A New Strategy for Tumor Antigen Discovery Based on *in Vitro* Priming of Naive T Cells with Dendritic Cells

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Abstract

We describe a method for discovery of new tumor antigens that uses dendritic cells (DCs) as antigen-presenting cells to prime autologous naive CD4+ and CD8+ T cells from healthy donors against tumor proteins and peptides. For the identification of HLA class I-restricted tumor antigens, peptides were extracted from tumor HLA class I molecules, fractionated by reverse phase-high performance liquid chromatography, and loaded onto *in vitro*-generated DCs to prime naive CD8+ T cells. Our results show that we were able to prime naive CD8+ T cells *in vitro* to several peptide fractions and generate specificity for the tumor. For the identification of HLA class II-restricted tumor antigens, fractionated by reverse phase-high performance liquid chromatography, and loaded on target cells bearing the same HLA molecules (3, 4). The "reverse immunology approach" uses peptide sequences derived from already known oncogenes or other putative tumor-associated genes that contain desired HLA anchor motifs (5, 6). All of these approaches depend on the availability of tumor-specific T-cell lines or clones derived from cancer patients and used to recognize the new targets. Most recently, the SEREX approach has been used where tumor cDNA expression libraries are screened with sera from cancer patients (7). These approaches have led to the identification of a panel of tumor antigens, primarily in melanomas (8–15). Very few tumor-specific antigens have been described in epithelial tumors, the best known being the HER-2/neu-derived peptides (16) and the core peptides of the MUC-1 tandem repeat (17). In addition to the overall number of tumor antigens being small, there is a concern that they were all identified using an immune response from individuals with cancer that has clearly not been effective as a tumor rejection response. Therefore, we devised and tested a new antigen discovery system that seeks to determine not what a cancer patient recognizes on his/her tumor but rather what a healthy immune system recognizes as foreign among numerous peptides and proteins derived from tumor cells. Our approach is based on *in vitro* cultured DCs that are used to prime autologous healthy naive T cells.

DCs have been shown to be the most potent APCs in the immune system, expressing high levels of MHC molecules, costimulatory molecules, and adhesion molecules essential for T-cell activation (18). Furthermore, DCs are capable of inducing primary T-cell responses *in vitro* to both viruses and synthetic peptides (19–22), whereas other APCs can only stimulate previously sensitized T cells (23). DCs have been successfully used to prime naive T cells *in vitro* against several known tumor antigens (24–30).

Studies have shown marked alterations in the signal-transduction molecules in the T cells of human cancer patients, such as alterations in expression of p56*ck* tyrosine kinase, T-cell receptor ζ-chain (31), and nuclear factor-κB p65 transcription factors (32). Therefore, in our approach we have used T cells from healthy donors. The results here illustrate the utility of this new approach for priming naive CD4+ and CD8+ T cells against proteins and peptides isolated from a breast epithelial tumor cell line and presented by DCs grown *in vitro*, and generation of specificity for the tumor. This has led to the isolation of candidate HLA class I- and HLA class II-restricted epithelial tumor antigens.

Introduction

Successful immunotherapy against tumors relies in part on the discovery of tumor-specific antigens that are able to stimulate effective immune responses in the host. Several approaches have been developed over the years for the identification of tumor antigens. The "genetic approach" uses tumor cDNA libraries transfected into target cells expressing appropriate HLA molecules (1, 2). The "peptide elution approach" uses peptides eluted from tumor HLA and loaded on target cells bearing the same HLA molecules (3, 4). The "reverse immunology approach" uses peptide sequences derived from already known oncogenes or other putative tumor-associated genes that contain desired HLA anchor motifs (5, 6). All of these approaches depend on the availability of tumor-specific T-cell lines or clones derived from cancer patients and used to recognize the new targets. Most recently, the SEREX approach has been used where tumor cDNA expression libraries are screened with sera from cancer patients (7). These approaches have led to the identification of a panel of tumor antigens, primarily in melanomas (8–15). Very few tumor-specific antigens have been described in epithelial tumors, the best known being the HER-2/neu-derived peptides (16) and the core peptides of the MUC-1 tandem repeat (17). In addition to the overall number of tumor antigens being small, there is a concern that they were all identified using an immune response from individuals with cancer that has clearly not been effective as a tumor rejection response. Therefore, we devised and tested a new antigen discovery system that seeks to determine not what a cancer patient recognizes on his/her tumor but rather what a healthy immune system recognizes as foreign among numerous peptides and proteins derived from tumor cells. Our approach is based on *in vitro* cultured DCs that are used to prime autologous healthy naive T cells.

