Immune monitoring of cancer vaccines
Report on a workshop held at the 9th NCI-EORTC Symposium on New Drugs in Cancer Therapy

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Both antibody-mediated and cell-mediated immunologic responses may play a role in prevention and treatment of neoplastic disease. Passive transfer of monoclonal antibodies or activated lymphocytes have produced tumor regression in animal models and in patients with advanced malignancies. However, these approaches are invariably expensive and often cumbersome, and efficacy has been limited. The identification of many tumor-associated or tumor-specific antigens, and new technologies for immunization, present the opportunity to induce anti-tumor immunologic responses in vivo, perhaps with greater efficacy and less expense. For these reasons, substantial resources are currently being devoted to the development of cancer vaccines.

The relative anti-tumor efficacy of antibody versus cell-mediated immune responses is not yet known, and perhaps both contribute to the anti-cancer immune response. However, in the past 5 years, the great majority of cancer vaccines entering into clinical trials have been designed to induce cell-mediated immune responses, based on several key developments in the field of cancer immunology, including: 1) the demonstration that immunotherapies, including cancer vaccines, have anti-tumor activity both in animal models and in patients; 2) the recognition of the critical role that T-cells play in these anti-cancer immune responses; 3) the recognition that antigens recognized by T-cells are actually peptide fragments of intracellular proteins that are transported to the endoplasmic reticulum, bound to MHC molecules, and then carried to the cell surface; 4) the recognition that these tumor antigens can be derived from normal proteins with limited normal tissue expression, or from proteins expressed only in tumor, such as mutated oncogenes and viral proteins; 5) the demonstration that these tumor antigens can be shared (expressed by tumors from different patients); and 6) the development of techniques to clone and manipulate these shared antigens to generate defined antigen vaccines. Another key development that has created substantial new interest in cancer vaccines has been the ease with which genes can be introduced into cancer cells to modulate their immunogenicity, in particular genes that express cytokines or other signals important in the presentation of antigen to, and activation of, T-cells. These techniques have allowed the generation of immunogenic cancer vaccines from the patient's own tumor, or from continuously maintained allogeneic cancer cell lines that express shared tumor antigens. In the case where autologous tumor is used as the immunizing preparation, the actual tumor rejection antigen or antigens need not be known.

Although several cancer vaccines have been shown to induce a low rate of objective tumor regression in patients with metastatic disease, most investigators in the field believe that the greatest utility of this approach will be to eliminate micrometastatic disease, or in the most optimistic sense, to prevent occurrence of malignancy in normal individuals. Unlike cytotoxic cancer therapies, it was previously considered improbable that a cancer vaccine which may be effective in the adjuvant setting will first demonstrate substantial anti-tumor activity against advanced disease. Thus the major dilemma for cancer vaccine development is to choose an appropriate endpoint in early trials, other than anti-tumor efficacy, that will justify further clinical development, ultimately leading to the large, expensive randomized clinical trials that are required to show efficacy in the adjuvant setting. The workshop at the 9th NCI-EORTC Symposium on New Drugs in Cancer Therapy was organized to discuss these early endpoints, particularly the methodologies that investigators are using to monitor in vivo immune responses to the antigen preparations used in current trials of cancer vaccines, and to discuss the implications of these results.

Presentations made by Drs. Drew Pardoll (Johns Hopkins), Ellen Rankin (Netherlands Cancer Insti-
tute), and Elaine Elder (University of Pittsburgh) discussed strategies for monitoring clinical trials of immunization with gene-transfected autologous tumor cells. As others have in the past, these investigators are assessing induction of immunity by measuring the pre- and post-vaccination delayed-type hypersensitivity responses to an intradermal challenge of parental (non-transduced) autologous tumor cells. To obtain additional information, biopsies of the intradermal parental tumor challenge sites are being performed some days after application to assess and characterize the histologic responses. Biopsies are also obtained from the intradermal injection sites of the gene-transduced tumor cells or from regional draining lymph nodes to determine the types of cells that infiltrate during induction of an immune response. In some instances multiple injections of gene-transduced tumor cells are placed during one treatment, either at the same dose or calibrated to produce different amounts of the transfected cytokine gene, in order to allow an assessment of histologic changes over time or cytokine dose-dependent histologic changes. Where possible, biopsies are also obtained from distant cutaneous or subcutaneous tumors to assess cellular infiltrates into tumor that result from induction of a systemic immune response. Finally, peripheral blood lymphocytes (PBL) are harvested at various times pre and post immunization in order to measure the frequency of precursor cytotoxic T-lymphocytes (CTL) against autologous tumor. The latter assays require the use of limiting dilution techniques which are expensive and labor-intensive.

