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13. ABSTRACT *(Maximum 200 words)*

We have devised a novel tumor antigen discovery system that utilizes dendritic cells (DCs) as antigen-presenting cells to prime naïve T cells against peptides and proteins isolated from a breast tumor cell line (MS) that does not express both the known breast tumor-specific antigens, MUC-1 and Her-2/neu. We isolated HLA Class I molecules from the tumor, and acid-extracted the peptides bound to the HLA Class I molecules. The peptides were then fractionated by reverse-phase HPLC, and individual fractions were collected and given to DCs to prime naïve $CD8^+$ T cells. Our current results show that we have been able to isolate HLA Class I molecules and acid-extract peptides with reasonable success, and we are in the process of doing specific bioassays with our priming cultures to identify positive fractions that may contain a new tumor-specific antigen. We have also done initial fractionations of the protein extracts from the tumor using reverse-phase HPLC, and used the individual fractions to prime naïve $CD4^+$ T cells. Our results indicate that there is an abundance of proteins in our extracts, and currently, we are in the progress of beginning specific bioassays to identify positive fractions.

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Henry Kaz 6/25/98

PI - Signature Date

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INTRODUCTION

The prospect of successful immunotherapy against breast tumors relies on the discovery of breast tumor-specific antigens and their ability to stimulate immune responses in the host. In the last few years, studies have centered on the elucidation of tumor-specific antigens in melanomas, leading to the identification of various tumor-specific antigens such as MAGE (1, 2, 3, 4, 5), BAGE (6), GAGE (7), and RAGE (8). However, the search for tumor-specific antigens in tumors of epithelial origin such as breast cancer has been very limited. Up to now, only two tumor-specific antigens from these tumors have been identified, i.e. Her-2/neu-derived peptides (9) and mucin (MUC-1) (10). Therefore, the need to identify more breast tumor-specific antigens is essential for the development of effective immunotherapeutic strategies.

Previous studies done to identify breast tumor-specific antigens have utilized tumor cells and tumor-reactive T cells isolated from cancer patients to generate secondary responses to the tumor antigens *in vitro*. However, the problem with this approach was two-fold: one was that it used tumor cells, which were poor antigen presenting cells (APCs) (11), and two, it used T cells from cancer patients, which have been shown to be defective in cancer patients (12). Therefore, it was difficult to generate any notable T cell responses to the tumor, thereby preventing the discovery of tumor antigens. The goal of this project is to create a new tumor antigen discovery system using human dendritic cells and autologous T cells from healthy donors to identify new tumor-specific antigens in breast tumors. We believe that the use of dendritic cells and T cells from healthy donors will bypass the two problems mentioned above and allow us to uncover new breast tumor-specific antigens.

Tumors are Poor Antigen-Presenting Cells

One of the main problems in studying human T cell responses to tumors is that tumors are poor APCs (11). Studies have shown that for T cell activation to occur, two signals must be provided by the APC (13, 14): "Signal 1" is delivered through the T cell receptor (TCR), and "Signal 2" is delivered via the costimulatory molecules CD28 and CTLA-4 (15). The ligands for these receptors, CD80 and CD86, have been found on APCs such as B cells (16), macrophages (16), and dendritic cells (17), but not on tumors (18). Without Signal 2, tumor antigens that are immunogenic may escape immune surveillance since the tumor-reactive T cells are unresponsive or anergic (19). Since previous antigen discovery systems (20) have utilized tumors as APCs, this may explain why limited tumor antigens have been detected to date.

Dendritic cells are Potent Antigen-Presenting Cells

In contrast to tumor cells, dendritic cells have been shown to be the most potent APCs in the immune system (21). Dendritic cells express high levels of cell surface molecules such as major histocompatibility complex (MHC) molecules, costimulatory molecules (CD80 and CD86), and adhesion molecules (CD54 and CD58) (21, 22, 23), all of which are essential for T cell activation. In addition, it has been shown that these cells can induce primary T cell responses *in vitro* to both viruses and synthetic peptides (24, 25, 26, 27, 28), whereas other APCs can only stimulate previously sensitized T cells (21). Furthermore, recent advances in propagating and differentiating DCs in vitro using various cytokine cocktails (29, 30, 31, 32, 33, 34) have allowed us to generate large number of DCs capable of presenting and stimulating T cells in vitro. The use of DCs as APCs instead of tumors should circumvent the problem of using tumors as APCs, and allow us to properly stimulate the T cells in vitro.

T cells from cancer patients are defective

Another problem in studying T cell responses to tumors is that the T cells obtained from cancer patients could be defective and non-functional (12, 35). Furthermore, studies have shown that marked alterations in the signal-transduction molecules were detected in the T cells of human cancer patients and tumor-bearing animals. These defects were characterized as decreases in expression of $p56^{lck}$ tyrosine kinase (36), the TCR-ζ chain (36, 37), and a loss of nuclear c-rel and members of the NK-κB family of transcription factors (38). A decrease in anti-tumor and cytotoxic function (39, 40) was also observed in the T cells of cancer patients. Since previous antigen discovery systems utilized T cells from cancer patients, this suggests that even in the presence of a potent APC and an immunogenic tumor antigen, a T cell response may still not be observed, leading to the loss of discovery of a new tumor antigen. Here we propose to circumvent this problem by using T cells *from healthy donors*, and with DCs as potent APCs, examine *primary responses* to potential tumor antigens in vitro.

