measures are routine, for the most part, for serologic studies, but have not been consistently applied to T cell-based methods of immunologic monitoring. First, is an assessment of "accuracy." Accuracy refers to the correctness and exactness of the test result. It is defined as the closeness of a test result to the true value and can only be calculated by comparison with a standard. Definitions of standards are difficult for T-cell assays. However, the use of known foreign disease antigens and the generation of T-cell clones specific for positive control antigens such as cytomegalovirus (CMV) and influenza (FLU) may provide standards for determining the accuracy of T-cell methodologies to be evaluated. A second important measure is "precision" or the reproducibility of the test. Precision is defined as the closeness of the test results to one another when using the same specimen. Precision is expressed as a standard deviation and coefficient of variation of multiple sample runs. The same sample can be run multiple times on the same plate or on the same day, defining interassay precision; or the same sample can run multiple times over several days, defining intraassay precision (within run and within day). Identification and assessment of known positive specimens is essential for this type of analysis. The "limit of detection" of a method is the capacity of the method to detect small amounts of a substance with some assurance and can be assessed by spiking a negative sample with known quantities of well-characterized T cells, for example, CMV clones in a CMV negative human leukocyte antigen (HLA) matched donor. The limit of detection of an assay is distinct from its "sensitivity," which is the proportion of true positives that test positive (i.e., high sensitivity equates to a low amount of false negatives). The sensitivity of an assay is the measurement most affected by background "noise." "Specificity" reflects the ability of a method to measure true negatives as negative (i.e., high specificity equates to a low amount of false positives). Interference assays

that dilute a defined T-cell population with one of another specificity (e.g., diluting a specific number of FLU T-cell clones with CMV clones and analyzing FLU-specific responses) would allow an estimation of the specificity of the analysis.

The "reliability" of a method describes the ability of the test to maintain accuracy, precision, sensitivity, and specificity despite changes in external factors such as technicians, instruments, or reagents. An assessment of reliability can be made using a well-characterized reference population. The reference population is the group of individuals assessed to determine the parameters of a "normal" value. If the well-defined values of this group fluctuate significantly with changes in the test, then, the assay is probably not reliable enough for repetitive use or application to the measurement of large numbers of samples.

In developing standard operating procedures, a working standard, controls, and acceptance criteria for each parameter should be provided. Even though it is not necessary at phase 1 or 2 (unless used for determination of patient trial eligibility), immunoassays will normally require validation at the time of initiation of phase 3 trials. This is not a trivial issue. From the Food and Drug Administration perspective, lack of a validated immunoassay for potency testing could lead to the nonapproval of a biologic licensing application.

Immunoassays or bioactivity endpoints may also prove relevant as surrogate markers in tumor-vaccine efficacy trials; however, these will only be of value if an effect on the surrogate does in fact correlate with a real clinical benefit. From the regulatory perspective, the strict definition of a surrogate endpoint is: "a laboratory measurement or a physical sign used as a substitute for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions, or survives and which is expected to predict the effect of therapy" (57 Federal Register 13234 and 13235, April, 1992). A new methodology or measurement will have to stand the test of time, because demonstration of substantial correlation with clinical benefit generally will include validation from adequate and well-controlled trials. The emergence of new technologies, some of which may have clear advantages over earlier assays, may compromise the interpretation of studies based on older methods. Furthermore, several disadvantages may accompany the use of surrogate endpoints as the basis for tumor-vaccine approval. For example, in compressing the extent and duration of phase 3 trials, important treatment related side effects may be masked. Such adverse events could unfavorably influence the final outcome of the trial and conclusions drawn about the therapy, especially when tumor vaccines are given chronically. At this time, immune-response parameters are more relevant as pharmacodynamic endpoints than as correlates of clinical response.

MECHANISMS OF IMMUNE DYSFUNCTION

Mechanisms of T-Cell Dysfunction in Cancer

T-Cell Dysfunction and Impaired Tumor Immunity

Although a number of immunotherapy strategies have resulted in increased frequency of tumor-specific T cells in the blood and in tumors, the generation of antitumor immunity has not correlated with clinical response. Tumors may evade immune surveillance by distinct mechanisms. Some of these are directed towards altering the signaling and function of T cells including T-cell anergy (antigen-specific unresponsiveness), TH2 bias, T-cell unresponsiveness (generalized dysfunction), and T-cell deletion (apoptosis). One or more of these mechanisms may represent a barrier to the development of an effective antitumor immune response.

T-Cell Anergy

T-cell anergy is induced in multiple murine tumor models as shown by defective interferon (IFN)-γ production and proliferation in response to specific and nonspecific stimuli. This appears to be an early event in tumor progression (2). The functional status of tumor-specific T cells has been examined in patients with cancer. Tetramer analysis has facilitated detection of tumor reactive T cells in blood and tumor. The combined use of tetramers and cytokine gene expression (real time PCR) or protein production has provided some insight into the functional activity of these cells. To date, a few studies have evaluated the functional activity of tumor-specific T cells in the blood after vaccination with melanoma antigen-derived peptides (3,4). The findings have been mixed. One study demonstrated that antigen-specific CD8+ T cells in peripheral blood did not produce cytokines, whereas other vaccine studies showed that tetramer-positive cells were capable of producing IFN-γ in response to peptide (3,4).

Antigen-specific T cells have also been detected by tetramer binding within melanoma tumors after peptide vaccination. Immunization was apparent in 8 of 11 lesions as shown by IFN-γ mRNA expression that appeared to correlate with high expression of the gp100 antigen by tumors (5). However, IFN-γ expression was

not accompanied by a significant inflammatory response or CD4/CD8 T-cell accumulation (5,6). Detection of these antigen-reactive T cells in the tumor did not correlate with clinical response. There are also data showing that MART-1 specific CD8+ T cells can traffic to tumor and be detected in lesions after adoptive transfer of antigen-specific T-cell clones (7,8); however, the functional status of these cells was not assessed.

The detection of tumor antigen-specific T cells in the blood and tumor in the absence of clinically detectable tumor regression, demonstrates the need for further functional analysis of these effector cells. Even though these studies and others demonstrate that tumor-specific T cells can infiltrate human tumors after immunotherapy, it is not clear whether these cells are typically activated and functional, in an anergic state, or undergoing apoptosis.

If there are functional defects and altered signaling in tumor antigen-specific T cells from patients with cancer, are they similar to those described for classic anergic T cells (9,10)? Anergic T cells display a proliferation defect and impaired production of interleukin (IL)-2. There are also data suggesting that anergy is characterized by alterations in T-cell signaling (11). Anergic cells have a lack of activation of lck, ZAP-70, Ras, ERK, AP-1, and NFAT molecules. The anergizing stimulus also appears to activate the protein tyrosine kinase fyn, increase intracellular calcium levels, and activate RAP1. There is upregulation of the cyclin-dependent kinase inhibitor p27kip1 that may block cell-cycle progression (11). Whether T cells that are unresponsive in patients with cancer fit this functional and signaling profile of anergic T cells has not been well addressed, nor has the difference in the functional status of tumor-specific T cells within tumors after vaccination versus adoptive transferred T-cell clones been studied. This is partly a result of the difficulty of assessing signaling events in tumor-specific T cells because of their low numbers. There are also data to suggest that although IFN-γ mRNA is expressed in tumors after vaccination, there is an absence of mRNA for IL-2, a critical growth factor for T cells (5). Additional techniques that allow assessment of function and signaling on small numbers of cells are needed. A summary of the mechanisms that may play a role in the induction of T-cell anergy in patients with cancer is provided in Table 1.

Defective DC-induced signaling of T cells caused by lack of costimulation or CD40 ligation accounts for anergy induction in murine tumors (12). In these animal models, antigen-presenting cells (APC) can mediate T-cell tolerance to self-antigens caused, in part, to a failure to activate APC via CD40. Tolerance of tumor-specific CD4 T cells can be reversed with in vivo ligation of CD40 (13). What role a defect in CD28 signaling and/or CD40 ligation plays in T-cell tolerance in patients with cancer is not known, because it has been difficult to detect tumor-specific CD4 T-cell responses. Recent studies in transgenic mice demonstrate that expression of a tolerizing antigen does not prevent the in vitro stimulation and recovery of T cells capable of mediating tumor destruction (14). Even though defective APC and CD40 signaling may be important to the induction of T-cell anergy in human cancers, other mechanisms are likely to account for the inability of activated effectors cells to induce tumor destruction.

TABLE 1. *Causes of anergy or Th2 biasing of the T cells from patients with cancer*

Defective DC stimulation of T cells:
No costimulation
No CD40 ligation; tolerance reversible with in vivo ligation of CD40
Persistent antigenic stimulation of T cells by tumor:
TGF-β
Drive the balance to Th2 via IL-10 as an intermediate
Inhibit Th1-type responses directly
IL-10
Downregulates the expression of Th1 cytokines
Regulates expression of TGF-β type II receptor
Clearance of antigen-specific IFN-γ producing T cells via Fas mediated-apoptosis

DC, dendritic cells; IFN, interferon; IL, interleukin; TGF, transforming growth factor; Th, T helper cell.

Dysfunction of antigen-specific T cells has been detected and studied in persistent infections where high doses of virus are thought to cause clonal exhaustion (15). The use of major histocompatability complex (MHC) tetramers has revealed a complex process where there is activation-induced cell death of T cells that recognize the nucleoprotein 396 of lymphocytic choriomeningitis virus. These cells become dysfunctional (loss of cytokine production), annexin V positive, and disappear. In contrast, T cells reactive against the glycoprotein GP33 become anergized, but do not undergo activation-induced cell death; their persistence (15) is a result of the different degree of antigen stimulation. Continuous antigenic stimulation by tumors may contribute to immune dysfunction in antigen-specific T cells in patients with cancer, although this possibility must be examined in detail.

Interleukin-10 plays a role in the induction of T-cell anergy by inhibiting the CD28 costimulatory pathway (16). However, IL-10 only blocks T cells stimulated by low numbers of triggered TCRs, a condition that is dependent on CD28 costimulation. Interleukin-10 does not affect T cells that receive a strong TCR signal and are

independent of CD28 cosignaling. Yet, there are no compelling data to suggest that this is a mechanism of tolerance induction in patients with cancer.

Shift to a TH2 Rather Than a TH1 Response

In animal studies, TH1 cytokines promote the development of an antitumor cell-mediated immune response (17). The production of IFN-γ is linked to the generation of an effective immune response responsible for rejecting tumors. Interleukin-2 is required for T-cell proliferation and acquisition of cytotoxic effector function. Interleukin-4 and IL-10 on the other hand, promote a TH2 response that is necessary for humoral immunity.

In animal models and human neoplasms, there is evidence that the tumor microenvironment can shift the balance to a predominately TH2 response. In animal studies, immunotherapeutic approaches that favor a shift back to a TH1 response can result in the development of an effective tumor-immune response (18). In patients with cancer, including those with renal cell or pancreatic carcinoma, there is also evidence that a TH2 response predominates in the peripheral blood with increased production of TH2 compared with TH1 cytokines as assessed by enzyme-linked immunosorbent assay (ELISA) (19,20). Measurement of intracellular levels of cytokines in patients with renal cell carcinoma (RCC) by flow cytometry demonstrated a change from a TH1 to a TH2 response with increasing stage (21). Preliminary findings also suggest that patients with RCC with progressive disease before therapy have a TH2 bias in MAGE-6 specific CD4+ T cells in the peripheral blood as assessed by enzyme-linked immunospot (ELISPOT) assay (Storkus, et al., unpublished data). A TH2 bias and barely or undetectable IFN-γ expression by the bulk CD4 population may reflect the regulatory influence of tumor antigen-specific CD4+ T cells in patients with cancer. It will be of interest to test whether vaccination can induce a TH1 response in the tumor-specific T cells, and what impact this might have on the clinical response.

Although factors causing a TH2 response in patients with cancer are not well defined, animal studies suggest that transforming growth factor (TGF)-beta and IL-10 may be involved in this process (Table 1). Transforming growth factor-beta may drive the balance to TH2 via IL-10 as an intermediate. Transforming growth factor-beta may also inhibit TH1-type responses directly in certain animal models (22,23). Interleukin-10 is known to play a role in down regulating the expression of TH1 cytokines and may augment the immunosuppressive activity of TGF-beta by regulating expression of TGF-beta type II receptors (17,24).

The ability of IL-10 to enhance TH2 responses and suppress antitumor immunity in certain animal models is confounded by observations indicating that IL-10 may promote antitumor immunity under other conditions (25–27). Thus, the role of IL-10 as an inhibitor of TH1 immunity is not entirely clear from the animal studies. However, in a limited number of studies in patients with cancer, there is correlative data linking IL-10 and TGF-beta expression and the presence of a TH2-type response in peripheral blood T cells. In patients with pancreatic carcinoma, coexpression of TGF-beta and IL-10 in tumor tissue was associated with elevated levels of both cytokines in the sera and with the predominance of a TH2 cytokine pattern in response to antiCD3 antibody stimulation (20). Serial sampling of fine-needle aspirates of identical metastases from patients with melanoma receiving IL-2-based vaccinations has been performed to assess changes in expression of IL-10, TGF-beta, and IFN-γ mRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR). Decreased levels of IL-10 in pretreatment tissues of responding lesions were noted, and IFN-γ transcript levels were found to increase after treatment in regressing as compared with nonregressing lesions (28). Studies like these will provide information on whether pretreatment expression of cytokines in the tumor will influence responses to immunotherapy. The use of array technology should also help define patterns of cytokine gene expression in tumors and their relation to treatment and treatment effect.

It is also possible that the shift to a Th2 response in tumor-bearing hosts, in some cases, is caused by the clearance of antigen-specific IFN-γ producing T cells. In an animal model, the elimination of TH1 cells was attributable to Fas-receptor mediated clearance (29). Whether clonal deletion of TH1-specific T cells occurs in patients with cancer and contributes to the TH2 phenotype has not been addressed.

Unresponsive T Cells in Patients With Cancer With Functional and Signaling Defects

Global immune dysfunction has been described in animal tumor models and in patients with cancer and is typically associated with advanced tumor burden. This dysfunction is characterized by a hyporesponsiveness to challenge with common recall antigens (30). This dysfunction is also defined by diminished T-cell function most pronounced in TIL and is characterized by impaired

proliferation and reduced cytotoxic effector function (31). There are also in vivo data that show that there is minimal induction of an inflammatory response involving expression of IFN-γ and IL-2 mRNA in the tumor microenvironment (31). Diminished T-cell function has also been observed in peripheral blood T cells. This is associated with reduced production of TH1 cytokines after stimulation of peripheral blood T cells with mitogens or antiCD3 antibody (31,32).

Alteration in the expression and/or function of T-cell receptor (TCR)-linked signaling events has been shown to occur in patients with cancer. The most studied defect in T-cell signaling is the reduction in expression of the TCR complex ζ chain, which is part of the TCR proximal signaling pathway that is necessary for T-cell activation and effector function (33). Reduced expression of the ζ chain has been observed in T cells from patients with a variety of tumors, including melanoma, ovarian and cervical cancers, and Hodgkin's disease (31,32). Reduced ζ chain levels have been detected by Western blotting and are reliably assayed by quantitative flow cytometry using intracellular antibody staining. Reduction in ζ chain expression has been correlated in some studies with a decrease in either cell proliferation or TH1-cytokine production (31,32). Data from several laboratories, but not all, suggest that decreased levels of ζ chain in T cells correlated with disease progression and diminished overall survival (34–36). Thus, ζ chain expression levels in T cells may be useful as a predictor for survival. However, current data suggest that ζ chain levels are not consistently useful for monitoring immunologic responses.

Several mechanisms have been proposed to explain the reduction in ζ chain levels as seen in Table 2. Hydrogen peroxide produced by activated granulocytes (blood) and macrophages (tumor) can decrease ζ chain expression in T cells, and decreased ζ chain expression can be prevented by the addition of hydrogen-peroxide scavengers (37,38). It is also possible that ζ chain degradation is partly caused by caspase activity induced in apoptotic T cells. In vitro studies have shown that induction of apoptosis in T cells coincides with ζ chain degradation that is blocked by the addition of a pan-caspase inhibitor and that the zeta chain can be a substrate for caspase-3 (39,40). A small subset of T cells in the peripheral blood and variable numbers in the tumor have been shown to have caspase activity and are annexin V positive (41,42). Even though apoptosis may partly explain ζ chain loss, it is not the only mechanism, because the number of T cells with low ζ expression is higher than the number of T cells that are apoptotic. Zeta chain downregulation has been reported in CD8+ T cells that proliferate in response to acute viral infections (43). In vitro studies have also shown that repeated stimulation via the TCR or by TNF can reduce the expression of ζ chains in T cells (32,44). Whether this occurs in patients with cancer remains to be determined.

TABLE 2. *Mechanisms by which tumors reduce zeta chain levels*

Hydrogen peroxide production by activated granulocytes and macrophages
Caspase activity in apoptotic T cells
Repeated stimulation through TCR or TNFR

TCR, T-cell receptor; TNFR, tumor necrosis factor receptor.

Defects in downstream TCR signaling have been demonstrated in T cells from patients with cancer along with reduced tyrosine phosphorylation of multiple substrates, including phospholipase C gamma (31,45). Quantitation of free intracellular calcium concentrations after mitogen stimulation revealed less mobilization of calcium compared with normal T cells. Whether these alterations are linked to defective ζ chain expression is not clear.