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3 The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; RP-HPLC, reverse phase-high performance liquid chromatography; TFA, trifluoroacetic acid; ATCC, American Type Culture Collection; IL, interleukin.
Materials and Methods

Cell Lines

MS (A3, B7, B7, C7, C7; DR15, DQ6 homozygous) is a breast epithelial adenocarcinoma cell line derived from the metastasis of a breast cancer patient. This cell line does not express either MUC1 or HER-2/neu (data not shown), the two major epithelial tumor antigens. MS-A2 is the same cell line that we stably transfected with the HLA-A2.1 plasmid (33) using the calcium phosphate precipitation method (Stratagene, La Jolla, CA). The B-lymphoma cell line Raji (A3, B15, C7 homozygous; DR3, DR10, DQ1, DQ2) was purchased from the ATCC (Manassas, VA). The chronic myelogenous leukemia cell line K562 was also purchased from ATCC. The melanoma cell line Mel 624 (A2, A3, B7) was provided by Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). The lung tumor cell line, 201T (A10, A29, B15, B44), which we also transfected with the HLA-A2.1 plasmid (designated 201T-A2), was a kind gift from Dr. Jill Siegfried (University of Pittsburgh). Naive CD4+, CD8+ T cells, DCs, and macrophages were derived from a leukophoresis product of a healthy platelet donor (A2, A29, B7, B44, C7, C7; DR15, DR7, DQ6, DQ2) obtained through the Central Blood Bank of Pittsburgh (Pittsburgh, PA).

Antibodies

Mouse antihuman HLA-DR (L243), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), and CD56 (Leu-19) were purchased from Becton Dickinson (San Jose, CA). Mouse antihuman CD45RO (UCHL1) and CD20 were purchased from Dako (Carpinteria, CA). Goat antmouse IgG antibodies were obtained from Zymed Laboratories, Inc. (South San Francisco, CA). W6/32, a mouse antihuman HLA class I antibody, was produced by the W6/32 hybridoma obtained from ATCC (Manassas, VA) and purified via a protein A-Sepharose column (Sigma Chemical Co., St. Louis, MO) in the laboratory.

Isolation of Tumor HLA Class I-bound Peptides

Isolation of HLA class I-associated peptides was similar to methods described previously (3, 34). MS-A2 tumor cells were grown in 10-chamber cell factories (Nalge Nunc, Naperville, IL) and expanded weekly until >1.5 × 10^6 cells were obtained. The cells were washed three times in ice-cold PBS, pelleted, and stored at -80°C for later use. Detergent lysis buffer (1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate and a mixture of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 μM iodoacetamide, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 3 mg/ml EDTA, and 0.04% sodium azide; Sigma) were used to solubilize the cells at 4°C for 1 h. The cell lysate was spun at 100,000 × g for 1 h to remove insoluble proteins, and the supernatant was filtered through a 0.22 μm filter (Millipore, Bedford, MA) to further remove debris from the suspension. The supernatant was then passed through a protein A-Sepharose anti-class I (W6/32) column (Bio-Rad, Hercules, CA) overnight. The column was then washed 30 times sequentially with low salt (150 mM NaCl, 20 mM Tris, pH 8.0) buffer, high salt (1 M NaCl, 20 mM Tris, pH 8.0) buffer, and Tris buffer (20 mM Tris, pH 8.0). Class I molecules were then eluted from the column using 0.2 N acetic acid, and peptides were extracted from the class I molecules by boiling in 10% acetic acid for 5 min. The released peptides were further purified using 5-kDa cutoff microconcentrators (Amicon, Bedford, MA), vacuum centrifuged to reduce the volume, and frozen at -80°C.