Rationale/Hypothesis

One of the main challenges in studying breast tumors is the difficulty in establishing epithelial tumor cell lines. However, our lab has obtained a breast tumor epithelial cell line, MS, which surprisingly does not express both HER-2/neu and MUC-1 (Figure 1). This formed the basis of this project, which proposes the use of a new antigen discovery system to identify and characterize new breast tumor-specific antigens. The novelty of this system is that it uses the most recent technological advances in *in vitro* cultures of human dendritic cells and human T cells to perform *in vitro* priming of naive T cells from healthy donors. This distinguishes it from other work done to date, which studies secondary responses from diseased patients using tumors as antigen presenting cells. We hypothesize that by using naive T cells from healthy donors along with this new and powerful *in vitro* priming system based on dendritic cells as antigen presenting cells, we could circumvent the problem of tumor-induced immunosuppression in vivo, and uncover potentially immunogenic breast tumor antigens which have eluded detection in previous studies.

The project continues to follow the original technical objectives:

Technical Objective #1: To use human dendritic cells to prime *in vitro* naive, autologous $CD8^+$ T cells to peptides eluted from HLA Class I molecules of the breast tumor, MS

Technical Objective #2: To use human dendritic cells to prime naive, autologous $CD4^+$ T cells to fractionated tumor cell lysates of the breast tumor, MS

Technical Objective #3: To clone the genes pertinent to the identified immunogenic breast tumor peptides or proteins

EXPERIMENTAL DESIGN & METHODS

Cell lines & Antibodies. The cell line used is MS, the breast tumor epithelial cell line that does not express both MUC-1 and Her-2/neu (Figure 1). Antibodies used: HC10, which recognizes

denatured HLA Class I molecules; W6/32, which recognizes native HLA Class I molecules; L243, which recognizes HLA-DR; BB7.2, which specifically recognizes HLA-A2.1.

Transfections. The MS tumor expresses an unusual HLA haplotype, being homozygous at the A3, B7, and C7 locus. Since HLA-A2.1 is the most common allele in the Caucasian population, it would be advantageous if the peptides extracted from the MS tumor were bound to HLA-A2.1. Thus, we transfected the HLA-A2.1 gene into MS using a calcium phosphate precipitation kit (Strategene). The HLA-A2 construct contained the entire HLA-A2.1 gene including its regulatory sequences and the neomycin resistant gene, and this was the result of subcloning the HLA-A2.1 gene into the EcoRI site of the vector pSV2neo (41). The cells were then placed in selective medium containing 1.2 mg/ml G418 (GIBCO), and after 2-3 weeks, the cells were stained for HLA-A2.1 expression using an HLA-A2.1 specific antibody, MA2.1, and analyzed by flow cytometry. Positive populations were then sterile sorted and further expanded in culture, and by 4-5 weeks, we have obtained a homogenous population of MS tumors expressing HLA-A2 (designated MS-A2).

Isolation of HLA Class I-bound peptides. The MS tumor cells were grown in 500 cm^2 flasks (NUNC) and expanded until $>10^9$ cells were collected. The cells were then washed three times in ice-cold PBS, pelleted and frozen at -80^0 C. Lysis buffer containing detergent (1% NP-40) and a cocktail of protease inhibitors (2 mM PMSF, 100 µM Iodoacetamide, 5 µg/ml Aprotinin, 10 µg/ml Leupeptin, 10 µg/ml Pepstatin A, 3 ng/ml EDTA, and 0.2% sodium azide) was then used to solubilize the cells. The cell lysate was then spun at 100,000 x g to remove insoluble proteins, and the supernatant was filtered through a 0.22 µm filter to further remove debris from the suspension. The resultant supernatant was our source of HLA Class I molecules. The HLA Class I molecules were then immunoaffinity purified using a Protein A-sepharose anti-class I (W6/32) column. The supernatant was also passed through a Protein A-sepharose anti-HLA-DR column to remove the predominant HLA Class II molecules. The column was then extensively washed with high and low salt buffer, and the Class I molecules were subsequently eluted using 0.2N acetic acid (pH 2.7). In order to extract the peptides from the Class I molecules, the eluate was boiled in 10% acetic acid (pH 2.1) for 5 minutes. The released peptides were then separated by centrifugation through a 5 kD cut-off filter (Amicon), vacuum centrifuged to reduce the volume to 150 µl, and frozen at -80^0C. The immunoaffinity purification of HLA Class I molecules was examined via a Western Blot using the HC10 Ab.