Studies in animal models and patients with RCC have demonstrated defective activation of the transcription factor NFκB in TIL and peripheral blood derived T cells (31,32,46). This leads to a failure in the nuclear accumulation of RelA/p50 dimers after T-cell activation as determined by Western blotting and by κB binding activity (gel mobility shift assays) (31). This defect is noted in T cells from 50% of patients with RCC but in less than 5% of patients with no evidence of disease after treatment and normal individuals (47). The absence of a quantitative flow-based method to detect NFκB has limited the assessment of whether defective activation of NFκB and other transcription factors correlates with clinical outcome. Studies from knockout mice defective in NFκB proteins demonstrate that NFκB is a cell survival factor that regulates expression of various antiapoptotic genes (48). There is also evidence for a preferential role of NFκB in TH1, but not TH2, T-cell responses (49). Whether the NFκB defect in patients' T cells is related to increased sensitivity to apoptosis or to a bias for a TH2 response needs to be assessed. Inhibition of NFκB activation may be partly caused by the presence of soluble products in the tumor microenvironment. Hydrogen peroxide production inhibited NFκB primarily in memory T cells, which correlated with reduced expression of TH1 cytokines (50). Gangliosides present in supernatants from RCC also suppressed NFκB activation in T cells (51). Moreover, tumor-induced inhibition of the lytic activity of tumor-specific T-cell lines correlated with suppression of NFκB translocation (52). Finally, in a transgenic animal model, the induction of tolerance to

self-antigens coincided with lack of AP-1 and NFκB activation (53). Whether impairment in transcriptional activity is noted in tumor specific T cells and is related to defective T cell function is not known.

Role of Apoptosis in T-Cell Dysfunction

Apoptosis of antigen-specific T cells is a major mechanism by which T-cell responses are downregulated after antigenic exposure (54). Repeated antigen stimulation of T cells increases their sensitivity to apoptosis. There is also growing evidence that apoptotic mechanisms regulate the development of an effective immune response to tumor cells. Varying numbers of tumor infiltrating T cells (5%–20%) show DNA breaks as defined by in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays (55,56) (Fig. 1). A greater percentage of TIL appear to be sensitive to apoptosis after in vitro culture for 24 hours (57,58). Apoptotic T cells have been reported in several different tumor types, and it is not clear whether the level of apoptosis relates to stage, grade, or clinical outcome. Spontaneous apoptosis of a small subset of T cells in the peripheral blood of patients with advanced melanoma and gastric cancer has been observed (31,41,42). Increased levels of T cells that are sensitive to activation-induced cell death have also been reported in the blood of patients with cancer (56).

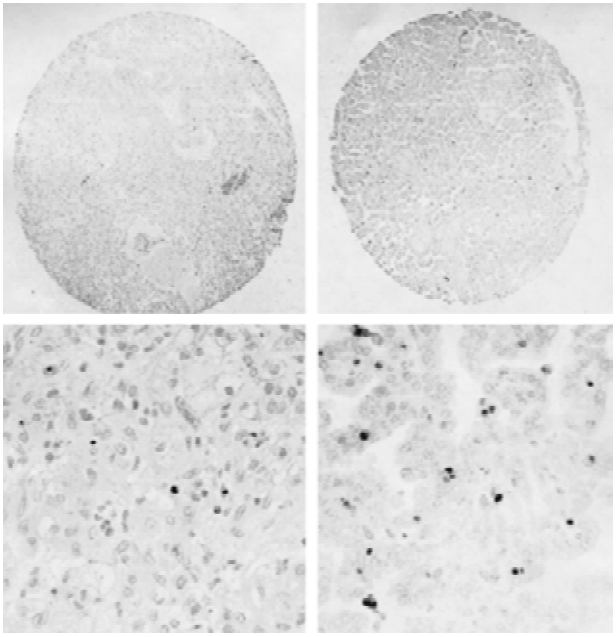

FIG. 1. In situ staining of renal cell carcinoma for DNA breaks. A tissue array was made containing clear cell and papillary tumors. The top figures are 4x magnification of a clear cell (left) and papillary kidney cancer (right). The bottom figures are the same tumors showing 40x magnification. Tissue sections were stained for DNA fragmentation using the TdT-FragEL (Oncogene, Boston, MA, U.S.A.) assay kit. The dark brown stained cells represent apoptotic lymphocytes.

Mechanisms for the observed apoptosis of T cells in patients with cancer are being investigated. While the Fas/FasL pathway appears to play an important role in the deletion of activated tumor-reactive T cells (59), FasL expression by specific tumor types has not been consistently observed. For example, melanoma-specific T cells are induced to undergo activation-induced cell death after MHC class I-restricted recognition of the tumor in a Fas-dependent fashion (60), notwithstanding the FasL-negativity of the melanoma lines used in the assays (60,61). Apoptosis of these tumor-specific T cells was prevented and normal immune functions maintained by the addition of pan-caspase inhibitors to the cultures, suggesting a role for T-cell fratricide in this process.

Some tumors do express FasL and are capable of inducing apoptosis of Fas-bearing T cells (62). Activated T cells as well as Fas death-receptor sensitive Jurkat cells undergo apoptosis when cocultured with allogeneic tumor-cell lines that are FasL positive (62). These T cells do not recognize the tumor in a MHC-restricted manner, though they are still susceptible to FasL-mediated killing. This mechanism is unlikely to be relevant for some tumor types, because not all express FasL (60,61). It is also possible that other tumor-derived products, including gangliosides, can increase the sensitivity of T cells to activation-induced cell death in the tumor microenvironment (58).

The spontaneous apoptosis of T cells reported in the peripheral blood of some patients may also occur via the Fas pathway (41,42). This is supported by the observation that the majority of apoptotic T cells express the Fas receptor (41). It is unclear whether Fas+ apoptosis-sensitive cells in the blood of patients with cancer include tumor-specific T cells.

There is evidence for immune dysfunction in T cells from patients with cancer, which is likely to be caused by multiple mechanisms. Functional and signaling abnormalities that affect a high percentage of the T-cell pool in the tumor and peripheral blood are often seen in patients with advanced disease. The functional status of tumor-specific T cells has been difficult to assess as a result of the relatively low numbers of these cells in the blood and tumor. Nevertheless, the utilization of techniques such as tetramer analysis, real time PCR, and ELISPOT have revealed that in certain cases, but not all, these cells may be functionally impaired. More effort should be made to develop methods to assess the functional- and signaling-pathway status of antigen-specific T cells in the tumor

before and after immunotherapy. Whether there is evidence for T-cell anergy (functional and signaling levels), a TH2 bias or increased sensitivity to apoptosis among tumor-specific T cells is not known. It will be important to determine which pathways of dysfunction are present in a given cancer, whether there is a common pattern in different tumor types, and whether there is evidence that the tumor- or antigen-specific T cell population is dysfunctional. Future vaccine studies should be designed to determine more precisely whether T-cell dysfunction (tumor specific and nonspecific) correlates with clinical outcome.

Defective Dendritic Cell Differentiation in Cancer

Failure of T cells from tumor-bearing hosts to recognize and eliminate tumor cells is a factor in tumor escape from immune control. The induction of an effective antitumor immune response requires host antigen presenting cells (APC) (63). Dendritic cells are the most potent APC. Dendritic cells, macrophages, and granulocytes arise from common myeloid progenitors. It appears that impaired balance between mature and immature myeloid cells is one of the hallmarks of cancer. There is now ample evidence that tumor growth in humans and mice is associated with accumulation of immature myeloid cells, monocyte/macrophages, and decreased number and function of DC. Accumulation of immature myeloid cells capable of inhibiting T-cell responses, could be a major factor responsible for immune suppression in patients with cancer.

The growth of many murine carcinomas, such as Lewis lung carcinoma (64), CT-26 (65), MCA-26 (66), and MCA-38 (SK, unpublished), colon carcinomas, Ehrlich (67), and D1-DMBA-3 (68) mammary adenocarcinomas, and at a lesser extent MethA, C3 (69), or CMS5 (70) sarcomas are associated with splenomegaly, extramedullary hematopoiesis, and marked accumulation of immature myeloid cells in lymphoid organs. Similar findings have been reported in patients with cancer. Decreased numbers of DC in the peripheral blood of patients with cancer has been associated with accumulation of cells lacking markers for mature myeloid and lymphoid lineages (71). Approximately one-third of these cells were shown to be immature macrophages and DC, and the remaining cells were immature myeloid cells at earlier stages of differentiation (71). The presence of these cells was dramatically increased in patients with advanced-stage cancer, but dropped considerably within 3–4 weeks after surgical resection of the tumor. This decrease is consistent with the generation of immature myeloid cells caused by the production of soluble factors by tumors. Resection of tumors also resulted in an increased number of immature Gr-1^+/Mac-1^+ myeloid cells and a decrease in the number and function of $CD3^+$ T cells in the spleen (70).

Several cytokines produced by tumors such as vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, IL-6, and IL-10 have been implicated in defective DC maturation and impairment of myelopoiesis in tumor-bearing hosts. Vascular endothelial growth factor is produced in large amounts by most tumors, and its production is closely associated with poor prognosis. Vascular endothelial growth factor stimulates the proliferation of endothelial cells and plays an important role in the formation of tumor neovasculature (72). AntiVEGF neutralizing antibodies block the negative effects of tumor-cell supernatants on DC maturation in vitro (73). Furthermore, an inverse correlation between the presence of DC and the expression of VEGF has been demonstrated within the tumor tissue (74) and peripheral blood (75,76) of patients with cancer. Vascular endothelial growth factor inhibits activation of the transcription factor NF-κB in hematopoietic progenitor cells, which is associated with alterations in the development of different hematopoietic lineages (77,78). Administration of recombinant VEGF to naïve mice resulted in an inhibition of DC development, associated with an increase in production of B cells and immature Gr-1^+ myeloid cells (78).

Granulocyte-macrophage colony-stimulating factor is another factor shown to be responsible for the stimulation of myelopoiesis in tumor-bearing hosts. Approximately 30% of 75 tested human tumor-cell lines spontaneously secrete this cytokine (79). Early studies have demonstrated that several experimental tumors can produce colony-stimulating factors that induce expansion of immature myeloid cells capable of inhibiting T cell-mediated immune response in vitro. Administration of antiGM-CSF and antiIL-3 antibodies in vivo abrogated accumulation of tumor-induced immune suppressive granulocyte-macrophage progenitor cells in mice with Lewis lung carcinoma. Recently, it was shown that long-term administration of GM-CSF to mice resulted in the generation of a cell population that morphologically resembled granulocyte-monocyte progenitor cells and expressed the granulocyte-monocyte markers Mac-1 and Gr-1 (79). These Gr-1/Mac-1 double positive cells can be differentiated in vitro into mature and fully functional APC in the presence of IL-4 and GM-CSF. It is likely that the combination of GM-CSF and VEGF may have a much stronger effect on inhibiting DC function than either of these growth factors alone. Granulocyte-

macrophage colony-stimulating factor stimulates myelopoiesis, whereas VEGF blocks maturation of hematopoietic precursor cells. Together they can impact on differentiation of myeloid cells and thereby contribute to tumor escape from immune control.

Other tumor-derived factors like M-CSF, IL-6, IL-10, and gangliosides have been also shown to be involved in defective DC differentiation in vitro (73,80–83); however, it appears that these factors do not stimulate myelopoiesis and mostly affect mature cells. Incubation of $CD34^+$ progenitor cells with IL-6 and M-CSF shifted cell differentiation from DC to monocytes (82); IL-10 prevents the differentiation of monocytes to DC but promotes their maturation to macrophages (80). Professional APC derived from IL-10 transgenic mice were found to have significantly suppressed capacity to induce MHC allo reactivity, cytotoxic T-lymphocytes (CTL) responses, and IL-12 production (84).

Dramatic accumulation of immature myeloid cells in tumor-bearing hosts raises the question about their role in cancer. Several groups recently have studied the functional significance of these cells. $Gr\text{-}1^+$ immature myeloid cells accumulated in tumor-bearing mice were able to inhibit IFN-γ production by CD8+ T cells in response to a specific peptide presented by MHC class I in vitro and in vivo (69). This effect was not mediated by soluble factors, and blockade of MHC class I molecules on the surface of $Gr\text{-}1^+$ cells completely abrogated the observed inhibitory effect. These tumor-induced $Gr\text{-}1^+$ cells were not able to inhibit the proliferative response of T cells to Con A or specific peptide presented by MHC class II molecules. This could be explained by expression of MHC class I molecules but lack of MHC class II and costimulatory molecules by $Gr\text{-}1^+$ cells. Stimulation of T cells in the absence of costimulation may result in T-cell anergy.

To investigate whether immature myeloid cells obtained from patients with cancer affect MHC class I-restricted responses, a CTL line specific for a FLU virus-derived peptide was generated from an HLA-A2-positive healthy volunteer (71). Addition of Lin^- $HLA\text{-}DR^-$ immature myeloid cells isolated from the peripheral blood of HLA-A2-positive patients with cancer specifically inhibited production of IFN-γ by $CD8^+$ T cells restimulated with DC pulsed with the specific peptide. Even though cancer progression is associated with increased production of immature myeloid cells as well as immature DC, this could be one of the mechanisms by which growing tumors may induce antigen-specific $CD8^+$ T-cell tolerance in the tumor-bearing host.

Bronte et al. described a similar mechanism of immunosuppression in mice (85,86). Immunization of naïve mice with tumor antigens resulted in loss of cytolytic activity and apoptosis of $CD8^+$ T cells upon restimulation in vitro with the same antigen. Depletion of $Gr\text{-}1^+$ cells prevented the apoptosis and restored the CTL activity. The induction of $CD8^+$ T-cell apoptosis was associated with adherent $Gr\text{-}1^+$ cells cultured for several days. In these conditions, immature myeloid cells acquire the F4/80 marker of monocyte/macrophages. Not only tumor growth but also acute, intense, antigenic stimulation may cause a dramatic alteration in $CD8^+$ T-cell function. Primarily, this may serve as a mechanism that limits expansion of activated T cells and prevents the development of autoimmunity. However, growing tumors may secrete factors like VEGF, GM-CSF, M-CSF, IL-6, and IL-10 that stimulate abnormal myelopoiesis, which results in immune unresponsiveness.

These results may have direct implication for cancer immunotherapy. Immature myeloid cells accumulated in patients with cancer can present antigen and inhibit immune responses to the antigens used for vaccination. This makes it necessary to not only measure immature myeloid cells in patients with cancer during immunotherapy, but also search for methods to overcome this inhibition. The most effective way to eliminate immature myeloid cells would be to differentiate them using growth factors and differentiation agents. One example of such an approach is all-trans-retinoic acid. It has been recently demonstrated in experiments in vitro that in patients with cancer, all-trans-retinoic acid eliminates immature myeloid cells and promotes DC differentiation (71). Similar effects were observed in tumor-bearing mice (69). This may provide a promising opportunity to improve the efficacy of cancer immunotherapy.

MEASURING T-CELL RECEPTOR FUNCTION

T-Cell Receptor/CD3 Complex Stimulation Analysis

Interaction between the antigen-MHC complex and the TCR/CD3 complex on the surface of T cells triggers a chain of molecular events resulting in T-cell activation (87). In vitro stimulation of T cells with antiCD3 monoclonal antibodies (mAbs) mimics APC-induced T-cell priming and leads to activation in an antigen-nonspecific manner.

It is well established that cancer is associated with T-cell dysfunction. Tumor-infiltrating and, to a lesser extent, peripheral blood lymphocytes from patients with advanced cancer demonstrate significant reduction in T-cell function and activation via the TCR/CD3 complex. Impaired production of IFN-γ, IL-2, and TNF-α by T cells in response to simulation with antiCD3 mAbs was

reported in patients with cancer (88–90). However, the production of IL-10 after stimulation with antiCD3 mAbs was normal or increased (89).

These data suggest that analysis of T-cell responses (proliferation and cytokine production) to stimulation with antiCD3 mAbs may be useful in evaluating T-cell function during vaccination. The limitations of this method are its lack of antigen specificity and variability in the sources of antibodies and other conditions of the assay, which may significantly affect the result.

The procedure for this assay includes activation of purified T cells for 3–4 days on plates coated with antiCD3 mAbs and detection of cell proliferation using incorporation of [^{3}H]-thymidine. In parallel, cytokines (IL-2, GM-CSF, IFN-γ, etc.) in cell supernatants can be measured using ELISA. The variability of the method is caused by different sources and concentrations of antibodies used by different laboratories. High concentrations of antibodies often result in hyperactivation of T cells, which would negate any differences between control and test samples. It is advisable to use suboptimal concentration of antibodies (0.5–1 μg/mL of antiCD3 mAbs). Another possible way to standardize the result of the assay is to report proliferation and cytokine production as folds-increase over background with no antibody. This would decrease the fluctuation of the data and make comparison between different laboratories easier.

This assay cannot determine whether a patient responded to the specific therapy or not; however, it may serve as a surrogate marker to determine whether T cells from patients are functionally competent. Consequently, it may be used in determining eligibility for vaccination and for monitoring of the progress of therapy.

At this time, there are few reports about the use of antiCD3 stimulation of T cells during clinical trials. Kim et al. (91) used antiCD3 stimulation to evaluate the response of patients with metastatic colorectal carcinoma to cellular treatment with expanded autologous lymph node cells. The responding patients' cells had higher levels of GM-CSF and IL-4 secretion in response to stimulation with immobilized antiCD3 in vitro (91). In a recent study, Li et al. (92) investigated immunologic effects of Bacille Calmette-Guerin as an adjuvant in autologous tumor vaccines (92). In patients with RCC, irradiated tumor cells alone or with Bacille Calmette-Guerin were inoculated intradermally. Seven–10 days later, draining vaccine-primed lymph nodes were excised. Lymphocytes from both groups were activated with antiCD3 mAbs and stimulated with irradiated autologous tumor. AntiCD3-activated T cells from patients treated with Bacille Calmette-Guerin admixed with tumor cells had greater release of IFN-γ without upregulation of IL-10 production than T cells from patients treated with tumor cells alone. Analysis of correlation between T-cell response to antiCD3 mAbs and clinical response to the treatment was not possible because of the limited number of patients enrolled.