Monitoring of trials using gene-modified autologous tumor cells must address a number of questions, most importantly the role of the gene transfection in modifying immunogenicity of the tumor, and the ability of the vaccine to induce tumor-antigen specific immune responses. The most rigorous attempt to address these difficult questions has been made by use of a novel study design in a trial of autologous GM-CSF transfected tumor cells in patients with renal cell carcinoma, presented by Dr. Pardoll. Investigators in this study had access to both a large amount of tumor and normal kidney cells from the primary renal cell carcinoma specimen. Patients were randomly assigned, using a double blind approach, to receive immunization with either transduced or non-transduced tumor cells. Patients were then assessed for DTH response against both normal autologous kidney cells and non-transduced tumor. After completion of the trial and breaking the blind, the investigators observed that patients who were immunized with the GM-CSF transduced tumor, in comparison to those immunized with non-transduced tumor, had greater DTH responses to both normal kidney and non-transduced autologous tumor. In addition, the DTH responses were greater in patients who received the highest cell doses for immunization. The data provided strong evidence that paracrine secretion of GM-CSF by tumor enhanced the immunogenicity of the tumor. Furthermore, biopsies of sites containing the GM-CSF transduced cells showed increased numbers of 'professional' antigen-presenting cells, lymphocytes and eosinophils compared to biopsies obtained from sites injected with non-transduced tumor cells. These observations were also made in the trial of GM-CSF transfected autologous melanoma cells presented by Dr. Rankin and in the animal models that formed the basis for these trials.

Perhaps the most relevant test of a vaccine approach is to determine whether immunity is induced against a tumor antigen, and data from the trial presented by Dr. Pardoll suggested that immunization had occurred against an antigen present in both normal kidney and tumor cells, since increased DTH reactivity to both was observed following the series of vaccinations. However, the possibility that the immune response was directed against some immunogenic component of enzymes or the media used in the preparation of the cells cannot be excluded. Delayed-type hypersensitivity reactions to parental tumor cells were also reported in the majority of metastatic melanoma patients immunized with GM-CSF transfected autologous tumor cells in the trial reported by Dr. Rankin. In both trials, additional measures to show induction of anti-tumor immunity included measuring precursor CTL frequency in PBL directed against autologous tumor cell lines as targets. Since many melanoma-associated T-cell antigens have now been isolated and cloned, it may be possible to monitor for specific responses to these defined antigens in melanoma vaccine trials as a marker for general immunologic response to unknown tumor antigens contained in the autologous tumor cell preparations (see below).