High Performance Liquid Chromatography (HPLC) fractionation of peptide extracts. The peptide extracts were fractionated by reversed phase HPLC on the Rainin HPLC separation system (Woburn, MA). The peptide extracts were concentrated to 150 ul on the SpeedVac, then injected into a Brownlee Aquapore column (2.1mm x 3 cm, 300 Å, 7 µm) and eluted with a 65 minute trifluoracetic acid (TFA)/acetonitrile gradient (v/v 0-15% for 5 minutes, 15-60% for 50 minutes, and 60-100% for 10 minuntes solvent B (60% HPLC acetonitrile in 0.085% TFA) in solvent A (De-ionized water in 0.1% TFA) with a flow rate of 200 ul/min). Fractions were collected at 1 minute intervals.

HPLC fractionation of protein extracts. Protein extracts obtained from the column flow through after HLA Class I purification was dialyzed against Tris Buffered Saline pH 7.2 (TBS), concentrated on a SpeedVac, and fractionated by reverse-phase HPLC using a Phenomenex

Jupiter C4 column (4.6 mm x 150 mm, 300 Å, 7 um). The proteins were then eluted with a 60 minute TFA/acetonitrile gradient (10-80% acetonitrile in 60 minutes) at a flow rate of 500 ul/min. Fractions were collected at 1 minute intervals.

Establishment of human dendritic cells (DCs) in culture. Peripheral blood mononuclear cells (PBMC) isolated from leukocyte research products (LRP; a byproduct of platelet donation) were centrifuged over Ficoll and washed extensively with phosphate buffered saline (PBS) to remove residual platelets. The PBMCs were then plated on a 6-well plate for two hours, after which the non-adherent cells were removed and frozen. The remaining adherent cells were treated with the cytokines granulocyte-macrophage stimulating factor (GM-CSF) and interleukin 4 (IL-4). These cells were cultured for 7 days in DC medium (AIM-V (serum-free medium) supplemented with Penicillin/Streptomycin and 2 mM L-glutamine) with 10 ng/ml rhGM-CSF and 26 ng/ml IL-4. Loosely adherent clumps of large cells were visible after two days in culture and peaked in number by days 5-7 of culture. These cells were predominantly DCs, as confirmed microscopically for the presence of cytoplasmic projections indicative of dendritic cell morphology. The DCs were then harvested on day 7 and purified from contaminating lymphocytes by immunomagnetic depletion using DYNAL magnetic beads. Purified dendritic cells were obtained by negatively selecting T cells, B cells, and NK cells using antibodies against the cell surface markers, CD3, CD19, and CD16, respectively.

Stimulation of purified $CD8^+$ T cells with fractionated peptide extracts. Approximately 2×10^4 purified dendritic cells were incubated overnight with 25% of each peptide containing RP-HPLC fraction in the presence of 1000 u/ml TNF-α. The dendritic cells were then used to prime 4.5×10^4 autologous naïve $CD8^+$ T cells in the presence of 2 ng/ml IL-1β, 20 U/ml IL-2, and 26 ng/ml IL-4. Purified naïve $CD8^+$ T cells were obtained by negatively selecting $CD4^+$ T cells, B cells, NK cells, and memory cells by panning, using antibodies against the cell surface proteins, CD4, CD20, CD56, and CD45RO. Macrophages were removed by plastic adherence and frozen for later use. After 7-10 days, the cultures were restimulated with peptide-pulsed autologous irradiated macrophages. The cultures were observed visually for T cell proliferation. The wells that have actively growing T cells were further expanded with irradiated MS tumors.

Stimulation of purified $CD4^+$ T cells with fractionated protein extracts. Dendritic cells were isolated and treated as described above. Purified naïve $CD4^+$ T cells were obtained by negatively selecting $CD8^+$ T cells, B cells, NK cells, and memory cells by panning, using antibodies against the cell surface proteins, CD8, CD20, CD56, and CD45RO. Macrophages were removed by plastic adherence. Individual fractions were incubated with 2×10^4 dendritic cells overnight in the presence of 1000 u/ml TNF-α. 4.5×10^4 autologous naïve $CD4^+$ T cells were then added to the DCs in the presence of the IL-1β (2 ng/ml), IL-2 (20 U/ml), and IL-4 (26 ng/ml). After 7-10 days, the cultures were restimulated with protein fraction-pulsed autologous irradiated macrophages. The cultures were observed visually for T cell proliferation.

PRELIMINARY RESULTS & DISCUSSION

1. MS tumor does not express MUC-1 and Her-2/Neu, the only two known tumor-specific antigens for breast epithelial tumors:

Since MS is a breast epithelial tumor cell line, we expected that it expressed either MUC-1 or Her-2/neu, the only two tumor-specific antigens known for epithelial tumors. Surprisingly, when we stained for MUC-1 and Her-2/neu expression by flow cytometry (Figure 1), we did not detect expression of these two molecules:

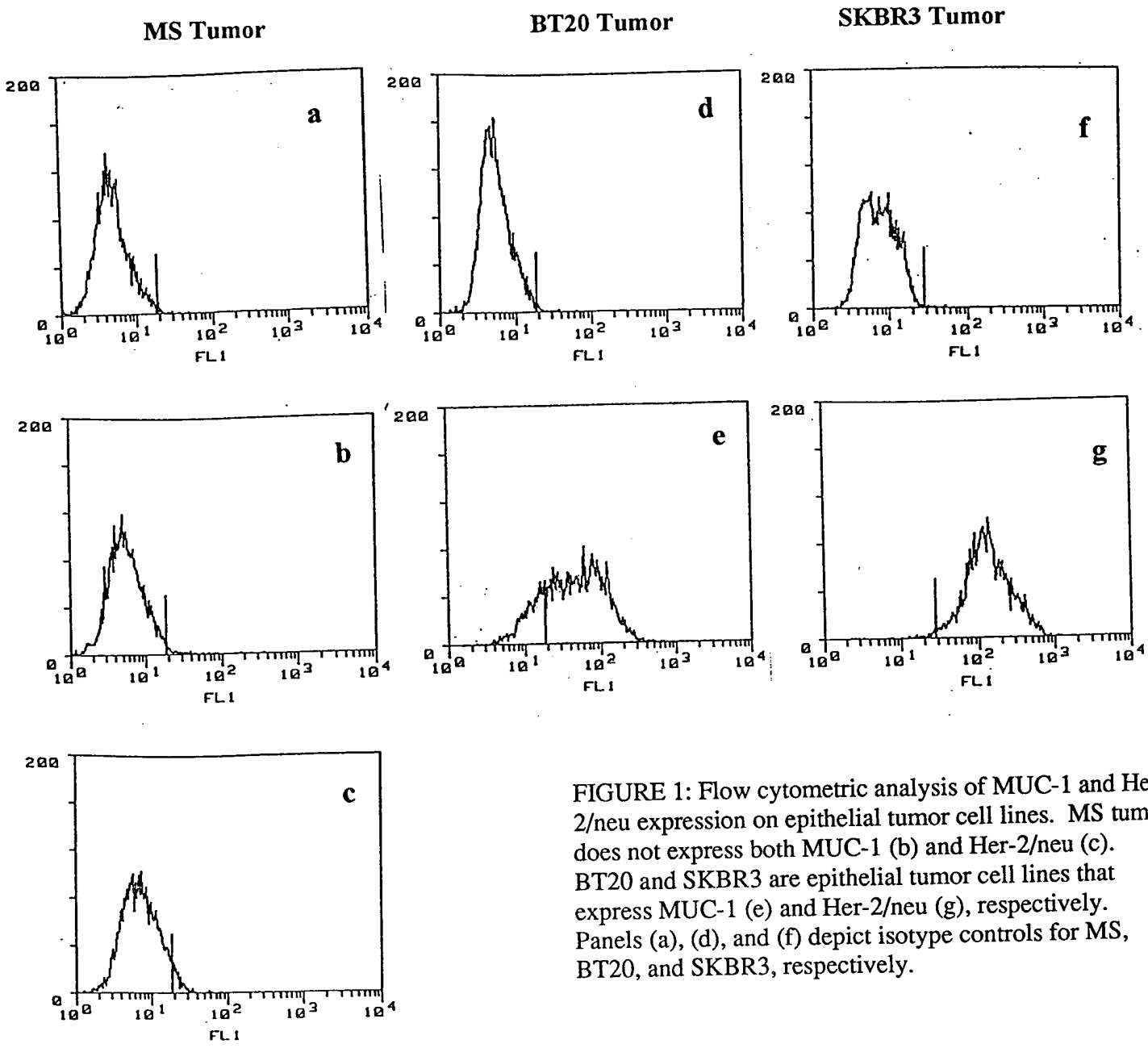

FIGURE 1: Flow cytometric analysis of MUC-1 and Her-2/neu expression on epithelial tumor cell lines. MS tumor does not express both MUC-1 (b) and Her-2/neu (c). BT20 and SKBR3 are epithelial tumor cell lines that express MUC-1 (e) and Her-2/neu (g), respectively. Panels (a), (d), and (f) depict isotype controls for MS, BT20, and SKBR3, respectively.

2. Transfection of HLA-A2.1 into the MS tumor (Relevant to Technical Objective #1):

We have stably transfected the HLA-A2.1 gene into the MS tumor. This will allow us to extract peptides bound to HLA-A2.1, which is the most common MHC allele in the Caucasian population. We are currently in the process of generating large numbers of MS-A2 tumor by injecting subcutaneously into the flanks of nude mice. When the tumors reach 1-2 cm in diameter, the mice will be sacrificed, and the tumors are excised, snap frozen, and the HLA-A2 molecules will be isolated using the methods described above, except that the immunoaffinity column will be specific for HLA-A2.1 using the anti-A2 Ab, BB7.2.