Analysis of T-cell responses to antiCD3 mAbs may be useful. This test is relatively easy to perform and standardize. It can potentially help in the evaluation of T-cell function before treatment (eligibility criteria) and in the monitoring of T-cell function during treatment. However, because it cannot be used for analysis of a specific immune response, its role will be rather limited. More data from clinical trials are needed to make a conclusion about its ultimate usefulness.

T-Cell Receptor Zeta Chain Analysis

The ζ chain is the signal-transducing chain of the TCR-CD3 complex expressed on T cells. Expression of the ζ chain is required for T-cell activation, and reduced levels or absence of ζ chain indicates defective T-cell activation (93). Reduced expression of the ζ chain has been identified in a large percentage of patients with different tumor types (94). In contrast to T cells isolated from inflammatory sites, tumor-infiltrating lymphocytes (TIL) have been shown to be hyporesponsive in proliferation and cytotoxicity assays, when used directly after isolation from the patient. These data suggested that the tumor microenvironment has negative effects on infiltrating T cells (95,96). Others have found decreased ζ chain expression after exposure to hydrogen peroxide produced by activated granulocytes and macrophages that was prevented by addition of hydrogen peroxide scavengers (37,38). The downregulation of ζ chains in T cells is not limited to TIL, but can also be measured in peripheral blood lymphocytes (PBL) and natural killer (NK) cells of tumor-bearing patients (97,98). Studies on ζ expression in PBL of patients with cervical intraepithelial neoplasia or cervical cancer (34) and Hodgkin's disease (35) have indicated that the ζ chain expression may correlate with disease progression, but this hypothesis could not be confirmed in the analysis of PBL from patients with breast cancer at early stages of their disease (99). A correlation between the expression of the ζ chain in the TIL of patients with head and neck carcinoma and their overall survival has been described (36). These data indicate that ζ chain expression in TIL may be a marker for survival in some tumor types. Additionally, ζ chain expression in PBL may be used as a marker for general immune function in patients with cancer and provides a potential stratification criterion for immunotherapy trials that use T-cell activation strategies (94). Finally, identi-

fication of ζ chain expression has been suggested as a tool for immunologic monitoring of patients receiving immunotherapy (100).

The most reliable detection method for ζ chain expression is quantitative flow cytometry (fluorescent-activated cell sorter [FACS]) using intracellular antibody staining. Flow cytometry can be performed on TIL or PBL, requires 10^5 CD3-expressing cells and can detect changes of ζ chain expression in small subpopulations of T cells. This method requires a flow cytometry apparatus with the ability to detect two fluorochromes. One will be required for detection of CD3 expression and specific gating of these cells; the other will be used to detect ζ chain expression. The flow cytometry data are expressed as mean fluorescence intensity and should be compared with an isotype-matched control monoclonal antibody that serves as an internal standard. The ratio between the mean fluorescence intensity of the ζ chain and isotype control can be reported as a normalized ζ chain expression level of an individual sample. All data should be obtained in patients before and after therapy and compared with ζ chain expression levels in T cells of normal sex and age matched healthy individuals. Zeta chain expression detection does not permit determination of immune specificity.

There is a lack of consistency in measurement of ζ chain expression in different patient studies performed to date. Different laboratories use different techniques and different cell samples (PBL, TIL, and TAL) that are either fresh or frozen. All of these factors contribute to the inability to compare results from different groups. Furthermore, no cross testing has been done by different labs; therefore, no interlab quality assurance is available.

The lack of ζ chain measurement validation may be a result of the unclear clinical relevance of the assay. Despite the suggestion that ζ chain levels could be used as a tool for immunologic monitoring (100), it is unlikely to be a marker for therapy outcome. Several studies did not show a predictive value for ζ chain expression regarding immune responsiveness. For example, patients with a complete tumor response after therapy have been shown to downregulate their ζ chain expression levels in PBL during therapy. In another study, there was no correlation between ζ chain levels and immune responses to a human papillomavirus peptide vaccine (101).

As stated previously, ζ chain expression levels in T cells may be useful as a population based epidemiological predictor and as a predictor for survival or disease progression (34,36). Although a definitive statement on the usefulness of TCR ζ chain analysis cannot be made without studying larger numbers of patients with different types of cancer, clinical data from patients with infectious and autoimmune diseases and theoretical arguments do not support the use of ζ chain expression analysis for the monitoring of immune responses during vaccine trials.

T-Cell Receptor Downstream Events

A critical downstream signaling event after T receptor engagement is the activation of transcription factors that regulate the expression of genes important to T-cell function and survival (102). Studies in patients with RCC as well as breast cancer have demonstrated that the activation of NFκB is impaired after CD3 stimulation (32,103, 104). Defective NFκB activation is also seen with PMA/ionomycin stimulation, showing that the decrease in NFκB activation is not simply a consequence of impairment in early signaling events in patients' T cells. Defective NFκB has been observed in T cells isolated from the tumor and peripheral blood.

The inhibition of NFκB may be caused by the tumor microenvironment. Tumor-induced inhibition of cytolytic activity correlated with suppression of NFκB translocation in a tumor-specific T-cell line (105). Hydrogen peroxide production by infiltrating macrophages and granulocytes inhibited NFκB in T cells and correlated with reduced Th1 cytokine production (37). Tumor-derived gangliosides from patients with RCC also suppress NFκB activation in normal T cells (51).

An issue that has not been adequately addressed is whether the defect in transcriptional activation (i.e., NFκB) correlates with immune dysfunction in T cells from tumor bearers. In patients with cancer, there is one report that showed no correlation between impaired NFκB activation in peripheral T cells and the induction of IL-2 production (21), but additional studies are needed using more quantitative methods (e.g., RT-PCR) to test whether defective transcription factor activation is linked to the reduced Th1 cytokine response (IFN-γ) that is observed in some patients with cancer (21).

Any attempt to establish a correlation between defective transcriptional activity, T cell function/survival, and clinical outcome is limited by the assay used to measure transcription function. Prior studies have used DNA binding to radiolabeled probes in an electrophoretic mobility shift assay to measure transcriptional activity. This assay requires approximately 8 million stimulated or unstimulated T cells. The probes correspond to NFκB, AP-1, and NFAT sites within regulatory regions of the appropriate genes. Standardization within a laboratory is achieved by the inclusion of a positive control in each gel and performing densitometry readings on the bands for comparison of data on multiple gels. Even though this assay is reproducible within a single laboratory, it would

be difficult to compare data from multiple groups. Western blotting of nuclear and cytoplasmic extracts from unstimulated and activated T cells has been used to detect translocation of NFκB (RelA) and p50 (106). The signal obtained in the cytoplasm before and after stimulation should be compared with the signals present in the nucleus. Standardization and quality control for Western blotting make it difficult to use this method for monitoring. The absence of a quantitative flow-based method is an obstacle to assessing whether any defect in NFκB nuclear translocation correlates with clinical outcome. However, nuclear translocation of NFκB can be assessed in small numbers of cells by immunostaining with purified IgG anti-p65 (RelA) antibody followed by incubation with Alexa Fluor 488 GAR (105). Nuclei are stained with propidium iodide and then analyzed by laser scanning confocal microscopy. It is possible that this assay can be used to assess transcriptional activity in small numbers of antigen-specific T cells derived from the blood and tumor of patients with cancer but at this time it is unclear that this analysis could yield sufficiently quantifiable results to warrant its inclusion as a recommended immune monitoring assay.

ANTIBODY ASSAYS

Background Information

Serologic methods for monitoring B cell function are more established and less complex than analysis of T-cell responses. However, cancer-vaccine monitoring has focused on the generation of cellular immunity. Even though infectious diseases have well defined serologic immunologic correlates of vaccine efficacy, serologic correlates have not been clearly demonstrated for tumor antigens in most cancer-vaccine systems. A comprehensive approach to the development of quantitative assays, which measure the amount of antigen-specific antibody and function of the antibody generated against a specific tumor antigen may serve as an adjunct to T-cell monitoring or even a predictor of the development of a T-cell response.

Immunologic correlates of efficacy for several infectious disease vaccines have been established. For some infectious diseases, especially those with relatively long incubation periods, seroconversion induced by vaccination is paralleled by the induction of immunologic memory. It is the induction of a memory response that provides the mechanisms for long-term protection even as antibody levels wane (107). Antibody levels, in some instances, may even serve as a reflection of a T-cell response. In many infectious disease models, the total level of antigen-specific immunoglobulin (Ig)G or A has been shown to correlate with protection from disease (107). A classic example is that of diphtheria vaccine antigen. The degree of protection against disease has been shown to correlate with the level of serum antibody against the toxin (107). Assays that measure antibodies are stringently standardized and although a level of 0.01 IU antiD/mL is accepted as protective, levels > 0.05 IU antiD/mL are considered to indicate optimal protection (108).

Immunologic correlates for viral antigen systems have also been developed. The induction of > 10-mIU antiHBs/mL has become accepted as a correlate of efficacy for Hepatitis B vaccines. This level of antibody has been associated with the generation of T-cell memory more than 5 years after primary immunization as validated by ELISPOT analysis and by the ability of a booster shot to elicit a rapid antiHBs response (109). Thus, the total quantitative level of antibody induced, particularly a class of antibody that indicates an Ig class switch and cognate T-cell help such as IgG or IgA, may serve as a surrogate for the development of a T-cell response and memory as well as a clinical measure of therapeutic immunity.

A "clinically effective" immune response to a tumor antigen is expected to be a T-cell response, historically, a cytotoxic T-cell response. Cancer-vaccine studies that seek to immunize with an antigen that is carbohydrate-based and, thus, may induce a predominantly humoral response, often measure tumor-specific antibody generation as a primary immunologic endpoint. Serologic responses to some cancer-restricted antigens (e.g. ESO-1) have been shown to be frequently associated with a detectable underlying T-cell response (110). Many clinical studies of cancer vaccines targeting specific antigens that are not carbohydrate based do not include analysis of humoral immunity. The assessment of a tumor-specific antibody response should be part of any cancer-vaccine study for several reasons. As has been proposed with infectious disease antigens, an antibody response may serve as a surrogate for the development of a T-cell response, particularly IgG and IgA, which require T-cell help for Ig class switching. Secondly, clinical studies indicate that antibodies to HER-2/neu or carcinoembryonic antigen (CEA) may be a marker for or can elicit a therapeutic response (111–113). Finally, an antibody directed to even an internal antigen, if it can bind the Fc receptor, may affect the developing immune response in a tumor bed through interaction with local APC. Therefore, the tumor antigen-specific antibody response that develops after active immunization is a worthwhile measure for analysis.

Antibody assays can be adapted in ELISA to measure a variety of parameters. First is the measurement of a polyclonal antibody response to a particular tumor antigen. Secondly, the identification of the antibody class involved in the initiation of the response, IgG, IgM, or IgA can be made. By changing the secondary antibody used for analysis, the IgG isotype may be determined, which has been shown in animal models to predict the T-helper phenotype involved in initiating the response. Finally, ELISA can be adapted to determine antibody avidity through one of several antibody dissociation methods. The ELISA can be adapted to impure sources of antigen via a "capture" method or can use purified antigen bound directly on the plate. Large numbers of patient serum samples can be analyzed for cancer-specific antibodies using ELISA. A sandwich-type ELISA is most commonly used. This technique requires a monoclonal or polyclonal antibody specific for the protein of interest and a source of protein. The antibody is fixed to solid support, generally by adsorption to a commercially available plate. A source of impure protein, such as a cellular lysate, is then incubated with the bound antibody. The protein of interest will be bound to the antibody on the plate while other impurities are washed away. With this technique, a reasonably pure source of cancer-related protein could then be probed with patient sera. Serum antibodies directed towards the protein of interest will bind to it and protein and become fixed to the plate. Irrelevant serum antibody will be washed away. After washing, wells on the plate are incubated with a labeled second antibody that is specific for human Ig. A substrate is then added that causes a photometric reaction and the assay is analyzed using spectrophotometric techniques. Capture ELISA has several benefits: (1) human cell lines that contain the protein of interest can be used as a source of protein as the initial step of the ELISA, because bound antibody will serve to "purify" the antigen, (2) assays are generally very specific, and (3) sandwich ELISA methodology is adaptable to the development of assays with newly determined antigens.

An alternative technique is known as indirect solid phase ELISA and uses a purified source of protein bound directly to the solid matrix. Patient antibody can bind directly to the protein and then be quantitated. In addition, using a uniform source of recombinant protein ensures lot quality, decreased lot-to-lot variation and is much more amenable to automation and the analysis of a large number of samples. Classically, antibody responses detected by ELISA are validated though direct visualization via Western blot analysis. Western blot has been used as a method to determine the presence of humoral responses to whole tumor-cell populations, but is not quantitative. An ELISA methodology for antibody analysis allows highly quantitative estimates of the magnitude of the humoral immune response.

Methodological Points to Consider When Evaluating Serologic Results

The ELISA has been widely used for measuring the concentrations of antibodies in human sera as a result of its simplicity, ease of automation, and objective analysis. The ELISA, including titers, international units, uses various methods to quantitate antibody concentrations and weight-based units (μg/mL). Variation in reporting of values makes comparison of ELISA results from different laboratories difficult. Simply comparing antibody concentrations expressed in titers may be misleading because the sensitivity of ELISA differ greatly depending on numerous factors such as incubation times, ELISA readers, and the quality of antibody conjugates (114). Therefore, although an antigen-specific positive control sera standard may be run on each plate, it is difficult to standardize assays across antigen systems unless standard curves (weight-based units) are used (114).

Even though capture ELISA is a specific assay, the sensitivity of the approach may be diminished for many reasons: (1) the binding monoclonal antibody may block the immunogenic epitope recognized by the patient's serum antibodies, and (2) human sera are known to have heterophile antibodies, directed towards different species, such as mouse and goat. Estimates of the prevalence of antimouse antibodies in normal sera have been reported to vary from 0.5%–40%. These naturally occurring antibodies may interact with the monoclonal or polyclonal binding antibody and thus, increase the background of the assay and decrease the sensitivity, e.g., patients with marginal but positive responses will be missed (115), and (3) sensitivity is dependent on the specific activity and affinity of the monoclonal and polyclonal antibodies available for that antigen. These problems can be overcome by utilizing a purified source of protein bound directly to the solid matrix, e.g., indirect solid-phase ELISA. However, the generation of large amounts of highly purified recombinant proteins will increase the cost of the assay and may not be possible for certain constructs.

Evaluation of the Robustness of Serologic Assays

Three different methods have been used to monitor antibody production in response to vaccination. Immu-

noblotting is the least informative method when evaluating antibodies against particular epitopes because it provides only semi-quantitative data when used with a densitometer. However, in the case of whole cell vaccination, Western blotting can be a very useful tool for identifying immunodominant proteins in vaccines that are capable of inducing a humoral response. Recently, ELISPOT has been used to evaluate levels of memory B cells (116,117) and to make estimations of antigen-specific B cell precursor frequencies. Enzyme-linked immunosorbent assay provides the best quantitative data and together with ELISPOT can provide an excellent picture on the generation of humoral responses to vaccines. Validation methods used to standardize antibody analyses are straightforward and include accuracy and precision measurements with a coefficient of variation of 10% or less.

Clinical Usefulness and History of Serologic Analyses

The generation of antibody responses has been assessed in a number of cancer-vaccine trials. Many studies have demonstrated that after vaccination, patients with higher titers of antigen-specific antibodies had increased disease-free and overall survival (118–123). Total quantitative IgG and IgM has been evaluated for a variety of tumor antigen systems after immunization. There is some suggestion that the generation of antibody immunity to tumors may have a clinical effect (119,124). Larger clinical trials have been conducted in situations where antibody responses have determined clinical efficacy of immunization strategies such as melanoma cell vaccines or antiidiotype vaccines designed to elicit serologic immunity. A recently reported trial of a comparison of high-dose IFN-α with immunization with GM2-KLH/QS-21 in patients with melanoma demonstrated superiority in survival for the interferon arm (125). However, the development of antibody titers to GM2 $\geq$ 1:80 after active immunization were associated with a trend toward improved relapse-free and overall survival. In addition to the measurement of total antibody generated, additional measurements such as isotype of the antibody response, affinity, avidity, epitope recognition, and functional tests such as neutralization and opsonization have been shown to be useful in predicting protection from clinical disease in various infectious diseases (107).

The functional characteristics of antibodies are also dependent on their isotype. IgG_1 and IgG_{2a} for example, differ in their abilities to fix complement and to bind to IgG Fc receptors (126). Determination of the functional capabilities of antibody isotypes is an area of intense investigation. For example, a recent analysis of the correlation between IgG concentration, isotype, and avidity to opsonophagocytic activity against *Strep pneumoniae* was made in infants after immunization with a variety of pneumococcal vaccines (127). Most of the antibodies generated were IgG_1, known to more efficiently activate the classic pathway of complement than IgG_2. In fact, an increased ratio of IgG_2:IgG_1 predicted little opsonization. Positive correlation of a particular isotype with a global measure of antibody function, such as avidity, would provide a rapid serologic system for potentially assessing outcome after active immunization.