HPLC

Fractionation of Peptide Extracts. The peptide extracts were fractionated by RP-HPLC on a Rainin HPLC separation system (Varian, Woburn, MA). The peptide extracts were concentrated to 150 μl via vacuum centrifugation and injected into a Brownlee Aquatep (Applied Systems, Inc., San Jose, CA) C_{18} column (column dimensions, 2.1 mm × 3 cm; pore size, 300 Å; particle size, 7 μm) on the HPLC. The peptides were eluted with a 65-min TFA/acetonitrile gradient (v/v 0–15% for 5 min, 15–60% for 50 min, and 60–100% for 10 min solvent B (60% acetonitrile in 0.085% TFA) in solvent A (deionized water in 0.1% TFA)) at a flow rate of 200 μl/min. Two hundred-μl fractions were collected at 1-min intervals, concentrated via vacuum centrifugation to 40 μl, and divided into four aliquots, three for the use in T-cell stimulation and 1 for mass spectrometry analysis.

Fractionation of Protein Extracts. MS tumor cells (>1 × 10^9) were lysed in detergent buffer, spun at 100,000 × g, and then filtered using a 0.22 μm filter as above. The supernatant was dialyzed overnight in Tris-buffered saline (TBS; pH 7.2; Sigma) to remove detergent. The protein extract was concentrated by vacuum centrifugation, and one-tenth of the extract (~1 × 10^9 cell equivalents) was fractionated by RP-HPLC using a Phenomenex Jupiter C_{18} column (column dimensions, 4.6 mm × 150 mm; pore size, 300 Å; particle size, 7 μm; Torrence, CA). The proteins were eluted with a 60-min TFA/acetonitrile gradient (v/v 10–80% solvent B (100% acetonitrile in 0.1% TFA) in solvent A (deionized water in 0.1% TFA)) at a flow rate of 500 μl/min. Five hundred-μl fractions were collected at 1-min intervals, concentrated by vacuum centrifugation to 100 μl, and divided into four aliquots, three for the use in T-cell stimulation and one for protein content analysis.

Subfractionation of Protein Fractions. Twenty-five % of a specific protein fraction obtained from the primary fractionation was further subfractionated by RP-HPLC using a Phenomenex Jupiter C_{18} column (column dimensions, 4.6 mm × 150 mm; pore size, 300 Å; particle size, 7 μm; Torrence, CA). The proteins were eluted with a shallow 10-min TFA/acetonitrile gradient (v/v 55–62% solvent B (100% acetonitrile in 0.1 TFA) in solvent A (deionized water in 0.1% TFA)) at a flow rate of 500 μl/min, and fractions were collected at 1-min intervals. The subfractions were then further concentrated by vacuum centrifugation, with 33% of the material loaded onto a 15% SDS-PAGE gel and visualized using a silver staining analysis kit (Bio-Rad), and 33% was loaded onto macrophages and used in a proliferation assay (see below). All solvents were HPLC grade and were obtained from VWR Scientific Products (West Chester, PA).

Generation of DCs in Vitro

DCs were cultured in vitro for 7 days as described previously (28). Peripheral blood mononuclear cells from a healthy donor were isolated after centrifugation over Lymphocyte Sep-
Purification of Naive CD8+ and CD4+ T Cells

Nonadherent cells obtained after plastic adherence for DC isolation was used as the source of naive CD8+ or naive CD4+ T cells. To purify naive CD8+ T cells, the cells were stained with anti-CD4, anti-CD20, anti-CD56, and anti-CD45RO antibodies for 45 min in cold PBS and washed in PBS supplemented with 5% human AB serum (Gemini Products, Calabasas, CA). Magnetic Dynal beads (Lake Success, NY) coated with goat antimouse IgG were then added to the cells for 45 min, and the contaminating cells were removed by magnetic separation. Flow cytometry analysis of the remaining cells showed that they were high HLA-DR+ and B7-2+.