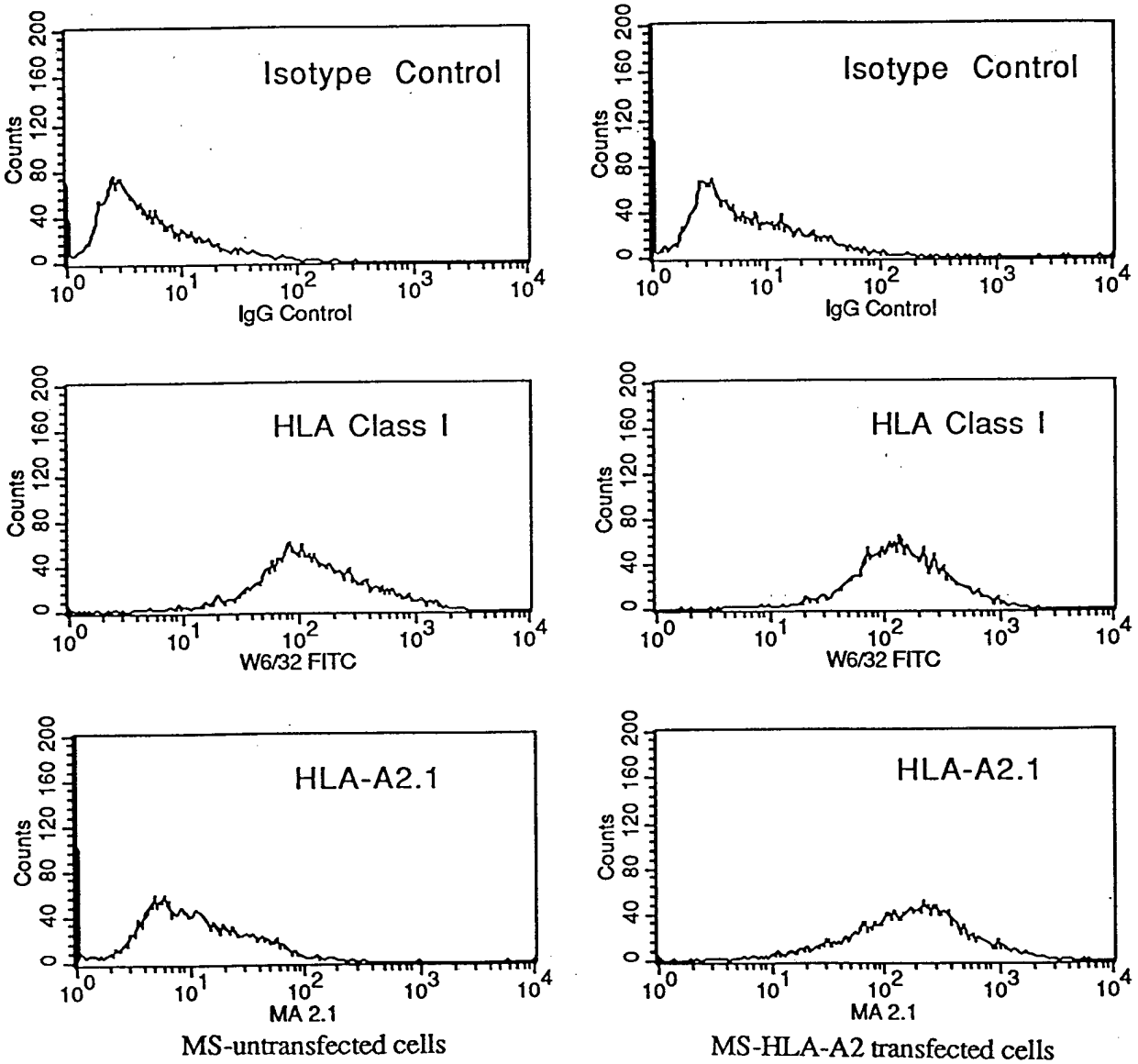

Figure 2: HLA-A2 transfection of MS tumor cells. The left and right columns depict MS tumor cells before and after transfection, respectively. The top two panels are isotype controls. The middle two panels are staining with W6/32, which stains for all HLA Class I molecules. The bottom two panels show staining with the MA2.1 antibody, which is specific for HLA-A2.1.