Comparison of Serologic Analysis With Cellular Cancer Immune Monitoring

Assessment of antibody immunity is an underutilized immunologic monitoring tool. Cytokines induced in the immune environment play a major role in selecting the isotypes of antibody that are produced during an immune response (126). Two dominant cytokines influencing the generation of specific antibody responses are IL-4 and IFN-γ. Studies have demonstrated that IL-4 can induce activated B cells to secrete IgE as well as IgG_1 and that IFN-γ can inhibit that secretion (128). In addition, IFN-γ has been shown to effect Ig isotype selection in T cell-dependent and T cell-independent systems by stimulating IgG_{2a} production (128). Most investigations evaluating the correlation between T helper subsets and IgG isotype have been performed in animal models. For example, in one study, mice were immunized with Hepatitis antigens and T helper cytokine secretion and IgG isotypic antibody responses were evaluated (129). A strong correlation between IFN-γ production and IgG_{2a}, and IL-4 production and IgG_1 was demonstrated. Furthermore, T-cell cytokine production and associated antibody responses could be modulated by in vivo cytokine treatment. Other investigators proposed using the development of a specific IgG isotype response as a surrogate for the T-cell helper subset stimulated during lipid vesicle immunization with ovalbumin antigen (130). In these studies, not only did IFN-γ/IgG_{2a} and IL-4/IgG_1 correlate, but both responses could be manipulated by the delivery vehicle of the antigen. No clear parallel has been made in the human system to IL-4 and IFN-γ control of IgG_1 and IgG_{2a} as it has in the mouse. Lack of established antigen systems with known correlations and difficulty in establishing quantitative isotype antibody analysis have contributed to the lack of data in patients.

T-CELL ASSAYS

General Methodological Aspects

Sample Requirements (Ex Vivo Analysis)

There is considerable interest in the use of frozen/thawed PBMC samples for monitoring of immunologic effects in clinical trials. These samples allow blood to be collected, stored, and analyzed in batches or shipped to a reference laboratory. This enables analysis of longitudinally collected patient samples in single assays, thereby limiting variability associated with interassay assessment. The care with which cells are frozen and thawed impacts the quality of the resultant data, particularly for cytokine-secretion assays, but also for tetramer staining. Tumor-specific T cells, especially those recently stimulated by vaccination, may be activated effector T-cells containing intracellular granzymes B, and thus, may be more susceptible to damage from freezing and thawing. Although high frequencies of specific T-cells (CD45RA+CCR7-GranzymeB+) can be detected in frozen PBMC, there are no studies on the influence of freezing on such T-cell populations. Furthermore, frozen cells may be able to react well to some antigens but not others; hence, there may be a need for testing and comparing fresh and frozen cells for each antigen to be studied. Encouragingly, aliquots of PBMC frozen in liquid nitrogen from healthy donors exhibited little change in precursor frequencies as measured by the ELISPOT assay when cells were thawed from various freezing times ranging from 59 to 158 days (131). The Adult AIDS Clinical Trials Group (ACTG) immunology website, http://aactg.s-3.com/immmeth.html, contains a consensus method for PBMC cryopreservation. Thawed cells may be "rested" in complete media for times ranging from several hours to overnight before analysis, because it reduces nonspecific IFN-γ release (132). The recovery after overnight culture should be 80%, and the viability should be at least 90%. If antigen-reactive T cells are analyzed and additional APC are to be used, this overnight culture should be performed in hydrophobic plastic materials, e.g., polypropylene tubes, to avoid monocyte adherence. Concern exists that specific CD8+ T cells in patients with cancer, particularly those with advanced stage disease, may exhibit heightened levels of "spontaneous" apoptosis ex vivo (133) depending on the thawing period after recovery. Furthermore, clumping of cells may also occur if there is DNA released from thawed cells. To compensate for these potential problems, viable cells may be enriched by discontinuous gradient centrifugation (Ficoll/Percoll) before staining, if necessary.

The cytokine flow cytometry (CFC) assay can be performed with whole blood. Whole-blood samples should be collected in sodium heparin, as other anticoagulants chelate calcium and severely compromise lymphocyte function. Whole blood should be stored at room temperature to avoid platelet activation and should be used within 8 hours of collection to obtain maximal responses. Longer storage times compromise antigen-presenting cell function (required when using whole protein antigens), and shipping can compound loss of function. Fresh PBMC and viably frozen PBMC also suffer loss of APC function over time, making stimulation with protein antigens inaccurate. Major histocompatability complex matched peptides, or peptide mixtures that do not require processing for binding to host MHC molecules, however, can still be used to successfully activate CD4+ and CD8+ T cells from previously frozen archived samples (134).

Use of Ex Vivo Restimulated Effector Cells

The threshold for IFN-γ production has been shown to be much higher shortly after restimulation with antigen (135); therefore, effector cells (particularly cloned T cells), should be used at the end of the restimulation cycle when production of IFN-γ is not detectable. Results from assays using fresh PBMC or T cells may be repeated with ex vivo restimulated samples. The failure to confirm the results after one stimulation would suggest either that the original result was incorrect or that the responding T-cells may be hyporesponsive to antigen or unable to proliferate as has been observed in several studies (136–138).

Assay Standardization and Quality Assurance

Enzyme-linked immunospot, CFC, and tetramer assays to identify tumor-specific T cells have beem refined over the past several years and, provided uniform standard operating procedures can be developed, these assays ultimately may be used for reliable immune monitoring of patient T-cell responses. Standard operating procedures should include a set of suggested controls for each specific assay. Even with a uniform standard operating procedures, however, it is still unclear whether data compiled from different laboratories involved in a multisite clinical trial can be compared with a high-degree of confidence. Barring the development of stringent validation criteria, these assays may be limited to application within reference laboratories at the current time.

Functional T-cell assays are subject to higher variation than assays that use phenotypic staining by FACS, as a result of assay complexity and the biologic variability associated with in vitro cell activation. Nevertheless, re-

cent evaluations have reported an overall coefficient of variation in a CMV-specific CFC assay to be within 20% in a multi-site study (see "Cytokine Flow Cytometry"). Similarly, a comparative study was performed in four European laboratories showing concordant frequencies of FLU-reactive T-cells determined by ELISPOT assay (139).

Limiting Dilution Analysis

Basic Principles

The two most commonly used in vitro methods for measuring antigen-specific T-cell responses include assessment of the proliferative T-cell response to antigen and a measurement of the ability of T cells to lyse labeled tumor. Incorporation of a radiolabel into DNA is used to assess antigen-induced clonal expansion and lytic function of helper T cells and CTL. There are two mechanisms by which CTL induce cell death in target cells. One involves the secretion of molecules that induce pore formation within the target cell membrane via perforin and granzymes B, and the other involves the induction of apoptosis via Fas-Fas ligand interactions (140). The most established assay of CTL activity is the chromium release assay (CRA). In this assay system, T lymphocytes are mixed with target cells that have been labeled with ^{51}Chromium (Cr). Spontaneous release of ^{51}Cr from the target is slow and thus, rapid release indicates destruction of the target-cell membrane. It is assumed that in vitro target killing is a reflection of the ability of CTL to kill in vivo although this assumption is difficult to prove. In addition to ^{51}Cr, cytotoxicity assays using fluorometric or colorimetric techniques have been developed to assess CTL activity. One such colorimetric compound that has been widely used in lysis assays is the tetrazolium salt, MTT, which is hydrolyzed by viable cells to form a blue crystal measurable in a microtiter plate reader (141). The use of florigenic compounds such as MUH (142) and AlamarBlue have been recently incorporated into a CTL assay giving exquisitely sensitive results compared with ^{51}Cr assay (143). Fluorometric methods of lysis assessment avoid the use of radioisotopes but are associated with longer assay times and considerable cost outlays for a microplate reader. No comparison of these two types of lytic measurements has been reported.

Incorporation of a radiolabel in DNA is a common method to assess a proliferative T-cell response to antigen. The method relies on the antigen-induced clonal expansion of T helper lymphocytes and CTL. Typically, cells are incubated in the presence of soluble antigen for 5 days at which time ^{3}H-thymidine is added for several hours. Afterwards, the radioisotope associated with the cells is measured, which represents DNA synthesis and thus, the mitogenic potential of the antigen. The lectin PHA is often added as a positive control, because of its nonspecific T cell-activating ability. This assay continues to be used extensively, because clonal expansion of a tumor antigen-specific T-cell population is the desired outcome of any vaccination protocol. Many groups use this assay as a surrogate for a Class II restricted response as the incubation of APC with soluble antigen requires antigen uptake and most likely, preferential processing of that antigen in the Class II pathway.

Methodological Points to Consider When Evaluating Limiting Dilution Analysis Results

In general, limiting dilution analysis (LDA) has some major limitations. To adapt the assay to be more quantitative requires the use of replicates of PBMC at different concentrations to generate enough data points for analysis. As immune responses to tumor antigens require estimation of precursor frequency along a wide range, the assays can be extensive. In addition, cellular function can be inhibited or blunted by cryopreservation. Furthermore, cell losses in cryopreservation compound the requirements of these assays. Many LDA include an in vitro culture step prior to the analysis. Thus, readouts may not be a true reflection of the actual precursor frequency. Finally, both assays require prolonged culture so that issues pertaining to serum and media screening and quality assurance programs as well as technician training must be addressed. Results of both of these assays are highly operator dependent.

The most common assay to measure CTL activity is the CRA. The CRA has several limitations including lack of reproducibility due to inconsistencies in choice and labeling of targets. In its basic form, effector T cells are mixed with labeled target cells at effector to target ratios of 100:1 to 1:1. The cells are incubated together for 4–6 hours and release of ^{51}Cr is measured in the supernatant. This protocol is appropriate for detecting lytic reactivity, but tells nothing about the cell type performing the killing other than that it is MHC class I restricted. Furthermore, when used in a nonLDA or bulk culture format, a strong killing response could mean a robust population of effectors or a larger population of weak effectors. The targets themselves are also a problem. They must be HLA matched and ideally one would use autologous tumors. Unfortunately, for many solid tumors under evaluation, autologous tumor cannot be easily obtained.

Proliferation based assays also have distinct limitations, such as the inability to determine what T-cell type

is responding, poor reproducibility, and a nonquantitative readout. Although the assays can be adapted to a LDA format, the number of PBMC needed to perform this type of analysis would require the use of leukapheresis specimens. Finally, evaluation of the blastogenic response requires that antigens used be exceptionally pure. This is a problem as many recombinant proteins are made in *E. coli* systems, and even small amounts of endotoxin can result in T-cell proliferation. When adapted to the LDA format, both of these assays require a tremendous amount of effort and large numbers of cells. Therefore, few studies have assessed the robustness of either system. In addition, the cellular nature of the assays makes it difficult to determine reproducibility without well-defined positive controls.

Clinical Usefulness of the Limiting Dilution Analysis

The CRA has been used as a method to determine immunogenicity and to monitor clinical trials. The limitations of CRA have led to the development of other methods of immunologic monitoring such as ELISPOT and cytokine flow cytometry. Although we expect that unimmunized volunteers or patients with cancer will not have detectable tumor antigen- specific immunity, this is not always the case. For example, a significant number of individuals without cancer as well as some melanoma patients can have immune responses to melanoma differentiation antigens (144–146). An evaluation of volunteers without cancer and patients with cancer is needed to assess the prevalence of immunity in an unimmunized melanoma population. Similarly, analyses might be necessary in patients with other malignancies. Immunologic readout against helper peptides and tumor-specific protein antigens has focused on proliferative responses. Although used as a measure to determine immunogenicity of tumor-related proteins and as a method to monitor immune responses in clinical trials, there has been no good correlation of antigen-specific proliferation with clinical benefit.

Enzyme-Linked Immunospot Assays

Principle of the Assay

The ELISPOT assay was originally established in 1983 for detection of antibody secreting cells (147) and was later adapted for the detection of antigen-specific T-cells (148,149). ELISPOT is not one assay but, in fact, many distinct assays, depending on the format of the assay selected. The ELISPOT assay is based on the principle of the ELISA. A 96-well nitrocellulose-bottomed microtiter plate is coated with an antibody that binds the cytokine of interest. To detect antigen-specific T-cells, either unseparated PBMC or isolated CD8+ or CD4+ T lymphocytes are incubated in the antibody-coated wells together with an antigen for 6–48 hours. In response to recognition of the antigen, T-cells specifically release the cytokine of interest, which is then bound by the well-coated antibody. After a washing step, which removes the cells from the wells, cytokine release is visualized by an enzyme-labeled detection-antibody and its corresponding chromogenic substrate, which has to be nonsoluble to become attached to the surface of a well. The end result is a set of colored spots, each of which represents the area where one cell secreting the cytokine of interest had been. The limit of detection by ELISPOT was reported to be 10–200 times lower than ELISA performed on culture supernatants (150). After short-term in vitro stimulation of PBMC, however, the secretion of cytokines in response to antigen recognition into the culture supernatants as detected with the ELISA can be used as a semiquantitative measure for specific T-cells (151).

Plates

Most groups use sterile nitrocellulose-bottomed 96-well plates; however, the use of Nylon-bottomed plates or plastic-bottomed plates is also possible and is reported to reduce background staining (152). The disadvantage of plastic-bottomed plates is that the spots are not stable, so the plates have to be analyzed immediately. Overlaying wells with BCIP substrate in 1% low melting agarose solves this problem (153). Leaking of the membrane on the bottom of the plate may also be a problem.

Serum

T-cell coincubation with antigen in the ELISPOT plate is usually performed in the presence of 10% serum. This is a critical point because the serum alone can stimulate IFN-γ secretion in T cells. It is therefore recommended that inactivated human serum be used not only for the assay but also for freezing and overnight resting. Different human serum batches should be tested before use to ensure that the serum alone neither stimulates IFN- γ secretion in T cells nor suppresses antigen-induced IFN-γ secretion. Most laboratories use human group AB serum; however, some use AIM-V serum-free media, as serum may nonspecifically stimulate APC used in the assay.

Antibodies

The choice of the antibody pair used for trapping and detection of cytokines is critical. Each antibody pair has to be carefully titrated chessboard wise to determine the optimal concentration. Several antibody pairs for ELISPOT assays are now available commercially, but not all are equally well suited.

Enzymes/Substrates

Most detection antibodies use alkaline-phosphatase or peroxidase as the developing enzyme for the chromogenic substrate. With the latter, endogenous peroxidase may give some diffuse background staining if cell debris is left on the membranes.

Read-Out Systems/Technical Requirements

Manual enumeration of spots can be laborious and is subject to human error, especially if multiple cytokine secreting cells are present in a single well or if the spots are very small. Therefore, computer-assisted image analysis systems have been adapted for an automated evaluation of ELISPOT plates (154,155). Such systems lead to a more objective analysis of spots and provide helpful parameters, thus allowing one to discriminate between spots and possible color sediments and measure the size of the spots reflecting the amount of cytokine produced by a single cell, which can be a function of T-cell avidity (135). Commercially available image analysis systems adapted for the evaluation of ELISPOT plates are still expensive, however, and not all of them yield reproducible results.

Analysis of Results

There are different ways to analyze and report ELISPOT results. Usually, frequencies of IFN-γ-secreting T-cells in unstimulated T-cells or in response to a control peptide like HIV are subtracted from T-cells reactive to the antigen of interest. If T-cell frequencies against single epitopes in peripheral blood are analyzed, usually 1 million PBMC are necessary to analyze whether the expected frequencies are < 100/1 million PBMC. These cells must be seeded into five or six wells to get optimal cell densities. One possible approach is to consider a T-cell response positive if the number of spots in six wells with peptide exceeds the number of spots in six control wells by 10 and the difference between the single values of the six wells containing peptide and the six control wells is statistically significant at a level of $p \leq 0.05$ using the *t* test. Other groups consider individual samples positive when at least a two- or three-fold increase in T-cell precursor frequency is noted compared with unstimulated samples. Samples taken after vaccination are considered positive when the frequency of post-vaccinated peptide-specific T-cell precursors is at least two or three times higher than the samples taken before vaccination, or when samples taken after vaccination are higher than ones taken before by at least two to three standard deviations from the background value.

Markers Analyzed

As a read-out in the ELISPOT assay most investigators now use IFN-γ production. For the detection of antigen-reactive T-cells in PBMC samples, measurement of IFN-γ release has the advantage in that unstimulated PBMC in many subjects do not secrete IFN-γ spontaneously (132); a small fraction of PBMC constitutively secrete TNF-α (149). Interleukin-2 and IL-4 are usually not suited for "ex vivo" analysis of specific CD8+T-cells because too few T-cells secrete these cytokines. Interleukin-5 was reported to be a suitable cytokine to detect Th2 responses (156). The detection of granzymes B release by ELISPOT assay is also possible (157). However, some have maintained that granzymes B is not well suited for "ex vivo" analysis because there is some spontaneous release and the spots are rather small (Scheibenbogen C, personal communication, November, 2001).

Effector Cells

T-cell responses can be analyzed in the whole PBMC population or after separation of T-cell subpopulations. If TAP-deficient T2 cells lacking endogenous MHC are used as APC, before the separation of CD8+T-cells, they may reduce the background IFN-γ release. Data from one multicenter trial suggest that concordant results are obtained if T-cell responses are either analyzed using whole PBMC or using CD8+ T-cells and peptide-presenting T2 cells (158). The most readily available source of lymphocytes for ELISPOT assays is the peripheral blood; however, data exist to suggest that T-cell responses in the blood may not always accurately represent the responses evident in draining nodes or tumor deposits. It is also possible to perform ELISPOT assays with lymphocytes obtained from these other sites. (159,160).

Antigen/Antigen-Presenting Cells

Most investigators analyze T-cell responses against MHC class I-restricted peptide epitopes. If T-cell re-

sponses are analyzed using PBMC, free peptide that is presumably presented by monocytes can be added directly. Variable frequencies of monocytes were shown to have no significant influence on the detectable frequency of peptide-specific T-cells (161). The TAP-deficient T2 cell line is frequently used as an APC, because this cell line exclusively expresses HLA-A2 molecules. However, T2 cells themselves induce some T-cells or probably NK cells to secrete IFN- γ. In a comparative study assessing T-cell response against FLU peptide in blinded samples with the IFN- γ ELISPOT assay, similar results were reported using PBMC and free peptide versus peptide-loaded T2 cells and separated CD8+ T-cells (158). The use of HLA-A2-matched allogeneic tumor-cell lines as target T-cells is a also feasible approach, because little or no alloreactivity is seen in a 20-hour ELISPOT assay (162). Tumor cells can be used as APC but have significant disadvantages. Tumors do not process or present antigens in a "normal" way, and they also secrete immunoinhibitory factors. Pass et al. reported that the frequencies of gp100 peptide-reactive T-cells detectable with the IFN-γ ELISPOT assay are 10–100 fold higher using T2 cells instead of tumor cells as APC (163). Investigators have also used HLA-A2-transduced insect-cells (164) and the C1R-A2 cell line as APC. The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or B antigens. The C1R-A2 cell line is composed of C1R cells that express a transfected genomic clone of HLA-A2.1 and is used when measuring response to HLA-A2 peptides. There have been no problems with high backgrounds using C1R-A2, which circumvents the potential problem of defective APC common with autologous PBMC (131). Also being evaluated is the use of autologous dendritic cells infected with recombinant poxvirus vectors expressing full-length antigen genes as targets to avoid the HLA restriction and provide a wider range of antigenic epitopes for presentation.