Priming Naive CD8+ T Cells to Tumor Peptides

To prime naive CD8+ T cells, 2 x 10^5 DCs were incubated for 2–4 h, first with 25% of each peptide containing RP-HPLC fraction (10 μL) and then overnight in the presence of 1000 units/ml tumor necrosis factor-α (Genzyme, Cambridge, MA) in 96-well, U-bottomed plates (Falcon, Franklin lakes, NJ). Autologous naive CD8+ T cells (2 x 10^5) were added the next day to the DCs in the presence of 2 ng/ml IL-1β (R&D Systems, Minneapolis, MN), 20 units/ml IL-2 (DuPont, Wilmington, DE), and 26 ng/ml IL-4 (Schering-Plough). The CD8+ T-cell cultures were fed every 3–4 days with 10 units/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. In addition, 10 ng/ml IL-7 (PharMingen, San Diego, CA) was included in the cytokine mixture after the second restimulation. The CD8+ T-cell cultures were restimulated every 7–10 days using autologous macrophages (obtained by plastic adherence) loaded with the individual protein fractions (T cells:macrophages, 10:1) and fed every 4–5 days with 10 units/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. Ten ng/ml IL-7 (PharMingen) were added to the CD8+ T-cell cultures after the second restimulation. For the third restimulation, MS tumor was irradiated for 7 min (2.18 J/cm^2) using a Spectra Mini II UV-B irradiator (Daavlin, Bryan, OH) and loaded onto macrophages overnight (apoptotic tumor:macrophages, 5:1) that were used as stimulators (T cells:loaded macrophages, 10:1) the next day.

Cytotoxicity Assays

Target cells (1–2 x 10^6) were labeled with 50 μCi of Na_251CrO_4 (Amersham, Arlington Heights, IL) for 90 min at 37°C. The labeled cells were then washed three times and plated at 1 x 10^5 cells/well in a 96-well, V-bottomed plate (Costar, Cambridge, MA) with various numbers of effector T cells. In addition, a 50-fold excess of unlabelled K562 (5 x 10^5) was added to the wells for 15 min prior to the addition of T cells to prevent the detection of lymphokine-activated killer activity in the assay. The plates were centrifuged and incubated for 4 h at 37°C. All determinations were done in triplicate. Supernatants were harvested using a Skatron harvesting press (Skatron Instruments, Sterling, VA) and counted on a Cobra II series auto gamma counting system (Packard, Meriden, CT). Maximum release was obtained by adding 50 μl of 1% Triton X-100 to the labeled target cells. Spontaneous release was calculated from the following formula: % specific lysis = (maximum release - spontaneous release). In blocking experiments, anti-MHC class I antibody (W6/32) was added to the labeled target cells for 30 min prior to the addition of the effector T cells.

Proliferation Assays

Autologous macrophages loaded with UV-induced apoptotic MS tumor cells (apoptotic tumor:macrophages, 5:1) were seeded in round-bottomed, 96-well microplates (Costar, Cambridge, MA) with primed T-cell cultures at a T cell:stimulator ratio of 20:1. For proliferation assays using tumor lysate, MS tumor cell lysate was generated as described above, and 1.75 x 10^6 cell equivalents were loaded onto 2 x 10^5 autologous macrophages for 2 h. T cells were added at a T cell:stimulator ratio of 10:1. For the proliferation assay using the subfraction 44, 33% of the subfraction was loaded onto 5 x 10^4 macrophages overnight and added to T cells with a T cell:stimulator ratio of 1:1 the next day. The wells were pulsed with [3H]thymidine (Amersham, Life Science) for the last 18 h of the 5-day period, harvested by a Skatron semiautomatic cell harvester (Skatron Instruments), and counted on a Wallac 1205 beta plate scintillation counter (Gaithersburg, MD). The results are expressed as mean values of triplicate determinations.
Mass Spectrometry Analysis