3. Isolation of HLA class I-bound peptides from the MS tumor (Relevant to Technical Objective #1)

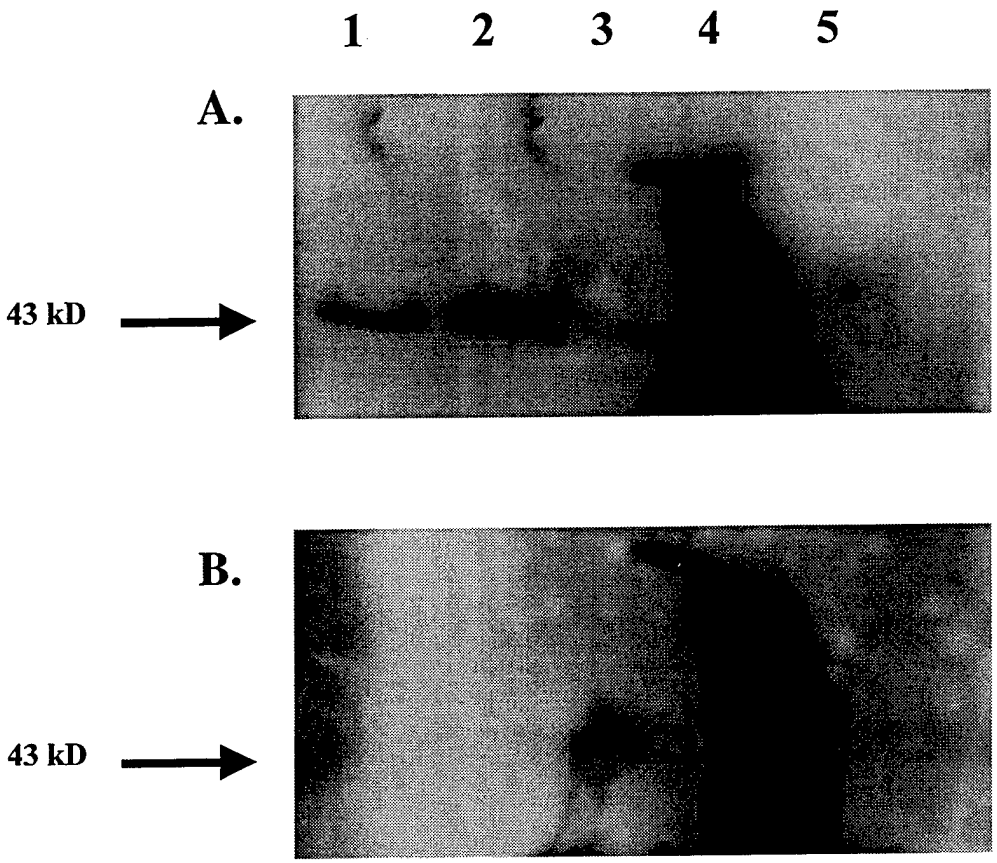

FIGURE 3: Western Blot Analysis of HLA Class I molecules during the immunoaffinity purification procedure of HLA Class I molecules and acid-extraction of bound peptides. Samples collected during various steps of the purification procedure were loaded onto a 12% SDS-PAGE gel, run under reducing conditions, and examined using the HC10 antibody. Samples were collected at these stages: *Lane 1*: After ultracentrifugation, but prior to passage through column; *Lane 2*: After filtration through 0.22 μm filters, but before passage through the HLA Class I column; *Lane 3*: After passage through column; *Lane 4*: Retentate of 5 kD cut-off microconcentrator; *Lane 5*: Filtrate (peptides) of 5 kD cut-off concentrator. Panel A: HC10 plus goat-anti mouse peroxidase conjugate secondary antibody ; Panel B: Secondary antibody alone.

The samples were collected at different stages of the peptide extraction procedure, and the presence of HLA Class I molecules was detected using a Western Blot. In Lane 1, since the HLA Class I molecules are in the supernatant, we expected to detect it in the supernatant. Before passage through our HLA Class I column, the supernatant was further filtered through a 0.22 μm filter to remove debris that might clog our column. We expect that HLA Class I molecules are still present in the supernatant at this point, as is illustrated in Lane 2. To show that our immunoaffinity column bound the HLA Class I molecules, we examined the column flow through for HLA Class I molecules, and as shown in Lane 3, the absence of the band at 43 kD indicates that the HLA Class I molecules were captured in the column. The Class I molecules were then eluted from the column, boiled in acid, and filtered through a 5 kD cut-off microconcentrator. The smearing of bands observed in Lane 4 is due to high levels of HLA Class I molecules retained in the retentate part of the microconcentrator, thereby leading to non-specific binding of the antibody in the Western Blot. We will determine the optimal amount of samples loaded per well to prevent overloading the wells in the future. In Lane 5, which consists of the filtrate portion of the microconcentrator, we did not expect detection of HLA Class I molecules, since it contains only molecules below 5 kD. These preliminary results suggest that we were able to immunoaffinity purify HLA Class I molecules from the MS tumor and acid-extract peptides with reasonable success, addressing Task 1 outlined in my *Statement of Work.* We will repeat Task 1 using MS-A2 cells.

4. HPLC fractionation of Acid-extracted peptides (Relevant to Technical Objective #1):

The acid-extracted peptides obtained above were then fractionated via reverse phase HPLC (see *Experimental Design & Methods)*, and individual fractions were collected. Below are profiles of a mock run and our peptide extract run (Figure 4A & 4B):