The ELISPOT assay is also suitable for determination of T-cell frequencies against proteins (160). Exogenous proteins are usually presented via the MHC class II pathway to CD4+ T-cells. A preincubation of PBMC with protein prior to plating the cells in the ELISPOT assay is required for efficient antigen presentation. Protein concentrations of around 1 microgram/ml are optimal, but this should be titrated for each protein. The concentration of monocytes can influence T-cell frequencies, although this is not seen when free peptide is added.

After in vitro sensitization, the APC in peripheral blood (monocytes, macrophages, B cells) have been depleted in culture. Instead of free peptide alone, autologous PBL or APC corresponding to the patient's HLA type, have to be used in ELISPOT assays with stimulated lymphocyte samples. The addition of fresh APC enables efficient presentation of peptide to all antigen-specific cells in the lymphocyte populations being evaluated (153,160). The impact of efficient antigen presentation in ELISPOT assays with PBL samples after sensitization with peptide is demonstrated in Figure 2.

Quality/Sensitivity of the Assay

Using T-cell clones it was shown that ELISPOT measurements could be made with single-cell resolution (135). Some initial attempts have been made to standardize the quantitation of antigen-specific T-cells by ELISPOT assay. An almost linear correlation between the number of spots and the number of peptide-specific T-cells either derived from peripheral blood or from a short-term T-cell line was demonstrated showing that ELISPOT quantification is reliable over a wide range (132). To evaluate the reliability of T-cell frequency analysis with the ELISPOT assay, a comparative study was performed in four European laboratories (158). Similar frequencies of FLU-reactive T-cells were reported in the ELISPOT assay by all four laboratories.

Positive Controls

As a positive control for T-cell cytokine synthesis and release, some groups have introduced mitogen-stimulated T-cells in the ELISPOT assay. Pokeweed mitogen, which stimulates IFN-γ release in a subset of 0.1%–1% of CD4+ and CD8+ T-cells, has proven to be a suitable positive control to the ELISPOT assay (165). Impairment or absence of pokeweed mitogen-induced IFN-γ release may indicate cell damage during the freez-

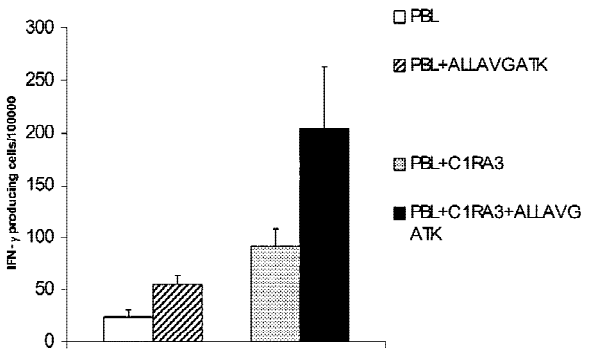

FIG. 2. Peripheral blood lymphocytes of VMM18 patients have been sensitized with ALLAVGATK peptide (40 mg/mL) once. After 2 weeks in culture with IL-2 (20U/mL), inteferon-γ enzyme-linked immunospot assay was performed using peptide alone or C1RA3 loaded with ALLAVGATK (40 ug/mL). 112 ALLAVGATK specific T cells were revealed when C1RA3 were used as antigen-presenting cells, but only 30 when peptide was used alone.

ing and thawing procedure prior to assaying. Phytohemagglutinin (PHA) is also a useful positive control mitogen. It stimulates IFN-γ release in approximately 0.1%–0.2% of PBMC from all individuals with low intra- (7%) and interassay (17%) variability. Several investigators use FLU-reactive T cells as a positive control for the T-cell reactivity during the course of vaccination in patients with cancer. Seventy-five percent of healthy individuals had detectable T cells reactive with HLA class I-restricted FLU peptide epitopes (165,166). Candida has also been used as recall antigen control when multiple HLA types are being assayed. A Candida response was detected in 91% (n = 35) of women assayed with cervical dysplasia regardless of HLA type (Lathey JL, personal communication, November, 2001).

Reproducibility of the Enzyme-Linked Immunospot Assay

Studies were conducted to validate the reproducibility of the ELISPOT assay when using frozen PBMC that were thawed and cultured overnight (i.e., for 18 hours) before assay (131). Aliquots of PBMC from two healthy HLA-A2 donors were frozen in liquid nitrogen to be used as standards. As shown in Table 3, aliquots were thawed from each of these donors at various times after freezing. Enzyme-linked immunospot assays were used to determine the reproducibility of measurement of a FLU peptide-specific T-cell precursor frequency. The mean ± standard deviation precursor frequencies for donors 1 and 2 (from six and seven determinations, respectively) were 1/20,197 ± 1/3054 (0.15-fold) and 1/7,657 ± 1/792 (0.10-fold), respectively. The results from both of these groups show that determination of precursor frequency for the FLU peptide, using this ELISPOT assay for IFN-γ, can be reproduced at different times after the PBMC are thawed. Furthermore, these PBMC samples from healthy donors have been used as internal controls to validate assays reported when determining the precursor frequency of PBMC obtained from cancer vaccine trials.

TABLE 3. *Reproducibility of precursor frequency determinations using the ELISPOT assay*

Donor	Time before thawing (days)*	Precursor frequency†
1	59	1/21,739
	63	1/19,718
	77	1/20,588
	116	1/16,280
	131	1/17,857
	158	1/25,000
2	59	1/6,931
	76	1/7,692
	84	1/7,954
	96	1/6,816
	129	1/6,931
	130	1/8,695
	133	1/8,571

* The time before thawing represents the number of days after the sample was initially frozen in liquid nitrogen before being thawed for the assay.

† Results are expressed as peptide-specific IFN-γ-secreting PBMC. Donor 1 mean ± SD = 1/20,197 ± 1/3,054; donor 2 mean ± SD = 1/7,657 ± 1/792. Healthy donors 1 or 2 were used simultaneously in each ELISPOT assay with patient samples.

Reprinted from *Cancer Immunology and Immunotherapy:* 49, Arlen et al., The use of a rapid ELISPOT assay to analyze peptide-specific immune responses in carcinoma patients to peptide vs. recombinant poxvirus vaccines: 521, Table 1: Copyright 2000, with permission from Springer-Verlag.

ELISPOT, enzyme-linked immunospot; IFN, interferon; PBMC, peripheral blood mononuclear cells; SD, standard deviation.

Detection of Naturally Occurring Tumor-Specific T-Cells in Unstimulated Peripheral Blood T-Cells of Patients With Cancer

A number of studies have reported the detection of tumor-reactive T-cells in the peripheral blood of patients with tumors using the ELISPOT assay (Table 4). Taken together these data suggest that the assay is suitable to detect low frequency functional T-cell responses against tumor antigens in PBMC.

Enzyme-Linked Immunospot Monitoring of Tumor-Specific T-Cells in Vaccination Trials

Several vaccination trials have been published describing the use of the ELISPOT assay alone or in combination with other assays for T-cell monitoring (129,131,167–177). Most investigators have analyzed T-cell responses against peptides (Table 5), but there are also reports showing the suitability of melanoma cells

TABLE 4. *Detection of naturally occurring tumor-specific T cells in peripheral blood by ELISPOT assay*

Tumor	Antigen	Reference
Melanoma	Tyrosinase/MART-1	Herr (149), Scheibenbogen (165), Bennouna (156)
Colon cancer	CEA, EpCAM, her2-neu	Marshall (172), Nagorsen (178)
Melanoma	various	Griffioen (137), Dhodapkar (138)
Melanoma	MAGE-A10	Valmori (179)
Melanoma/sarcoma	NY-ESO	Jager (180)
CLL, melanoma	surviving	Andersen (181)
CEA-pos. carcinomas	CEA	Arlen (131)

CEA, carcinoembryonic antigen; CLL, chronic lymphocytic leukemia; ELISPOT, enzyme-linked immunospot.

TABLE 5. *Antigen-specific vaccination trials monitored by ELISPOT assay*

Antigen/adjuvants	Correlation of T-cell and tumor response	Reference
MART-1/IFA	ELISPOT no ELISA yes (prolonged freedom from relapse)	Wang (177)
Tyrosinase/QS21	no (no tumor response)	Lewis (182)
Tyrosinase/GM-CSF	yes	Scheibenbogen (139)
Dendritic cells with various melanoma peptides	yes	Banchereau (176)
Dendritic cells with MAGE-1/MAGE-3	no	Toungouz (183)
Dendritic cells with MAGE-3 peptide	no (most patients mounted T-cell response)	Jonuleit (184)
rV-CEA prime	yes (increased survival)	Marshall (172)
Avipox-CEA boosts	yes (correlated with survival)	Slack (185)
rV-PSA prime	yes (PSA stabilization)	Eder (170)
Avipox-PSA boost		
CEA	yes	von Mehren (173)
Avipox-CEA/B7		
Avipox-CEA/B7	yes	Horig (171)

CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; GM-CSF, granulcoyte-macrophage colony-stimulating factor.

(163,174) or idiotype protein in patients with myeloma (175) for T-cell monitoring. For example, the ELISPOT results from Figure 3 demonstrate that avipox-CEA can be given at least four times with resulting increases in CEA-specific T-cells.

Correlation With Clinical Outcome

Some information is available regarding the correlation between T-cell response induction as demonstrated by ELISPOT assay and clinical outcome (Table 4). In patients immunized with a polyvalent melanoma vaccine, the induction of specific T-cells reactive with HLA class I binding peptides derived from MAGE-3 and/or

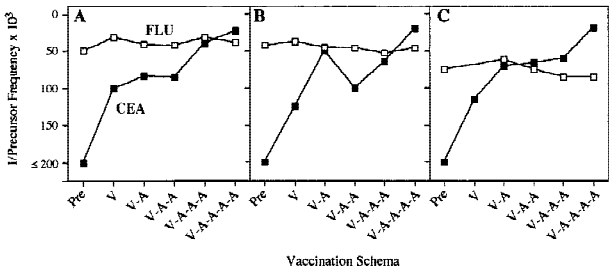

FIG. 3. The impact of carcinoembryonic antigen (CEA)-based vaccination on CEA-specific T-cell responses. Three patients received rV-CEA (V) followed by three vaccinations with avipox-CEA (A). Depicted are T-cell responses to the 9-mer influenza (FLU) peptide (□) and to the 9-mer CEA peptide (■) using the enzyme-linked immunospot assay. Reprinted from *Journal of Clinical Oncology:* 18, Marshall et al., Phase I study in advanced cancer patients of a diversified prime and boost vaccination protocol using recombinant baccinia virus and recombinant nonreplacing avipox virus to elicit anti-carcinoembryonic antigen immune responses, 3969, Table 3: Copyright 2000, with permission from Lippincott, Williams & Wilkins.

MART-1 was found to correlate with prolonged freedom from recurrence (174). In a phase I study with MART-1 peptide in high-risk melanoma patients, T-cell induction against the vaccine peptide, determined by ELISA after two in vitro stimulations but not by ELISPOT assay, was associated with prolonged freedom from relapse (177). In a randomized phase II trial conducted by the same group with gp100 and tyrosinase peptides, IFA +/– IL-12, no correlation between T-cell induction determined by ELISA after one or two in vitro stimulations or by tetramers and relapse-free survival was found (151).

A recent study in which 18 patients with melanoma were vaccinated with peptide-loaded DC showed a good correlation between tumor regression and T-cell response analyzed by ex vivo and recall ELISPOT assay (176). In a phase I clinical trial with ALVAC-CEA-B7, three patients experienced clinically stable disease that correlated with increasing CEA-specific precursor T-cells, as shown by in vitro IFN-γ enzyme-linked immunoassay spot tests (Fig. 4). In a phase II study vaccinating patients with stage IV melanoma with tyrosinase peptides and GM-CSF, specific T-cells were detectable in four of fifteen patients, including the only patient with a mixed response, one of two patients with stable disease, and two patients with prolonged freedom from recurrence (139).

A recent study (172) examined whether changes in CEA-specific T-cell responses (using the ELISPOT assay) as a result of CEA-based vaccination may be prognostic for survival. Eighteen patients (12 HLA A2+) were randomized to receive monthly vaccines with CEA-vaccinia (V) as priming with CEA-avipox as a boost (VAAA, n = 9, 6 A2+) or CEA-vaccinia as a boost (AAAV, n = 9, 6 A2+). Eleven of 12 HLA A2+ patients

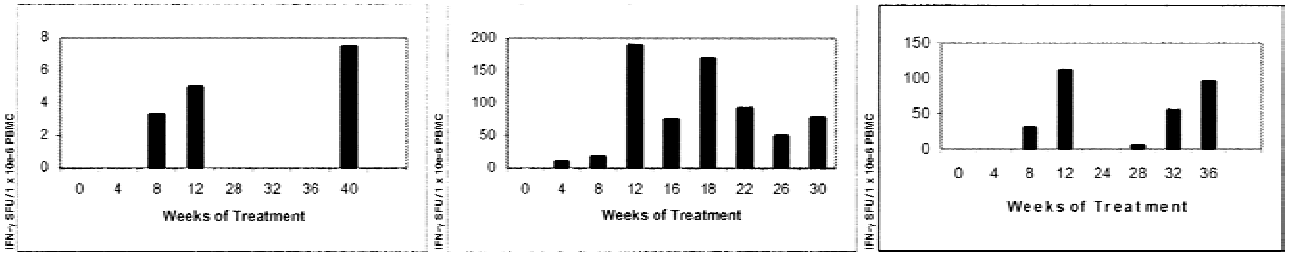

FIG. 4. Sample of enzyme-linked immunospot assay results from three patients immunized with an ALVAC-carcinoembryonic antigen (CEA)-B7.1 virus. Note that none of the patients exhibit CEA-specific T cells before vaccination, whereas all three patients demonstrate significant precursors after three vaccinations. Interestingly, these patients appear to lose the response after stopping treatment, but could be boosted with additional vaccinations. All three patients exhibited stable clinical disease throughout the vaccination period. Reprinted from *Cancer Immunology and Immunotherapy:* 49, Horig et al, Phase I clinical trial of a recombinant canarypoxvirus (ALVAC) vaccine expressing human carcinoembryonic antigen and the B7.1 co-stimulatory molecule: 510, Figure 4: Copyright 2000, with permission from Springer-Verlag.

had complete immunologic data for analysis. Samples for CEA-specific T-cell responses (analyzed with an overnight ELISPOT assay) were obtained q28d before each vaccination. To test the association between T-cell responses and clinical outcomes in the A2+ patients, several potential prognostic factors were explored separately using Cox regression models with continuous data where possible. Each model included one prognostic factors controlling for disease status (NED or advanced) at the time of study entry. These prognostic factors were (1) pre- and (2) post-4 month values, (3) the ratio of post-/pre-CEA-specific T-cell responses, and (4) treatment order (VAAA versus AAAV). The analysis demonstrated that both higher posttreatment levels of CEA-specific T-cell responses as well as ratios of post-/pretreatment levels were associated with increased survival after accounting for disease status ($p = 0.04$ and $p = 0.03$, respectively). Estimates of survival at 2 years are 67% (+/– 19%) and 0% for patients with post-T-cell titers at 4 months of $> 1/80000$ and $\leq 1/80000$, respectively. Patients with a ratio of T-cells post-/pre-treatment > 2.5 had survival estimates of 60% (+/– 22%) compared with 17% (+/– 15%) for ratios ≤ 2.5. Treatment with VAAA resulted in longer survival than treatment with AAAV with five of nine patients randomized to the VAAA arm still alive compared with zero of nine in the AAAV arm ($p = 0.05$). The survival estimates for all patients treated ($n = 18$) at 2 years are 56% (+/– 17%) and 11% (+/– 10%) for VAAA and AAAV, respectively. The improved survival was independent of HLA A2 status. Survival duration was unrelated to pretreatment T-cell levels ($p = 0.77$) or disease status at study entry ($p = 0.66$). In summary, in a small sample of patients with colorectal cancer, an association was shown between the intermediate endpoint-CEA-specific T-cell response using the ELISPOT assay, the absolute number of T-cells, as well as the ratio of pre- and posttreatment values and survival.