Twenty-five % of the RP-HPLC peptide fraction was concentrated by vacuum centrifugation to near dryness and resuspended in 5 µl of 0.1 M acetic acid. One µl of this material was loaded onto a microcapillary C18 column (150 mm × 75 µm inside diameter) and eluted with a 20-min linear gradient [v/v 0–80% solvent B (0.1 M acetic acid in 100% acetonitrile) in solvent A (deionized water in 0.1 M acetic acid)]. Flow rates for the nanospray probe (186 nl/min) was achieved by coupling the Rainin HPLC system with an Accurate microflow processor (LC Packings, San Francisco, CA) for flow splitting. The nanospray probe was operated at a voltage differential of +3.2 keV. The source temperature was maintained at 30°C. Mass spectra were obtained by scanning from 300-1500 every 3 s and summing individual spectra on a Fisons Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Loughborough, United Kingdom).

Results

Identification of HPLC Fractions Containing Immunogenic Tumor Peptides. CD8 + T-cell cultures were primed and restimulated with HPLC fractions as described in “Materials and Methods.” Because of the low amount of peptide, later restimulations were done using macrophages loaded with irradiated MS-A2 tumor cells. Monitoring the CD8 + T-cell cultures with an inverted microscope over four restimulations clearly showed that although there was T-cell proliferation in all wells, several of the CD8 + T-cell cultures were expanding at a much higher rate, suggesting the presence of immunostimulatory peptides in the fractions used for priming in these wells. Most of the unstimulated CD8 + T-cell cultures reached senescence after 8 weeks in culture.

Fig. 1 shows the result of one priming experiment in which after the fourth restimulation, we were able to test all of the T-cell cultures for their ability to recognize the original tumor, MS-A2, from which the tumor peptides were derived. Of the 65 CD8 + T-cell cultures primed on the individual peptide fractions, 12 (fractions 15, 22, 30, 32, 37, 38, 43, 44, 50, 51, 52, and 63) exhibited strong cytotoxicity against the tumor. Because the peripheral blood lymphocyte donor and the tumor were mismatched at the HLA-A3 allele, we also used the Raji tumor cell line, which was matched only at the HLA-A3 allele with the peripheral blood lymphocyte donor, to ensure that the cytotoxic CD8 + T-cell cultures were not alloreactive. None of the cultures that killed MS-A2 tumor cells recognized the Raji targets.

The 12 cytotoxic CD8 + T-cell cultures were also tested for their ability to recognize another epithelial adenocarcinoma, a lung tumor, 201T-A2, to look for shared tumor antigens. As shown in Fig. 2, a CD8 + T-cell culture, primed with peptide fraction 32, recognized again the original tumor and also the lung tumor. Because the lung tumor and the breast tumor shared only the HLA-A2.1 allele, this suggested that the peptide being recognized was a shared antigen restricted by HLA-A2.1.

To determine the extent of reproducibility of this approach, we repeated the acid extraction, peptide fractionation, and priming procedure. We decided to pool several consecutive HPLC fractions for two reasons: (a) to compensate for small shifts in fraction number between HPLC runs; and (b) to reduce the total number of T-cell cultures in vitro, making the approach less labor intensive. Naive CD8 + T cells were primed on pooled...
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Fig. 3  CD8+ T cells primed with pooled peptide fractions recognized the original tumor and were HLA class I-restricted. A, CD8+ T cells primed with peptide fractions 41–46 recognized the original tumor, MS-A2, and were blocked by the anti-MHC class I antibody, W6/32. B, CD8+ T cells primed with peptide fractions 61–65 recognized the original tumor (MS-A2) and not an HLA-matched tumor, Mel 624.