Figure 4A: Mock Run

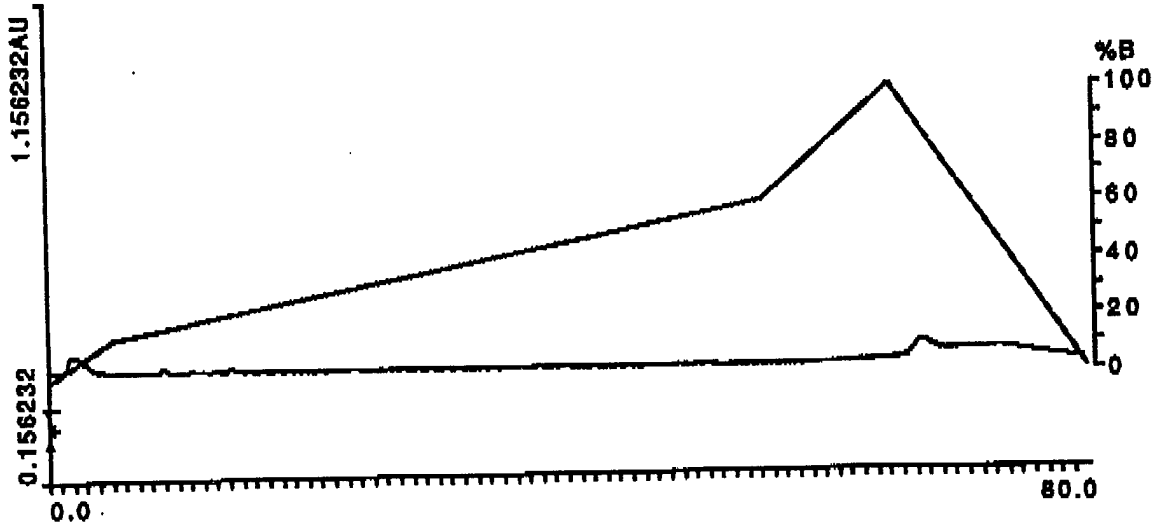

Figure 4B: Peptide Extract Run

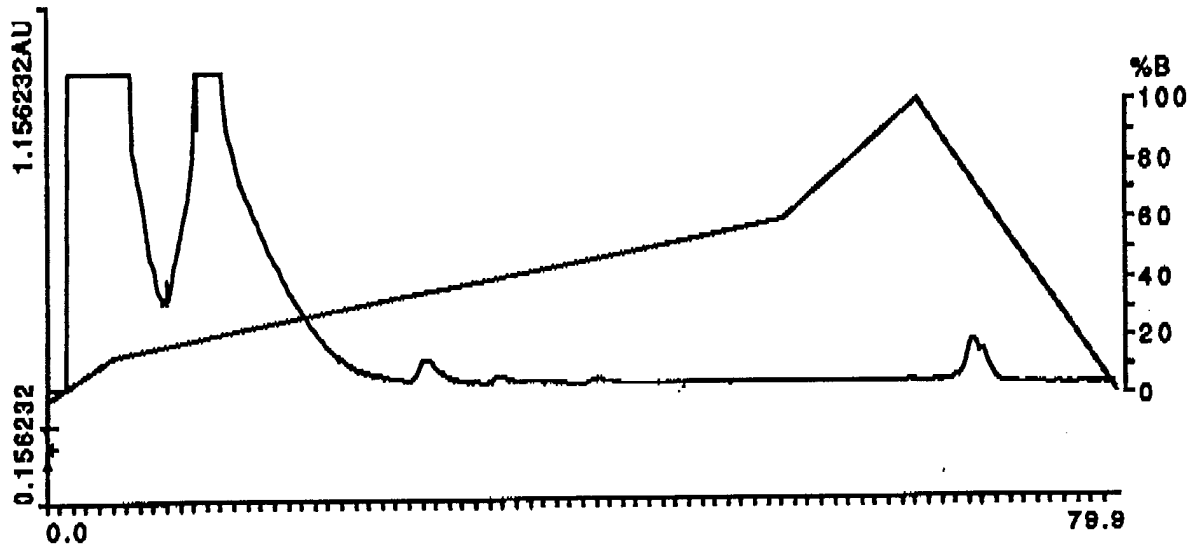

Figure 4: HPLC Profiles of peptide extracts bound to HLA Class I molecules of the MS tumor.. A) Mock run of samples. B) Profile of peptide extracts. Peptide extracts were loaded onto an Aquapore Brownlee C18 column (2.1 x 3 mm, 300 A, 7 um) and eluted with a 60 minute Acetonitrile/TFA gradient as described in *Experimental Design & Methods.* The gradient is depicted in the figure. Individual fractions were collected at 1 minute intervals. We collected a total of 72 fractions.

The results shown here suggests that there might be peptides and/or proteins in our peptide extract, since we detect a few peaks in our profile. However, since the cut-off value of our filter was 5 kD, it is possible that the peaks we see here are peptides/proteins between 1-5 kD that have passed through the filter, and not our potential tumor antigen (s), a 1 kD 9-amino acid peptide (s) that binds to the HLA Class I molecules. In addition, we can not rule out the possibility that there may be many peptides in our extract that are below the sensitivity of our HPLC machine, so the absence of peaks may not be truly reflective of what we have in the extract. Thus, we proceeded to the Task 2, which is to start priming T cell cultures.