Cytokine Flow Cytometry: A method for Analysis of Antigen-Specific T-Cell Subsets

Basic Principles

Cytokine flow cytometry or intracellular cytokine cytometry (ICC) is based on direct detection of intracellular cytokine expression with fluorochrome-conjugated anti-cytokine antibodies after short periods of activation with various stimuli (Fig. 5). Stimulation can be performed with mononuclear cells isolated from PBMC (186), whole blood (187,188), lymph nodes, or other biologic fluids. A total incubation period of 6 hours is optimal for achieving high levels of cytokine-secreting cells for IL-2, IL-4, IFN-γ, and TNF-α, as well as for achieving maximal cytokine staining intensity (188). Cytokine secretion is disrupted during the latter portion of the incubation (usually the last 4 hours) with the addition of drugs that inhibit cytokine secretion such as monensin or brefeldin A (189). The cells are then fixed using paraformaldehyde or similar agents. Permeabilization of cell membranes is

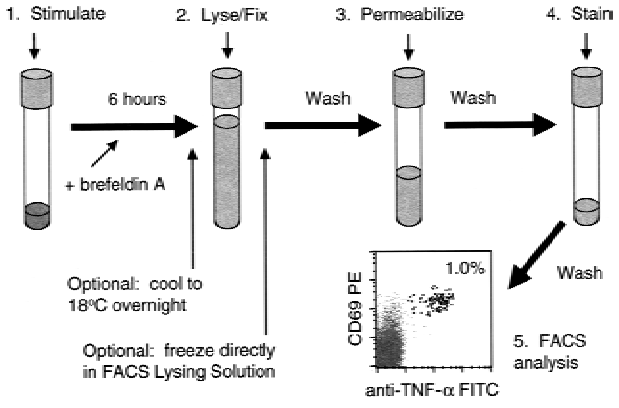

FIG. 5. Schematic of cytokine flow cytometry procedure. Note the optional stopping points that can be used to automate handling of clinical samples and batch them for later staining and analysis.

achieved using nonionic detergents, followed by intracellular staining using mixtures of antibodies that recognize determinants in fixed and permeabilized cells.

Unstimulated leukocytes normally do not express cytokine. Because background constitutive cytokine expression is rare (usually $\leq$ 0.05% of CD4 or CD8 T cells), very low frequencies of positive stimulated cells can be detected. In addition, because of the short incubation time in the presence of a secretion inhibitor, bystander effects or cytokine absorption by soluble or cell-surface receptors does not compromise the cytokine immune profiles.

T-cell responses in antigen-specific CFC assays are dominated by memory cells, as determined by phenotyping of cytokine positive cells for markers such as CD45RA versus CD45RO, CD27, CD44, and CD95 (187). CD4+ T cells dominate the response to intact protein antigens, although some $CD8^+$ responses can be detected (187,190,191), particularly with higher antigen doses. On the other hand, optimal peptide epitopes or peptide mixtures can be used as antigens to efficiently induce $CD8^+$ T-cell responses (3,192–195). Figure 6 illustrates CD4+ and CD8+ T cell cytokine responses in normal whole blood cultures responding to whole protein and peptide CMV antigens. Altered in vitro T cell cytokine responses to nominal antigen as a consequence of vaccination or disease status can be measured using this procedure (134,196–203).

Significant Methodological Issues

Antibodies selected for intracellular staining (e.g. anti-cytokine mAbs) need to possess high affinity and specificity for epitopes that survive the particular fixation and permeabilization conditions used. Altering the fixation and permeabilization conditions might alter the performance and optimal titer of surface and intracellular staining antibodies. Many commonly used monoclonal antibodies to cell surface determinants from a number of vendors can be used with fixed and permeabilized cells (e.g., CD3, CD4, CD8, CD69, and cytokine-specific antibodies). In situations where antibodies to cell-surface markers do not work well under fixation and permeabilization conditions, it is necessary to add a separate surface staining step prior to fixation and permeabilization. Also essential is the use of highly purified fluorochrome conjugates of antibodies used to detect intracellular targets. Particularly, the absence of free fluorochrome and overly-conjugated antibody is important to minimize backgrounds and retain the high avidity of antigen binding in intracellular staining necessary for optimal flow cytometric detection of rare antigen-specific events.

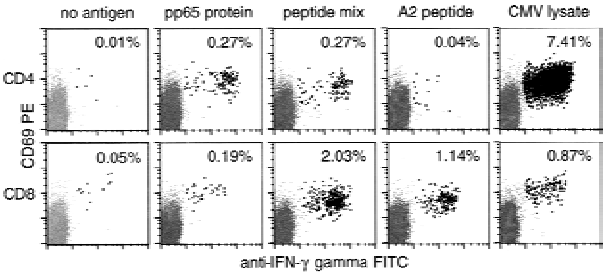

FIG. 6. Typical example of cytokine flow cytometry responses with various types of cytomegalovirus (CMV) antigens. CD3+CD4+ gated lymphocytes are shown in the top row, and CD3+CD8+ gated lymphocytes are shown in the bottom row. "Peptide mix" refers to a mixture of 138 overlapping 15-amino acid peptides spanning the CMV pp65 protein. "A2 peptide" refers to the HLA-A2-restricted pp65-derived peptide, NLVPMVATV. Reprinted from *Journal of Immunological Methods:* 255, Maecker et al., Use of overlapping peptide mixtures as antigens for cytokine flow cytometry: 30, Figure 1B: Copyright 2001, with permission from Elsevier Science.

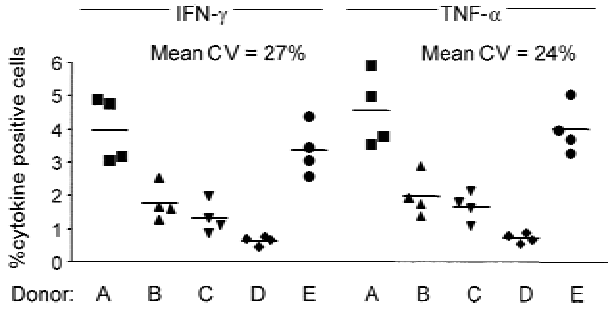

FIG. 7. Results of a multi-site study of cytokine flow cytometry reproducibility. Whole blood from five donors (A-E) was shipped at room temperature via overnight courier to four participating laboratories. Each lab activated, processed, and analyzed the samples independently and reported their results as percent cytokine-positive CD4+ cells responding to CMV lysate (symbols). Bars represent the means of the four labs' results for each donor and cytokine. Mean coefficients of variation (CV) are shown.

Cytokine Flow Cytometry Assay Validation

Functional assays in general are subject to higher variation than phenotypic staining, due to assay complexity and the biologic variability associated with in vitro cell activation. Nevertheless recent evaluations have reported an overall coefficient of variation in a CMV-specific CFC assay to be within 5% for intraassay variability (188) and within 20% in a multi-site study (Fig. 7). Interestingly, it was determined that much of the interassay variability could be attributed to differential gating during analysis. For example, $CD4^{dim}$ or $CD8^{dim}$ lymphocytes can often be found as a very minor proportion of the total $CD4^+$ or $CD8^+$ cells. However, these cells tend to be highly enriched for activated cells, since

they have undergone down-modulation as a result of recent antigenic stimulation. Thus, inclusion or exclusion of these cells can result in a difference of up to 1.5-fold in the percentage of cytokine-positive cells assessed. With experience, however, consistent gating can certainly be achieved. In the future, automated gating algorithms may also be available to minimize this source of variability.

Application to Clinical Samples

Cytokine flow cytometry assays are a potentially powerful tool for analyzing antigen-specific T-cell responses in a quantitative manner. Standard functional assays tend to rely on longer stimulation times to amplify specific populations, allowing time for apoptosis and/or proliferation, and thus compromising the ability to quantitate precursor cell frequencies. In addition, the ability to perform CFC assays in whole blood within 8 hours, with minimal hands-on time, make CFC assays highly feasible as clinical monitoring tools. Finally, the multiparametric information obtained from flow cytometry allows for qualitative as well as quantitative information about the nature of immune responses to specific antigens.

The CFC assay has been used to characterize human T cell responses to a number of infectious disease agents including HIV (197,198,204–206), CMV (134,188,190, 193,195,196,199,200,207,213), EBV (214,215), and others (190,216–222). The largest amount of published data using CFC in clinical settings is in the area of HIV-specific immune responses. Pitcher et al. (197) showed that patients with HIV could be stratified into those that maintain a detectable CD4 Th response to HIV antigens by CFC assay and those that do not. All HIV+ nonprogressors tested were in the former group, showing CD4+ Th cell IFN-γ and TNF-α responses to HIV p55 *gag* of 0.1% or greater. About half of those with progressive disease showed similar CD4+ Th responses, but half did not; likewise with individuals who had been on highly active antiretroviral therapy (HAART) for less than 6 months. Perhaps most interestingly, individuals treated with long-term HAART were uniformly low (< 0.1%) in their CD4 Th response to HIV antigens.

Another potential area of clinical application for CFC assays is in the development of new vaccines, and the refinement of existing vaccines. As part of a recent clinical study of vaccination with a gp120-depleted inactivated HIV immunogen, CD4+ T cell CFC responses to HIV were followed in 18 patients (198). Dramatic increases in the frequency of HIV-specific CD4 cells were demonstrated in 15 of 18 patients after three vaccinations. In another example, varicella immunization has been shown to induce significantly lower CFC responses in older adults than in young adults; and such responses in older adults are not boosted by secondary immunization (190).

Several studies have also demonstrated that CFC assays can be used to detect responses to tumor antigens: MART-1 and tyrosinase epitopes in melanoma (3), MUC-1 mucin in various solid tumors (203), and immunoglobulin idiotype in vaccinated multiple myeloma patients (201). These reports demonstrate that the sensitivity of CFC assays is sufficient to detect positive responses to tumor antigens, although the response frequencies are often lower than with chronic infectious disease antigens. It remains to be seen whether CFC assays are predictive of clinical responses in vaccinated patients with cancer. In part, this is dependent on the use of these assays in studies with a vaccine that has at least some clinical benefit. Because there are currently no Food and Drug Administration-approved cancer vaccines, identifying vaccines that have the most potential for clinical efficacy is of paramount importance.

Clinical Relevance of Cytokine Flow Cytometry Assays

The ability to quantitate frequencies of functional antigen specific T cells has enabled investigators to assess the relationship between the strength of CD4+ and CD8+ T cell responses and immune protection in a number of disease models. Thus, the response to infection of Lymphocytic Choriomeningitis virus in mice is associated with strong CD4 T cell cytokine responses, which are correlated with long-term memory (223). In a murine model of Leishmania, protective vaccination is associated with the development of IFN-γ-producing CD8 T cells, as measured by CFC (224,225). In a primate model of AIDS, control of viral rebound after structured therapy interruption is strongly correlated with anti-viral CD8 T cell cytokine responses (226). Such responses are also correlated with protection from mucosal challenge after vaccination (227). In humans, susceptibility to CMV-associated end organ disease in HIV-infected individuals was shown to correlate with the loss of CD4+ T-cell IFN-γ responses to CMV antigens (196,199,200). Even though such studies demonstrate correlation of CFC responses with disease outcome, there have been no proven thresholds established for protective immunity as measured by a functional assay in any disease system. In fact, the precise correlates of immunity are still poorly defined for most disease and vaccine systems.

The determination of a positive CFC response is dependent upon the level of background in the negative control sample, and upon the sample size. In fact, a sta-

tistical calculation can be made using these variables, such that a given difference between sample and control can be deemed significant with a particular power and confidence level (228). In other words, one obtains greatest sensitivity in the presence of low backgrounds and large sample sizes. For example, a sample size of 25000 events is sufficient to distinguish a 0.1% population as positive over a background of 0.03%, with 90% power and $p < 0.05$. However, at a background of 0.05%, more than 50000 events would be needed to distinguish the same positive population with the same power and confidence. In practice, achieving backgrounds of less than 0.05% is practical, save for the occasional donor who presents with a significant spontaneous cytokine-secreting population. Thus, it is not impossible to routinely identify positive populations on the order of 0.1% or even slightly less. On the other hand, populations significantly lower, on the order of 0.01%, will not be distinguishable from background, because they approach the average level of spontaneous cytokine-secreting cells in peripheral blood.

One factor that could still compromise the ability to detect significant antigen-specific T-cell responses after vaccination in clinical samples is the suboptimal timing of sample acquisition. This issue is being addressed in experiments examining the dynamics of the tetanus toxoid (TT-)-specific T-cell recall response (229). In these studies, healthy volunteers were vaccinated with TT, and CFC measured the TT-specific T-cell response directly in the peripheral blood after vaccination. The CD4 T-cell response peaked 1 week after vaccination at 0.4% of CD4+ T cells. Remarkably, we also noted a CD8 T-cell response, which peaked at about 0.5% of CD8+ T cells; however, this response peaked as much as 3 weeks later than that of the CD4 response. These data provide a time frame for sampling the peripheral blood to examine antigen-specific T-cell responses to vaccines and support the hypothesis that clinically significant T-cell immunity should be detectable directly from peripheral blood. The detection of spontaneous cytokine secreting cells, in fact, has the lowest limit of detection for any functional assay.

Exploration of the clinical use of CFC assays has been most extensive in HIV disease, where it has been shown to correlate with disease stage (197), and where it has been used to follow vaccine responses (198). Cytokine flow cytometry has also been used to detect responses to cancer vaccines (201,203). The use of CFC with either specific antigens or polyclonal stimuli to assess T cell responsiveness in other disease settings is less well explored. However, the opportunities to enhance patient care by more carefully monitoring the immune system should encourage future studies aimed at validating the use of such monitoring.

Major Histocompatability Complex-Peptide Tetramer Analysis for Cancer-Vaccine Monitoring

Basic Principles of the Tetramer Assay

Soluble recombinant MHC-peptide tetramers are becoming an increasingly useful tool capable of not only identifying and enumerating antigen-specific T cells, but also of providing functional information when combined with other methodologies. Tetramers may be generated using now well-established procedures (http://www.emory.edu/WHSC/TETRAMER/protocol.html). Briefly, recombinant MHC class I heavy chains that incorporate a short C-terminal substrate peptide for BirA-mediated biotinylation are produced in *E. coli*, purified from inclusion bodies, folded in the context of synthetic peptides and β2-microglobulin and biotinylated. Based on the specific application, fluorochrome-labeled (FITC, PE, PerCP, etc.) streptavidin is then added to purified (gel filtration and anion exchange) MHC/peptide "monomers" to form soluble tetrameric complexes. Fluorescent MHC/peptide tetramers when incubated with a polyclonal mixture of T cells, under the right conditions, will bind those T cells bearing MHC/peptide-specific TCRs and may be detected by fluorescent imaging systems, such as flow cytometry. While most published information has been gathered using MHC class I-peptide tetramers for CD8+ T cell screening, MHC class II-peptide tetramers have also been developed for the assessment of CD4+ T cell responses (230–232). The ability to monitor CD4+ and CD8+ tumor-specific T-cell responses, particularly in the face of active therapy, will likely prove critical to efforts to define appropriate laboratory surrogates in the cancer setting.

Factors Affecting Major Histocompatability Complex-Peptide Tetramer Staining

In practice, each new tetramer must be tested for specificity and titered before use. It is important to use tetramers at optimal concentrations to maximize the signal-to-noise ratio determined via flow cytometry, particularly when looking for rare T cell populations (< 0.1%). In general, the consensus is that the lower limit of detection for currently used tetramer-based assays is approximately 1/8000–1/10000 (i.e., 0.01%–0.0125%) (221,233–236). In most cases, quality-control requires the generation of specific T cell lines or clones to validate the specificity and high signal-to-noise ratio re-

quired for optimal performance of tetramer-based analyses. Because MHC/peptide tetramers are noncovalent complexes (i.e., MHC heavy chain, β2-microglobulin, peptide, and streptavidin), they "degrade" at variable rates that appear to depend in large part on MHC-peptide affinity (Lee P, personal communication, November, 2001). Hence, a given lot of MHC-peptide tetramer may be stable anywhere from 2 years (for most viral peptide antigens) to as little as 3 months (for certain low-affinity "self" peptides), necessitating periodic retesting of the reagent's efficacy by retitration analyses on a regular (every 6–8 weeks) basis.

There are a number of technical factors that may impact on the quality of tetramer data. In addition to the inherent instability of the peptide-MHC interaction, the concentration of cells and tetramer used, timing and temperature used for staining, the counterstain (fluorochrome labeled antiCD8, antiCD3, etc.) implemented in multiparameter analyses, and contaminant non-T-cell populations present in bulk populations may dictate the interpretation of results.

Typically, at least one million PBMCs are used per staining condition and as many events as possible (10^5–10^6) are collected for analysis. Staining proceeds using tetramers (often PE-conjugated) at their "optimal" quality-controlled concentrations, generally for 15–30 minutes at room temperature, together with, or followed by, addition of anti-CD8-FITC (and anti-CD4/14/19-Cy5PE, optional probes for "negative" selection of events). Cells are then washed extensively (2–3 times). Propidium iodide (PI) may be added before FACS analysis (optional) to exclude dead cells. Multiparameter analyses are generally performed using flow cytometry. Lymphocytes are gated based on their forward and side-scatter, dead and "sticky" cells are excluded based on PI staining and/or with "dump" (negative selecting) antibodies, and the remaining cells are assessed for CD8 verus tetramer staining status. As the percentage of CD8+ T cells in total PBMC can vary widely between samples, tetramer-positive events are generally "normalized" as a percentage of total CD8+ T cells or data may be reported as the absolute number of tetramer+/CD8+ cells per quanta (i.e., μl-mL) of donor blood. Only cell populations that are clustered well and display a clear separation from the CD8+/tetramer-negative T-cell population in two parameter analyses are considered to be "real" events for "higher" avidity T-cell populations. In contrast to pathogen-specific T cell systems, however, cancer-reactive T cells may be of lower overall avidity perhaps due to tolerance mechanisms invoked against "self" epitopes that tumor cells frequently present in their MHC complexes. As a result, some caution should be taken so as not to discard events at or near the level of background as "noise," since they may indeed be informative of "low" avidity antitumor T cells. As described below, the specificity of tetramer staining of T cells may be confirmed by inclusion of "competitors" such as antiCD3 antibodies (127).

The concentration of tetramers used to stain cells is critical in determining the optimal signal-to-noise range for a given probe. Importantly, the percentage and fluorescence intensity of tetramer-positive events appear to increase in proportion to tetramer concentration, until a plateau is reached. Thus, staining with suboptimal tetramer concentrations may lead to an inaccurate assessment of specific T-cell frequencies. This is particularly important when making comparisons between samples or across multiple tetramers.