To evaluate the content of these 12 immunostimulatory fractions, we analyzed them by electrospray ionization nanospray mass spectrometry. We obtained a panel of peptide species (Table 1) conforming to mass:charge ratios of 700–1300 daltons, indicative of HLA class I-binding peptides. These results showed that we were successful in extracting peptides from HLA class I molecules, and that there were immunostimulatory peptides in the HPLC fractions that were capable of stimulating naïve CD8+ T cells to proliferate and expand in vitro.

Identification of HPLC Fractions Containing Immuno-
genic Tumor Proteins. We have devised and tested a sim-
ilar strategy to identify HLA class II-restricted tumor anti-
gens by analyzing immunostimulatory properties of fractionated tumor proteins. CD4+ T-cell cultures were primed and restimulated as described in "Materials and Methods." By the third restimulation, macrophages loaded with apoptotic MS tumor cells were used to stimulate the CD4+ T-cell cultures. Similar to the CD8+ T-cell cultures, observation of the CD4+ T-cell cultures with an inverted microscope over five restimulations showed that not all of the CD4+ T-cell cultures were growing equally well, suggesting that the CD4+ T cells were responding to immunostimulatory proteins present in some of these fractions and not in others.

Most of the unstimulated CD4+ T-cell cultures reached senescence after 10 weeks in culture. Fig. 4 shows the results of one priming experiment in which after the second restimulation, we tested all of the T-cell cultures for their ability to recognize the original tumor, MS, from which the proteins were obtained. Autologous macrophages were loaded with apoptotic tumor and used in a 5-day proliferation assay as stimulators of the primed CD4+ T-cell cultures. Of the 52 CD4+ T-cell cultures, 14 (fractions 5, 10, 11, 12, 13, 22, 28, 35, 37, 38, 39, 40, 46, and 51) proliferated in response to macrophages loaded with apoptotic tumor. We also tested the CD4+ T-cell cultures for cytotoxicity against the original tumor via a CTL assay. None of the T cells tested killed the original tumor (data not shown). Some of the positive CD4+ T-cell cultures were also tested for their ability to recognize autologous macrophages loaded with tumor lysate. As shown in Fig. 5, CD4+ T-cell

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Table 1  Mass spectrometry analyses of immunostimulatory peptide fractions


Fig. 4 Identification of 12 primed CD4+ T-cell cultures that recognized the original tumor, MS, from which the proteins were obtained. The primed CD4+ T cells were tested in a proliferation assay using macrophages loaded with UVB-induced apoptotic tumor (T cells:macrophages, 20:1) after the second restimulation. Bars, SD.

cultures primed with protein fractions 5, 6, and 15 were able to proliferate to macrophages loaded with tumor lysate, consistent with results shown in Fig. 4.

To determine the content of the immunostimulatory protein fractions, we evaluated the protein fractions using SDS-PAGE and silver stain analysis. Because of a large number of proteins in each fraction, the fractions of interest were further subfractionated by RP-HPLC. An example is shown in Fig. 6, where we analyzed the immunostimulatory protein fraction 44 from the second HPLC run that corresponded to fraction 46 from the first run (Fig. 4), because of slight variations in HPLC fraction number between runs. From fraction 44, we derived 10 subfractions and analyzed them for protein content (Fig. 6A) and immunostimulatory capacity (Fig. 6B). We detected immunostimulatory capacity in 4 of the 10 subfractions (44.1, 44.2, 44.4, and 44.7). Silver stain analysis detected two prominent bands, one at 17 kDa, and the other at 19 kDa in fraction 44.4 (Fig. 6A). We are in the process of sequencing these bands, as well as determining the immunostimulatory proteins in the other HPLC fractions.
Discussion

We have devised and tested a new approach to tumor antigen discovery that uses DCs as APCs to prime naïve CD4+ and CD8+ T cells against tumor proteins and peptides. This approach has advantages over previous approaches in that it seeks to explore antitumor responses that can be generated in healthy, immunocompetent individuals to tumor antigens presented by professional APCs. This new approach eliminates the use of T cells from cancer patients and the use of tumor cells as APCs. Studies have shown that T cells in cancer patients may be defective, and tumor cells are poor APCs (35, 36). In addition, by using T cells from healthy donors, we are relying on a T-cell repertoire that has not been affected by the presence of the tumor.