Presentations by Drs. Thierry Boon (Ludwig Institute for Cancer Research), Ulrich Keilholz (University of Heidelberg), and Francesco Marincola (Surgery Branch, National Cancer Institute) were directed towards monitoring of T-cell responses to defined antigens, in particular CTL responses to MHC class I restricted peptide epitopes of melanoma antigens. Patients in the trial presented by Dr. Marincola were immunized with the HLA-A2 restricted 9-amino acid peptide derived from the MART-1/Melan-A melanoma protein, admixed with a modified Incomplete Freund's adjuvant. PBL were obtained pre-treatment and following the second and fourth immunizations. The PBL were then stimulated in vitro with the MART-1 peptide (the influenza HLA-A2 matrix peptide was used as a control) and IL-2. After multiple in vitro stimulations, each consisting of a 7-day culture period, the PBL were admixed with target cells (T2 cells pulsed with the peptide, or autologous tumor) and tested for cytotoxicity and secretion of interferon-gamma. Dr. Marincola observed that reactivity against MART-1, as measured in these assays, could be found in normal patients and melanoma patients prior to immunization, although the number of in vitro stimulations required to detect specific lytic activity in healthy patients was greater than that required in melanoma
patients. In 13 of 18 patients immunized 2–4 times with the MART-1 peptide, a significant increase in specific lysis against MART-1 was observed compared to pre-immunization values. Dr. Steven Rosenberg et al. at the Surgery Branch, NCI, have subsequently reported successful immunization against peptide epitopes of gp100, another melanoma protein. Of particular significance, patients immunized with gp100 peptides modified at a single amino acid have developed potent CTL responses against the unmodified gp100 peptide and gp100 containing tumor cells that can be detected after a single in vitro exposure of PBL to the immunizing or the unmodified gp100 peptide. Objective regression of metastatic tumor has been observed in these studies, although the frequency has been low. To address potential mechanisms for the discordance between induction of immune responses to the tumor antigens and antitumor activity, Dr. Marincola noted that monitoring of patients within the Surgery Branch peptide vaccine studies has also included pre and postimmunization biopsies of metastatic melanoma to determine the level of expression of HLA-A2 and the MART-1 and gp100 proteins. Not surprisingly, metastatic melanoma appears to have heterogeneous expression of MHC class I molecules and of the melanoma antigens.

Keilholz et al. have been developing Elispot assays for quantifying the frequency of precursor CTL within a sample of PBL. PBL are harvested and first undergo a period of in vitro culture and expansion with the peptide. A predetermined number of these PBL (or the CD8+ subset) are subsequently placed on 96-well nitrocellulose plates that are coated with an anti-human IFN-gamma antibody to which approximately 10^5 PBMC (or T2 cells), the peptide target, and IL-2 are then added. Since the cells are essentially fixed on the plate in a single layer, any CTL precursor in the PBL sample with specificity for the peptide presented by the PBMC or T2 cells will be activated and will produce interferon-gamma at a fixed spot on the plate. A rabbit anti-human IFN-gamma is added to the plate, and the plate is then developed with a subsequent reaction that produces a colored spot at the site of rabbit IgG. The spots are counted and the frequency of peptide specific CTL within PBL is calculated. Dr. Keilholz reported that this procedure was developed and validated using samples obtained from normal donors and tested against CTL peptide epitopes of influenza and cytomegalovirus. The Elispot assay offers the opportunity to quantify CTL from PBL without any prior in vitro culture and expansion with peptide, depending on the frequency of peptide-specific CTL within the PBL sample. Validation of the Elispot assay as a means to measure immune responses to antigen in vaccine trials of melanoma peptides is underway.

Perhaps the most surprising data of the workshop was presented by Dr. Boon regarding their studies of immunization with the HLA-A1 restricted MAGE-1 and MAGE-3 peptides, respectively. The initial studies have administered the peptides subcutaneously without an adjuvant, a strategy that would appear suboptimal for induction of immunity. Monitoring consists of assessing DTH responses to an intradermal challenge with the immunizing peptide. CTL reactivity is assessed against peptide-pulsed target cells following two 7-day in vitro stimulations of PBL with peptide. Perhaps as expected, the investigators did not observe DTH responses to the peptide skin test, and they were unable to detect evidence of CTL reactivity directed against the immunizing peptide. However, 3 of the 7 patients treated with the MAGE-1 vaccine were reported to experience pain and inflammation in distal tumors, although none met the criteria for objective response. Dr. Boon also reported that in the MAGE-3 trial, among 21 melanoma patients currently evaluable for response, they observed 1 minor response, 1 partial response, and 2 complete responses. Sites of responses included skin and lung. One patient with lung metastases had initial progression of his lesions, followed by a delayed response with continued immunizations. Similarly, a patient with in-transit metastases did not begin to display a response until 4 months after the third injection (injections are given in 30-day intervals), and subsequently achieved a complete response with a second course of therapy. The presentation stimulated substantial discussion regarding the value of immunologic monitoring as an endpoint in clinical trials, particularly since the immunologic monitoring had so far failed in the setting of objective tumor responses to the immunization. It also provided a basis to reassess the actual value of an immunologic adjuvant administered with peptide in induction of immunity, and provided additional clinical evidence, similar to previous reports with older vaccines, that objective tumor responses to immunotherapy (vaccines) in advanced disease may require repeated immunization, and may be delayed or occur after initial evidence of progression or stabilization of disease.