Based on an expanding literature, the staining temperature appears to significantly impact the degree of specific tetramer staining (235–237). Tetramer staining of T cells on ice (or at 4°C) appears to allow for significant formation of low avidity (frequently cross-reactive) interactions, thus decreasing the signal-to-noise ratio. In contrast, tetramer staining performed at room temperature (23°C) or 37°C, appears to favor predominantly higher avidity tetramer-TCR interactions, thereby reducing background staining of low avidity (specific or cross-reactive) T cells.

Another important variable is the specific "counterstaining" antiCD8 (or CD4) antibody used in multiparameter analyses. Several reports have suggested that tetramer staining may be affected by the concentration of antiCD8 (or CD3) antibodies used, and even by the particular clone of antibody used (234,235,238,239). Indeed, depending on the CD8 determinant recognized by the antibody, antiCD8 counterstaining might block, have little impact on, or even augment the intensity of MHC/peptide tetramer staining of T cells (236,238,239). In careful titration experiments, the percentage of registered tetramer-positive events may vary depending on the concentration and type of antiCD8 antibodies concentration. Clearly, this provides another major area of variability when comparing data obtained between samples tested at unrelated laboratories using nonidentical reagents and protocols. The recent development of MHC/peptide tetramers based on mutant class I heavy chains that fail to bind to the CD8 coreceptor (Gammon S, personal communication, November, 2001) may reduce "noise" affiliated with low-avidity or cross-reactive T cells and should provide a clearer resolution for at least higher avidity (CD8-independent) specific T cell clonotypes in a bulk population.

As mentioned above, exclusion of certain non-T cell

types is also important to the resolution of tetramer-based analyses. Certain cells (such as monocytes) appear to "stick" to tetramer reagents, and can significantly increase the population "background" staining observed in flow cytometry testing. To reduce the severity of this practical problem, one typically includes antibodies to markers not present on cells of interest to exclude these cells in data interpretation.

Tetramer Analyses: Healthy Donors Compared with Patients

While early studies of substituted "heteroclytic" MHC/peptide tetramers (particularly the HLA-A2.1/MART-1 26–35 tetramer) indicated arguably "high" frequencies of "specific" peripheral blood CD8+ T cells in HLA-A2.1+ donors (240), this result appears to represent an exception, rather than a rule for most tetramer-based testing in the cancer setting. In general, MHC/tumor peptide tetramers detect only low background frequency events (< 0.01%) when used to stain freshly isolated normal donor peripheral blood T cells (157,235,237). In marked contrast, tumor-specific T cells can be frequently detected directly from the blood of patients with cancer ([3] and Storkus et al., unpublished data), and these frequencies can be significantly enhanced as a result of patient vaccination (151,159,184, 237,241,242).

Clinical Relevance of Tetramer-Based Assays

What is the clinical relevance of this assay? At present, it is too early to say, given the limited number of clinical trial reports using MHC-peptide tetramers for immune monitoring. Even though it is clear that tetramer-based analyses can detect and quantify pre- versus posttherapy changes in specific peripheral T-cell frequencies, this differential only infrequently correlated with clinical outcome in cancer-vaccine trials (4,151). However, there are also exceptions (228), which clearly support the more extensive and systematic evaluation of "optimized" tetramer-based immune monitoring. Based on our current understanding, it would be expected that clinical responders derive from the cohort of patients that display increases in their tetramer+ T-cell frequencies after treatment; however, the mere circulation of high frequencies of tumor-reactive T cells does not guarantee tumor regression (4,6,242). There is clear promise for the application of tetramers as a "front-line" clinical immune monitoring system; however, many more prospective trials employing these assays will need to be performed to determine the clinical relevance of tetramer-based analyses.

Limitations of the Assay

Two points should be emphasized. First, MHC-peptide tetramers stably bind to TCR exhibiting a certain minimal avidity. Hence, functional and potentially clinically important T cells may be missed in these assays, depending on the staining conditions (temperature, concentration of tetramers, anti-CD8 antibodies, etc.). Indeed, there have been reported cases where epitope-specific T cells fail to be effectively imaged using the appropriate MHC/peptide tetramers (Gajewski T, personal communication, November, 2001). Secondly, many clinically important peptide epitopes may bind MHC with sufficiently low affinity that precludes the technical production of tetramer probes (ineffective folding, etc.) (243). Under these conditions, TCR cross-reactive peptide "super"-agonists must be pursued to construct stable tetramers in order that effective screening might be accomplished.

Major histocompatability complex-peptide tetramers may ultimately serve as the foundation for a legitimate laboratory monitoring system for T-cell responses in patients with cancer; however, current enthusiasm for this possibility must be moderated by the lack of stringent validation in a large number of clinical trials. At the current time, MHC-peptide tetramers must be considered a highly promising research tool with clinical intent. The ability to produce an "off-the-shelf" probe, combined with the high-throughput and sensitivity of flow cytometry, clearly makes this assay system attractive for potential application in future trials involving large patient populations. Based on anticipated inter-assay variability at the current time, particularly in multiparameter analyses, this monitoring system may be best applied by a central screening laboratory supporting the single- or multi-site performance of immunotherapeutic approaches.

Quantitative Reverse Transcription Polymerase Chain Reaction Assays

Principle of the Quantitative Reverse Transcription Polymerase Chain Reaction Assay

The qRT-PCR assay is based on the principle that amplification of cDNA by the polymerase chain reaction follows a strict mathematical equation whereby with each cycle of amplification two copies are made from each individual. Thus, the amount of cDNA amplified

after a given number of cycles will be directly proportional to the log2 of the starting amount of template. It follows that, if the amount of amplified cDNA throughout various amplification cycles could be quantitated, the starting amount of template could be extrapolated. This quantitation is achieved with a gene-specific nucleotide probe complementary to a region of DNA nested between the PCR primers. This probe is labeled with a fluorochrome and also with a quencher that can absorb fluorescence. During amplification the probe is removed from the DNA strand and degraded by the 5′-3′ exonuclease activity of Taq DNA polymerase and the fluorochrome is separated from the quencher yielding one unit of fluorescence for each cycle of amplification. By recording incremental fluorescence at each PCR cycle it is, therefore, possible to calculate the starting amount of cDNA template. In particular, by titrating known amounts of the relevant cDNA used as templates a standard curve can be constructed that allows quantitation of the number of cDNA and, indirectly, RNA copies in a given specimen (244). The use of a recombinant standard is strongly recommended because this gives absolute information about a given transcript copy number and, therefore, simplifies comparisons among different laboratories. Thus, by qRT-PCR it is possible to gather quantitative information about gene expression in any given specimen assuming that the tissues were handled properly so as to preserve the quality and quantity of RNA.

Quality, Sensitivity, and Validation of the Assay

Among the technical variables to be considered in the interpretation and standardization of qRT-PCR results are: first, the selection of a reference gene against which to normalize the test results and, second, the strategy adopted for discriminating a positive from a negative result. Even though β-actin or other classic "housekeeping" proteins may be suitable reference genes for the study of cancer tissues, they may not be appropriate for studying the response of specific T-cell subsets to immune stimulation because they do not take into account variations in frequency of the cells targeted by the stimulus. For example, if the targets of the stimulus are CD8+ T cells, possible variations in the numbers relative to other cell subsets in a given population (i.e., PBMC) could be normalized using CD8 mRNA as reference that is likely not to be sensitive to the stimulus applied within the time frame of the assay. The selection of the normalizing factor and knowledge of its kinetics should be tailored according to the particular experimental conditions.

The other point still debated is the definition of a "positive result". Kammula et al. considered a positive result to be a 2–3-fold increase above the mean expression of test genes in a relatively large number of irrelevant specimens. This was found to be > 5 standard deviations above the mean expression in irrelevant specimens (5). Independent from its definition, most laboratories agree that a positive result should be confirmed by reproducing the same experiment at least once. Other technical issues related to the application of qRT-PCR to the field of immunogenetics and immune monitoring have been recently discussed (245).

Advantages and Disadvantages of Quantitative Reverse Transcription Polymerase Chain Reaction Assays

Since this method is dependent on logarithmic amplification, qRT-PCR allows detection of minimal amounts of RNA in small samples. In addition, by being a sequence-based method, it allows the study of the expression of any gene for whom the sequence is known independent of the availability of antibodies or other markers specific for individual gene products. Therefore, qRT-PCR can be considered the method of choice for the rapid and reproducible measurement of gene expression in small samples (246,247). In addition to its sensitivity, qRT-PCR also provides flexibility of analysis since cDNA is quite stable and it is possible to preserve clinical material almost indefinitely for the future analysis of expression of genes whose relevance to the clinical situation was not known at the time of the original analysis. A further improvement in the sensitivity of this method came from the recent development of an mRNA amplification method that maintains the proportional expression of various genes within a given sample (248). The addition of this preliminary step allows analysis of the expression of a practically infinite number of genes present in any tissue sample without causing significant distortion of their relative expression (249). This improvement has rendered qRT-PCR an extremely valuable tool for the validation of gene expression estimates derived from cDNA arrays (250).

Compared with other methods, qRT-PCR has some specific disadvantages. One important limitation of this method is the lack of discrimination among cell subsets present in a given sample responsible for differential gene expression. Theoretically, this could be overcome under special conditions. For instance, by using purified cell specimens or micro-dissected material (251), it is possible to improve the cellular specificity of the analysis. Unfortunately, this is not always feasible or practical and, therefore, other strategies need to be implemented to address this problem. Kammula et al. have suggested the use of cell specific reference genes to normalize the cal-

culated expression of a gene believed to be expressed only by a particular cell population (5). For example, peripheral blood mononuclear cells (PBMC) could be directly stimulated ex vivo with the same peptide used for vaccination, allowing antigen presentation to occur among the sample cells. Since the peptide in question has only HLA class I binding characteristics it could be postulated that the main affect of such stimulation would occur on CD8 expressing cytotoxic T cells. Upon cognate stimulation, cytokine (i.e., IFN-γ) transcript expression is theoretically induced only in the vaccine-induced T cells. Thus, IFN-γ expression could be estimated as a proportion of copies of cytokine messenger RNA over that of general house-keeping genes such as β-actin but also normalized according to the abundance of expression of CD8 messenger RNA more directly proportional to the frequency of the cells targeted by the test in this particular case (5). This complex strategy gives an approximate estimation of gene expression for a cell population but does not yield information about gene expression in individual cells within the population. For instance, Neilsen et al. noted that only a small percent of vaccine-induced T cells (as enumerated by tetrameric HLA/epitope complexes) produce IFN-γ upon cognate stimulation (as demonstrated by intracellular FACS staining for cytokine expression) (6). This detail could not have been obtained using qRT-PCR. This limitation may have particular significance in some studies where such level of discrimination is relevant, whereas in other monitoring circumstances it might not be as important. In particular, qRT-PCR may be useful when semi-quantitative analysis of gene expression is sufficient but a broad range of genes need to be analyzed for whom no antibodies are available for surface or intra-cellular staining. An important issue to be taken into account is the kinetics of expression of different transcripts in response to a given stimulation. For example, Kammula et al. noted that IFN-γ, GM-CSF, and IL-2 have very similar kinetics of expression with a peak transcript level by qRT-PCR approximately 3 hours after stimulation. This, however, does not apply to other genes such as TNF-α whose peak expression occurs at a later time point (5). Thus, the kinetics of expression of various genes (whether test genes or putative housekeeping genes) needs to be assessed in relevant experimental conditions before the test is applied.

Another major limitation of qRT-PCR is that it does directly measure the expression of proteins but of mRNA encoding proteins. This problem may not be significant in certain situations where the expression of a given gene is used more as a marker of cellular activation in response to a given stimulus rather than for estimation of its down-stream effects. For instance, Kammula et al have noted that several gene transcripts are rapidly and specifically upregulated on cognate stimulation of T cells (5). Such genes include not only cytokines such as IFN-γ, GM-CSF, TNF-α, IL-2, etc., but also surface markers such as CD25 and CD69. Although estimation of messenger RNA expression does not guarantee expression of the corresponding proteins, it yields accurate information about the level of responsiveness of a given cell population to a cognate stimulus through transcriptional activation of the responsive gene.

Application of Quantitative Reverse Transcription Polymerase Chain Reaction to Measuring T-Cell Responses Against Tumor-Associated Antigens

The qRT-PCR technique was originally used to measure virus loads in patients for diagnosis in different diseases such as CMV, EBV, and HBV (252–254) and for monitoring viral infections in transplanted patients (255,256). Others have used qRT-PCR in follow-up examinations for treating patients with hematological cancers (257). Still others have looked for evidence of micro-metastases in blood, lymph nodes and bone marrow, by measuring the expression level of cancer specific markers (258). However, little information, is available in the literature about the utilization of qRT-PCR for immune monitoring, as this methodology as been only recently applied to this field. Although various authors have applied this technology to the analysis of immune related markers in vivo in small samples, most of these studies were not specifically related to immune monitoring of vaccinated patients (246,247,259–261). Investigators at the National Cancer Institute Surgery Branch identified T-cell reactivity toward epitopes used for active-specific vaccination of melanoma patients by measuring IFN-γ transcript levels in PBMC obtained before and after treatment and stimulated ex vivo with the vaccine-relevant epitope (5). Evidence of vaccine induced sensitization of circulating lymphocytes obtained with qRT-PCR correlated with results obtained with classic in vitro sensitization methods (244) as well as T-cell phenotyping with tetrameric HLA/epitope complexes and intra-cellular cytokine detection by FACS analysis (6,241). A significant advantage to the use of qRT-PCR for immune monitoring is its flexibility. In addition to monitoring immune reactivity against individual tumor-associated peptides restricted by specific HLA molecules, qRT-PCR can also be applied to analyze immune reactivity against whole proteins, mixtures of proteins or even whole tumor cells without knowledge of the relevant peptides or restriction elements. For instance, to

monitor reactivities raised in patients with cancer of various HLA types by immunization against a tumor-associated protein, autologous antigen presenting cells manipulated to transiently express the protein of interest have been used to stimulate cytokine mRNA production from PBL collected from patients before, during and after therapy (Topalian et al., manuscript in preparation).

An important application of qRT-PCR in our experience has been the analysis of tumor specimens obtained from fine-needle aspirates. With this strategy, Morcellun measured dynamic changes in expression of tumor antigens (249), cytokines and other immune cell specific markers (262) during immunization. Although some of these markers could have been assessed by immunohistochemistry, for others there was no available antibody. In addition, the limited amount of material obtainable with fine needle aspiration would not have allowed the preparation of a sufficient number of cytology slides to study more than a few markers, while the RNA extracted from individual fine needle aspirates and amplified according to Wang's method (248) allowed the study of a virtually unlimited number of genes (263).

In conclusion, the qRT-PCR represents a useful tool for the monitoring of patients with cancer undergoing immune manipulation. This tool offers unique advantages and should be considered as part of a repertoire used to design a comprehensive immune monitoring strategy.

COMPARISON OF DIFFERENT T-CELL ASSAYS

Comparison of the Limiting Dilution Analysis With Other Methods Of Cancer Immune Monitoring

There have been several comparative studies of LDA with newer methods of immunologic monitoring. One limited study comparing chromium-based LDA with ELISPOT demonstrated a high degree of correlation (158). In a single-center study, frequencies of FLU-reactive T-cells determined with the IFN-γ ELISPOT assay correlated with frequencies derived from the LDA (166), but were several-fold higher than the CTL precursor frequencies determined by LDA in this study. An explanation for this discrepancy may be that the expansion of precursor CTL in the LDA is extremely sensitive to the culture conditions used to propagate the T-cells.

A number of studies have compared tetramer analysis with (LDA) (264–268). Typically, tetramer analyses have provided frequency estimates that exceed those detected using LDA, although tetramer analysis provides enumeration without functional information.

Although LDA will likely be replaced by immunologic monitoring methods that use less clinical material and are more amenable to standardization, such assays may have usefulness as secondary tools for analyzing immunity. As an example, in a recent trial employing a MAGE 3 peptide vaccine, LDA culture was used to isolate low-level antigen specific precursors and then quantitate the T-cell response by tetramer (168). A MAGE 3 specific precursor frequency of 1:40000 was detected in a patient showing a partial regression of bulky tumor after vaccination suggesting that low level T-cell responses can potentially result in clinical benefit.

Comparison of the Proliferation Assay With Enzyme-Linked Immunospot Assay

Flanagan et al. compared responses to multiple malarial peptides using ex vivo ELISPOT, cultured ELISPOT, and lymphoproliferation assays (156). All three assays demonstrated immune responses; however, different peptide recognition patterns were observed in the three assays with little correlation between them. There was a trend for the lymphoproliferation, but not the ELISPOT data to correlate with antibody responses. Thus, ELISPOT and lymphoproliferation assays appear to measure different aspects of the immune response.

Comparison of the Newer Methods of Cancer Immune Monitoring

Newer assays of T-cell specificity and function have been introduced that have distinct advantages over proliferation and cytotoxicity assays. Immunofluorescent staining with MHC-peptide tetramers is a simple and rapid method for enumerating T cells specific for single epitopes. However, it is not a functional test, as it reveals only the specificity of a population of T cells as determined by their ability to recognize a peptide bound to a particular MHC molecule. This limits the clinical usefulness of tetramers as monitoring tools, because they are restricted by epitope and by MHC allele, and because specificity does not always correlate with function especially in patients with advanced malignancies (3,185, 269).