We have used this approach in search of new epithelial tumor antigens that have been limiting to date, mainly because of the difficulty in generating tumor cell lines and consequently tumor-reactive T-cell lines (8). For the identification of HLA class I-restricted tumor antigens, we showed that we were able to prime naïve CD8+ T cells to tumor peptides and generate specificity to the original tumor from which the peptides were derived. In some cases, the primed CD8+ T cells also recognized another tumor of the same tissue type, suggesting the existence of shared tumor antigens. We also showed that we could reproduce the whole procedure, demonstrating that there were indeed tumor peptides present in similar fractions that conferred immunostimulatory capacity.

To determine the composition of immunostimulatory peptide fractions, we used electrospray ionization mass spectrometry to look for masses that would conform to what is expected of HLA class I-associated peptides that have mass:charge ratios between 700 and 1300 daltons. The advantages of using the mass spectrometer to identify HLA class I-associated peptides have been documented extensively (37). It offers higher sensitivity for detection of small amounts of peptides. We were able to identify a panel of peptides that fit the criteria of HLA class I-binding peptides, suggesting that these immunostimulatory fractions contained peptides that could be MHC class I-restricted candidate tumor antigens. The identification and sequencing of these naturally processed peptides is currently ongoing. Fraction 50 (Fig. 1) has already yielded a new tumor antigen not found by other approaches to date.

For the identification of HLA class II-restricted tumor antigens, we demonstrated that we were able to prime naïve CD4+ T cells to fractionated tumor proteins and generate specificity against macrophages fed the original tumor, both in the apoptotic form and the tumor lysate form. Not surprisingly, SDS-PAGE and silver stain analysis of each of the positive protein fractions yielded an array of protein bands (data not shown), requiring further subfractionation and confirmation of immunostimulatory capacity. Subfractionation of one of these protein fractions yielded two predominant proteins of 17 kDa and 19 kDa, narrowing our search to two specific tumor antigen candidates. Investigations are currently under way in our laboratory to further subfractionate and sequence other positive protein fractions identified by in vitro priming of CD4+ T cells.

Although we expect that this new approach will be capable of identifying numerous new tumor antigens, it is not without its disadvantages. As with all of the other methods used previously, it is very labor intensive. Furthermore, it is very dependent on the ability to grow and expand tumor peptide or tumor protein-specific T cells. In our experience, we have been able to generate only 3–5 × 10^6 CD8+ T cells and CD4+ T cells/HPLC fraction, which severely limited the number of cytotoxicity and proliferation assays that could be done to evaluate their specificity. This problem is becoming less critical, however, with the advent of new functional assays that use very few T cells, such as ELISPOT (38). The technical complexities of using the mass spectrometer to detect and sequence peptides from these fractions could also be a daunting task, although we were moderately successful in identifying a panel of peptides at the 100 fmol level.

In summary, by devising a system that relies on healthy donors, we have used the full immune potential of the donor’s T cells and DCs as reagents to screen for new tumor antigens, and we have detected a number of candidate tumor antigens that are further characterized. Although we focused our search on epithelial tumor antigens, the strength of our approach is its universal applicability to all tumors, including those that do not readily grow in vitro. Although we have used a tumor cell line as a source of peptides and proteins, they can just as easily be derived from pieces of tumor removed at the time of surgery. In this approach, the peptides and proteins that are found to be immunostimulatory are already known to be naturally processed and presented, an important factor in the design of cancer vaccines.

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References


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