Overall, the presentations provided an excellent summary of techniques being used to monitor vaccine studies, preliminary results of these efforts, and problems that still face this field. The Surgery Branch, NCI investigators provided convincing evidence that CTL responses to peptide vaccines in melanoma can be detected in vivo and at least partially quantified. The Elispot assay presented by Dr. Keilholz may simplify these laboratories procedures and allow for more precise measurement of the CTL precursor frequency against the peptide epitopes pre and post immunization. These assays can be used to monitor new trials attempting to optimize immunization with these melanoma antigens. Monitoring of CTL responses to peptides in vitro can also be used to determine if gene-transduced autologous tumor cells are effective means of immunizing patients with melanoma. If so, the data can be extrapolated to gene-transduced autologous tumor cell vaccines in other diseases (for example, renal cell carcinoma) where the actual tumor rejection antigens are unknown.
Now that some cancer vaccines have been shown to generate an immune response to a tumor antigen in patients, the relevant question becomes what further evidence is required to proceed to a phase 3 trial in the adjuvant setting? One option is to require a correlation between vaccine-induced immune response and some clinical outcome, for example, tumor regression in advanced disease. However, conditions present in advanced disease that may cause 'resistance' to vaccine immunotherapy, such as the size of the tumor, barriers or pressure gradients within the tumor that prevent access by effector cells, possible increased heterogeneity in antigen and MHC expression, and subtle systemic or local tumor-induced immune dysfunction, may be less prevalent in the adjuvant (micro-metastatic) setting. Thus, many investigators believe that lack of substantial anti-tumor activity in the advanced disease setting (and by inference, poor correlation between immune response and anti-tumor response) may not preclude substantial activity of the vaccine in the adjuvant setting. In any case, the level of comfort for proceeding to adjuvant studies has been increased by the observation of anti-tumor responses in many of the current trials, which is beyond initial expectations, and particularly surprising in the case of the MAGE peptides, which were administered without adjuvant and did not induce a CTL response sufficiently strong to be detected with 2 in vitro stimulations of PBL with peptide.

Many new technologies for presenting peptides (for example, loaded on dendritic cells) or their corresponding protein antigens (for example, viral vectors) that are only now entering the clinic may substantially improve the ability to induce and thus detect CTL responses. Some may argue that adjuvant trials should await assessment of these new, perhaps more potent immunization methods. However, these trials will take some time to complete, and at this time there is no clear correlation between magnitude of an immune response and clinical anti-tumor responses, particularly in a micro-metastatic setting. Therefore, based on data presented at this workshop, a reasonable argument could also be made that there is enough information (tumor responses and/or induction of immunity) regarding the melanoma peptides to consider a phase 3 adjuvant trial now or in the near future. Such a study would most likely employ a polyvalent vaccine (perhaps the modified gp100 peptides, the MART-1 peptides, and the MAGE peptides, with consideration for use of other peptides such as those derived from tyrosinase) based on the demonstrated heterogeneity of antigen expression in metastatic tumor. The results of clinical trials of vaccines in advanced disease also suggest that the vaccine should be administered over a prolonged period of time, based on the delayed anti-tumor responses observed in some patients. Certainly data that will become available in the next 12 months will answer many of the important questions raised and discussed at this meeting.

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