Enzyme-linked immunospot and CFC assays are similar in that they both measure the production of cytokines by individual T cells as a surrogate for function. They differ in the cell processing requirements for assay set-up (CFC can be performed on whole blood; ELISPOT requires isolation of PBMC or even CD4- or CD8-depleted PBMC). These assays also differ in their turnaround time (8 hours for CFC and 24–48 hours for ELISPOT). Fi-

nally, the detection systems of the two assays differ (flow cytometry for CFC versus microscopy for ELISPOT). Enzyme-linked immunospot assays can achieve low limits of detection, up to 1 in 300000 in one report (270). If spontaneously activated cytokine-producing cells are in fact present at 0.01%–0.02% (1 in 5000–1 in 10000), such limits of detection should not be possible in an assay that measures cytokine production. However, it is possible that such spontaneous cytokine secretion is transient in nature and is therefore detected in CFC but not detected in the longer duration ELISPOT assays. If the above assumptions are true, in the absence of background-reducing strategies for CFC assays, ELISPOT could represent a potentially more sensitive assay than CFC. However, a lower limit of detection is not useful if the assay underestimates the positive responses to an antigen.

Comparison of Cytokine Flow Cytometry and Enzyme-Linked Immunospot Assay

In comparative studies, the frequencies of cytokine-positive cells obtained by CFC have been several-fold higher than those obtained by ELISPOT (214,271). Using T-cell clones, peptide-reactive T-cells found to be positive by intracytoplasmatic staining were also detected by ELISPOT, and the lower detection limit was moderately in favor of the ELISPOT approach (155). In one study, PBMC samples from six subjects were analyzed for the frequency of FLU-reactive CD8+ T-cells by flow cytometry detecting either intracellular IFN-γ (IC-FC) or secreted IFN-γ (S-FC) and by IFN-γ ELISPOT assay. The frequency of FLU peptide-reactive T-cells determined by IC-FC and ELISPOT assay showed a high interassay reproducibility and a close correlation between both assays. Little or no IFN-γ production was observed in unstimulated PBMC samples using intracellular IFN-γ FC or ELISPOT assay. In contrast, using S-FC, a high number of IFN-γ-secreting CD8+ T-cells were detected in unstimulated PBMC. The frequency of FLU-reactive CD8+ T-cells determined by S-FC did not correlate with those detected by IC-FC or ELISPOT assay (272).

Comparison of Cytokine Assays With Tetramer Staining

A number of studies have extensively compared tetramer and cytokine ELISPOT analyses (265–268,273). Typically, tetramer analyses have provided frequency estimates that exceed those detected by ELISPOT. Even though this may, in part, be a result of issues of "higher backgrounds" associated with certain MHC-peptide tetramers, it may also be a result of the fact that cytokine ELISPOT assays detect functional memory/effector T cells that responded to cognate antigen. Because this population of "primed" T cells represents a subset of all T cells bearing TCR that can bind a MHC-peptide complex, while MHC-peptide tetramers can detect "nonresponsive" (i.e., naïve, anergic, or hyporesponsive") and "functional" T cells, it is not surprising that tetramer assays provide a higher estimate. When combined with a functional readout (intracellular or membrane captured cytokines) in multiparameter assessments, however, tetramer estimates fall more in line with the results of LDA and ELISPOT assays. A study by Rubio-Godoy et al. (273) showed that a tyrosinase 368–376 peptide-specific T-cell clone detected by IFN-γ ELISPOT in patients with melanoma was not detectable by staining with the corresponding A2/peptide multimers, but was cytolytic. This was explained by a faster TCR/pMHC complex dissociation rate of this clone.

Cytokine flow cytometry frequencies, in the absence of anergic cells, tend to be similar to those obtained by MHC tetramer analysis (266,274).

PERSPECTIVES

Current Recommendations for Trial Monitoring

In summary, each assay has advantages and disadvantages. The recommendations agreed on for monitoring of cancer-vaccine trials during the Workshop are listed in Table 6. These recommendations take into account the principles of the assays as well as the current state of development of individual assays towards standardization and validation. The latter is expected to change over the coming years. These recommendations are further limited by the paucity of vaccine trials that have systematically used two or more immune monitoring assays, an approach that would facilitate comparison of results. This situation also is likely to change in the near future. In principle, three situations with cancer vaccines have to be considered separately when devising a suitable immune-monitoring plan: (1) vaccinations aiming to induce a response against defined CD8 T cell epitopes, (2) vaccinations aiming to induce a response against a specific protein, and (3) vaccinations with antigens that are at least partially undefined.

The immune monitoring recommendations for these three situations are discussed separately below.

Before including a patient in a vaccine trial, the ability of the individual patient to mount an immune response should be assessed. This could be accomplished with

TABLE 6. *Assays recommended for trial monitoring*

ELISPOT
Lowest limit of detection ($1/10^5$ PBMC with tumor antigens)
Several reagents are critical
Needs dedicated readout equipment
CFC
Low limit of detection ($1/10^4$ PBMC with tumor antigens)
Needs skilled person to perform analysis
Tetramer + functional assay
Intermediate limit of detection ($1/10^{3-4}$ PBMC with tumor antigens)
T-cell subset analysis is optimal
Needs skilled person to perform analysis
Antibody
Useful for protein responses
PCR
Needs more data, very flexible, least in-vitro manipulation, least material requirements
TCR dysfunction
Additional information provided, but relevance needs to be proven

CFC, chlorofluorocarbons; ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; TCR, T-cell receptor.

assays measuring general T-cell function such at ζ chain expression (see "T-Cell Receptor Dysfunction in Cancer"). While providing potentially useful information, these assays are at present insufficiently standardized and validated to be used as eligibility criteria for vaccine trials. Additional prospective assessments of the ability of these assays to predict for the robustness of an immune response to a cancer vaccine need to first be performed.

Monitoring of T-Cell Responses Against Defined CD8 T-Cell Epitopes

The assays that are principally suitable for this purpose include ELISPOT, CFC, tetramer, qRT-PCR, and LDA assays. The recommendation from the Immune Monitoring Workshop is to use, whenever possible, a combination of two of the following assays: ELISPOT, CFC, and tetramer assays.

The ELISPOT assay is currently the best characterized. One inter- and numerous intralaboratory comparative studies have been published, as detailed above. Furthermore, the ELISPOT assay has the highest reported sensitivity to detect a T-cell response against most tumor antigens, although this may not be the case with T cells producing low levels of cytokines (Keilholz U, unpublished data). Reliable analysis is relatively easy to perform, but requires constant care because several critical reagents can vary from batch-to-batch. Furthermore, an automated plate reader is required for objective analysis.

The CFC assay exploits the same T-cell properties as the ELISPOT assay, however considerably fewer studies analyzing T-cell responses to tumor antigens are published with the CFC. Most of the current experience with the CFC assay is with viral diseases, which may not be relevant to many of the less immunogenic tumor-derived self-antigens. Cytokine flow cytometry commonly employs multicolor staining. Therefore there is a need for a dedicated and specially trained person to perform the flow analysis, because gating of cells during event acquisition and setting of markers to discriminate negative and positive staining requires experience and a high level of expertise. Theoretically, intelligent interactive software solutions could be developed to ease this process and reduce operator variability.

The tetramer assay is currently less well standardized and is less sensitive than the ELISPOT assay and probably the CFC. For each T-cell epitope an individual tetramer has to be produced and characterized. However, once a specific tetramer is available, the assay has the advantage over ELISPOT and CFC assays that it allows the detection of specific T-cells regardless of their ability to produce cytokines. For the same reason, however, the tetramer assay is unable to distinguish between functional and dysfunctional T cells, or between T cells producing Th1 or Th2 type cytokines. Therefore, it is currently recommended that the tetramer assay be used in conjunction with one of the two functional cellular assays (ELISPOT or CFC).

The qRT-PCR assays are also of great interest because they require the least in-vitro manipulation. Two hours of stimulation are sufficient to elicit cytokine transcription, but not cytokine release, making intercellular variability during the incubation period unlikely. Furthermore, qRT-PCR is very flexible. Once the cDNA is generated a large number of different cytokines can be tested using the material obtained from a single experiment. A major disadvantage of the qRT-PCR assay is that it is not a single cell assay and therefore cannot quantitate T cell frequencies or characterize specific T cells. Because of the lack of standardization, qRT-PCR assays cannot currently be recommended as a sole assay for monitoring of T cells in peripheral blood. On the other hand, the use of the qRT-PCR assay for assessment of tissue samples is of special interest, since the number of cells recovered from tissue is usually insufficient to allow the performance of ELISPOT or CFC assays. Consequently, the combination of qRT-PCR to assess for reactive cells and peptide specific cell quantification via tetramer staining may ultimately prove to be extremely valuable for in situ analysis of tissue specimens.

The disadvantage of the LDA is the requirement of several rounds of in vitro restimulation resulting in quan-

titative and functional biases. Also the amount of work required to perform the analysis represents a major practical limitation. For these reasons, the LDA is not recommended for trial monitoring.

In summary, if single epitope-specific T cells are to be monitored in peripheral blood, the ELISPOT assay is recommended as a sensitive assay to detect functional T cells. If possible, all samples should be further analyzed with a second assay, either CFC or tetramers. The CFC assay would be a confirmatory assay, and the tetramer assay would allow detection of the antigen specific T-cells that didn't produce the cytokine used in the read-out of the ELISPOT assay. Cytokine flow cytometry as well as tetramer analyses allow, in cases where there is a T-cell response of sufficient magnitude, detailed further characterization of epitope specific T cells (see "Current Developments").

Monitoring of a Response Against a Specific Protein

In protein vaccine trials, antibody responses, CD4 T-cell responses, and CD8 T-cell responses may be induced, signifying a higher level of complexity. The role of each of these components for tumor rejection is currently unknown and may vary from one protein to another. Therefore, in protein vaccine trials, all three types of immune responses are of interest. For analyzing antibody responses, the specific recommendations given in the "Antibody Assays" section of this work are applicable. Assessment of CD4 responses is usually performed with proliferation assays, with the limitations discussed in the section describing LDA (see "Limiting Dilution Analysis"). Variations of the ELISPOT and the CFC assay have been developed to enable assessment of CD4 responses and will likely replace the proliferation assay in the future. Vaccination with proteins may also induce (albeit usually with low efficacy) CD8 responses, which can be monitored in a way analogous to what was recommended for monitoring of responses against defined CD8 T-cell epitopes. No further recommendations were made during the Workshop because of the limited availability of published information on immune monitoring for clinical trials utilizing protein vaccines.

Monitoring of Responses Against Partially Undefined Antigens

In case of vaccination with modified tumor cells, with tumor-cell lysates, or with DC-tumor cell fusions, a variety of antigens may differentially induce immune responses, including antibody responses, CD4, and CD8 T-cell responses. Under these circumstances the principles detailed under the "Current Recommendations for Trial Monitoring" section apply, with the vaccine preparation used as the target material rather than a specific protein. To dissect the immune response to complex vaccines, efforts should be undertaken to characterize at least a limited number of antigens in the vaccine preparation as a way of monitoring the specific components of the immune response. Furthermore, it should be noted that under the conditions described in the respective sections the ELISPOT and CFC assays might also work with whole cellular targets.

Current Developments

The workshop and this report are oriented toward the quantitatation of CD8 T-cell responses towards tumor antigens presented by MHC class I. In addition to the quantitation of a T-cell response, further characteristics of antigen-specific T cells are important, namely their level of functional differentiation, including lymph node homing, proliferative capacity and lytic effector function, and their expression of receptors governing T-cell migration into specific peripheral tissues. Reagents to investigate these parameters are becoming increasingly available and are likely to be incorporated into future immune monitoring strategies.

For functional T-cell differentiation, three CD8 T-cell subsets can be distinguished by virtue of expression of the lymph node homing receptor CCR7 and the CD45 isoform RA as proposed in a model by Sallusto and Lancavecchia (275). Naive T cells are typically CCR7+CD45RA+, central memory T cells are CCR7+CD45RA−, and peripheral memory T cells are CCR7-CD45RA−. According to this model, naive T cells home to T-cell areas of lymph nodes and require antigen priming by DC to become functionally active. The central memory T cells are capable of directly migrating into inflamed tissue and proliferating upon antigen exposure, but lack immediate effector function. Peripheral memory T cells also can migrate directly into inflamed tissue and can then respond directly to antigen exposure with production of IFN-γ and IL-4. A fourth T-cell subset, termed terminally differentiated lytic effector T cells, has recently been added to this model (276,277). These T cells are CCR7−CD45RA+, are able to migrate into inflamed tissues, and are capable of efficiently lysing target cells without further stimulation. The classification of T-cell subsets according to their level of functional differentiation may provide very useful information and may differ from one vaccination protocol to the other and even from one antigen to the other. The respective antibodies necessary to perform these subset analyses are

available and can easily be incorporated into CFC and tetramer assays.

A far less well-defined topic is the expression of homing and chemokine receptors on T cells. The degree of expression of these receptors appears to determine tissue migration (278). This expression of homing and chemokine receptors on T cells may be of great importance. Because immune monitoring is usually performed using peripheral blood T cells, determining their capacity to specifically extravasate into other compartments may be critical to understanding the clinical efficacy of a particular vaccine.

For the future, the organizers and authors hope that these Workshop proceedings will help provide a uniform language and a common experimental approach for clinical and translational research in the field of cancer vaccines. The Society for Biological Therapy is dedicated to providing forums for discussion of further advances in this area and is planning to organize a second Immune Monitoring Workshop in the future.

APPENDIX 1

Immune Monitoring Workshop Participants by Breakout Session Group

Flow Cytometry for Cytokine Secretion:

Herbert Kim Lyerly, M.D. (Co-Chair), Vernon Maino, Ph.D. (Co-Chair), Paul Chapman, M.D., Thomas Davis, M.D., Robert Dillman, M.D., Susan Doleman, Susan Hand, Ph.D., Eddy Hsueh, M.D., Michael Lotze, M.D., James Mier, M.D., John Neefe, M.D., Sattva Neelapu, M.D., Craig Slingluff, Jr., M.D., Paul Sondel, M.D., Ph.D., Edwin Walker, Ph.D., Louis Weiner, M.D., and Jon Wigginton, M.D.

Enzyme-Linked Immunospot/Enzyme-Linked Immunosorbent Assay:

Carmen Scheibenbogen, M.D. (Co-Chair), Ulrich Keilholz, M.D. (Co-Chair), Jeffrey Schlom, Ph.D. (Co-Chair), Jean-Claude Bystryn, M.D., Carter Cliff, John Dunne, Ph.D., Lawrence Fox, M.D., Ph.D., Frank Haluska, M.D., Ph.D., Stephen Hodi, M.D., Lori Jones, Ph.D., Howard Kaufman, M.D., Janet Lathey, Ph.D., Jonathan Lewis, M.D., Ph.D., Philip Livingston, M.D., Cristina Musselli, M.D., Ph.D., Laurie Stephen, Ph.D., Crystal Sung, Ted Trimble, M.D., Theresa Whiteside, Ph.D., and Robert Wiltrout, Ph.D.

Real Time Polymerase Chain Reaction:

Francesco Marincola, M.D. (Co-Chair), Kathleen Beach, M.D., David Essayan, M.D., Jared Gollob, M.D., Elizabeth Jaffee, M.D., Reiner Laus, M.D., Mike Perricone, Ph.D., Knut Sturmhoefel, Ph.D., Suzanne Topalian, M.D., Pierre Triozzi, M.D., Nancy Valente, M.D., and Frank Valone, M.D.

T-Cell Receptor Function:

James Finke, Ph.D. (Co-Chair), Dmitry Gabrilovich, M.D., Ph.D. (Co-Chair), W. Martin Kast, Ph.D. (Co-Chair), Keith Douglas, Jeff Edelson, M.D., Stephen Fields, Ph.D., Oscar Kashala, M.D., Ph.D., Stephanie Kenis, Samir Khleif, M.D., Robert Martell, M.D., Ph.D., Augusto Ochoa, M.D., Nicholas Restifo, M.D., Steven Rosenberg, M.D., Ph.D., Scott Saxman, Ph.D., and Peter Wettstein, Ph.D.

T-Helper and Antibody Assays/Limiting Dilution Analysis:

John Kirkwood, M.D. (Co-Chair), Nora (Mary) Disis, M.D. (Co-Chair), Mark Albertini, M.D., Priscilla Ayers, Neil Berinstein, M.D., Soldano Ferrone, M.D., Ph.D., Bernard Fox, Ph.D., James Mulé, Ph.D., Rathinam Selvan, Ph.D., Vernon Sondak, M.D., Michael Vasconcelles, M.D., and Hassan Zarour, M.D.

Tetramer Analyses:

Peter P. Lee, M.D. (Co-Chair), Walter Storkus, Ph.D. (Co-Chair), Thomas Gajewski, M.D., Ph.D., Susan Gammon, Ph.D., MBA, Cheryl Guyre, Peter Hersey, M.D., Ph.D., Barb Hickingbottom, Tina Kuus-Reichel, Ph.D., Ping Law, Ph.D., Bill Rees, Jeffrey Sosman, M.D., John Thompson, M.D.

APPENDIX 2

List of Authors for Specific Sections Lecture Summaries

Mechanisms of T-Cell Dysfunction:

James H. Finke, Charlie Tannenbaum, Patricia Rayman, Amy Richmond, Eric His, and Ronald Bukowski

Defective Dendritic Cell Differentiation in Cancer:

Sergei Kusmartsev and Dmitry I. Gabrilovich

Breakout Session Reports

Measuring T-Cell Receptor Function in Cancer:

W. Martin Kast, Dmitry I. Gabrilovich, and James H. Finke

Antibody Assays and Limiting Dilution Analysis:

Mary L. Disis and John M. Kirkwood

Enzyme-Linked Immunospot Assays:

Carmen Scheibenbogen and Jeff Schlom

Cytokine Flow Cytometry:

Vernon C. Maino, Holden T. Maecker, Paul J. Mosca, and Herbert Kim Lyerly

Tetramer Assays:

Peter P. Lee and Walter Storkus

Real Time Polymerase Chain Reaction Assays:

Francesco M. Marincola and Suzanne Topalian

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