We have started priming naïve $CD8^+$ T cells using dendritic cells as APCs and our individual fractions as antigen. We have yet to demonstrate reproducibility using this column, since we have peptides only for one run. We will address this issue once we extract more peptides. We are currently in the 4^{th} week of restimulation, and since we ran out of antigen, we have switched to using irradiated MS tumors as our source of antigen. We have detected visually proliferating T cells in some wells and not others. However, since the cell number is still quite low, we are waiting for the wells to reach higher cell number before performing

bioassays to test the specificity of these T cells. This addresses part of Task 2 outlined in my *Statement of Work.* We will repeat Task 2 using MS-A2 cells.

5. HPLC fractionation of protein fractions (Relevant to Technical Objective #2):

The column flow through obtained from the HLA Class I and Class II immunoaffinity columns were dialyzed against TBS to remove detergent, and then concentrated by SpeedVac. We decided to use a C4 column (4.6 x 150 mm, 300 Å, 7 um) and reverse-phase chromatography as our initial fractionation procedure (see *Experimental Design & Methods).* Individual fractions were collected, and below are profiles of a mock run and two protein extract runs (Figure 5A, 5B, & 5C):

Figure 5A: Mock Run

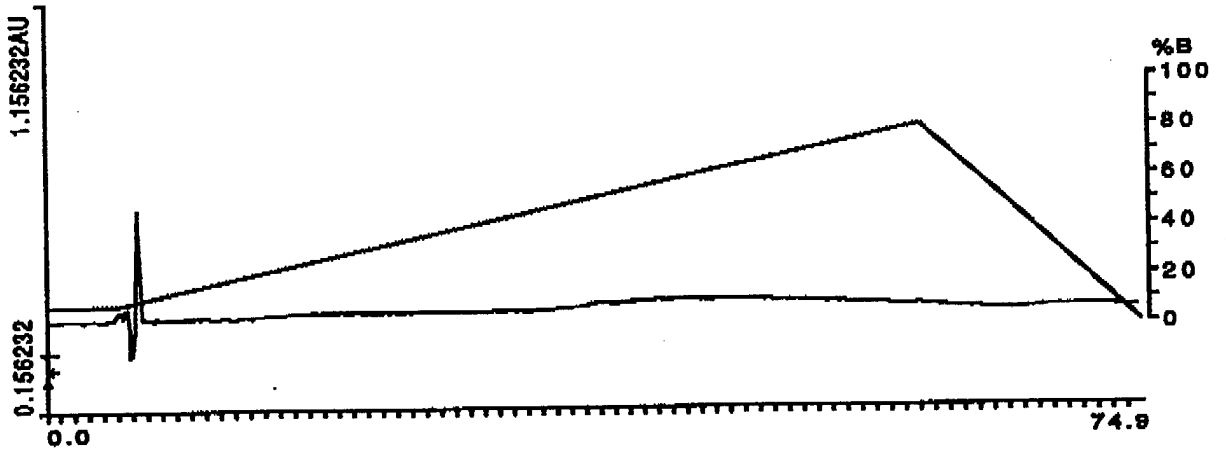

Figure 5B: Protein Extract Run I

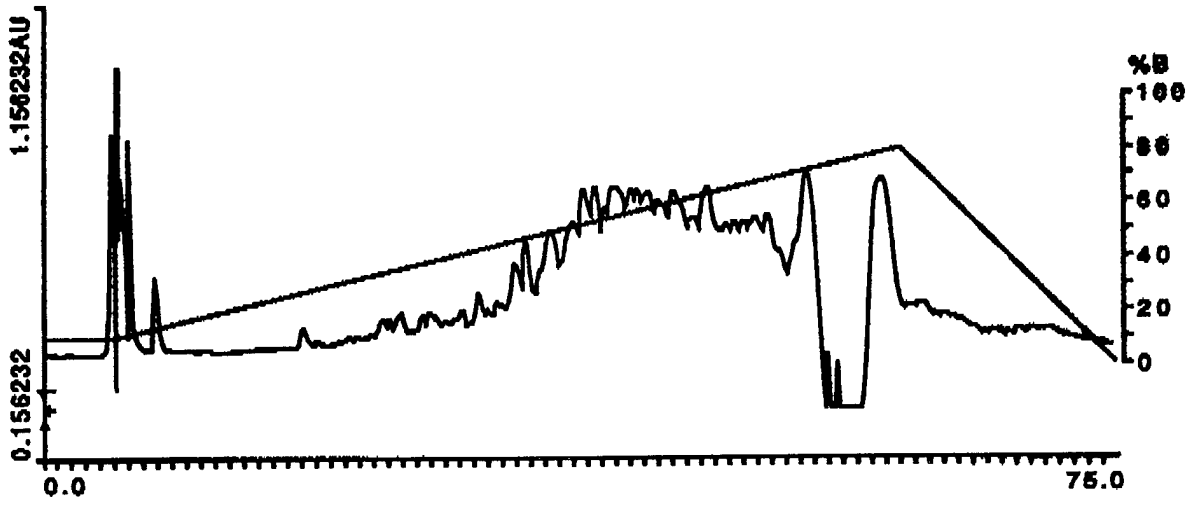

Figure 5C: Protein Extract Run II

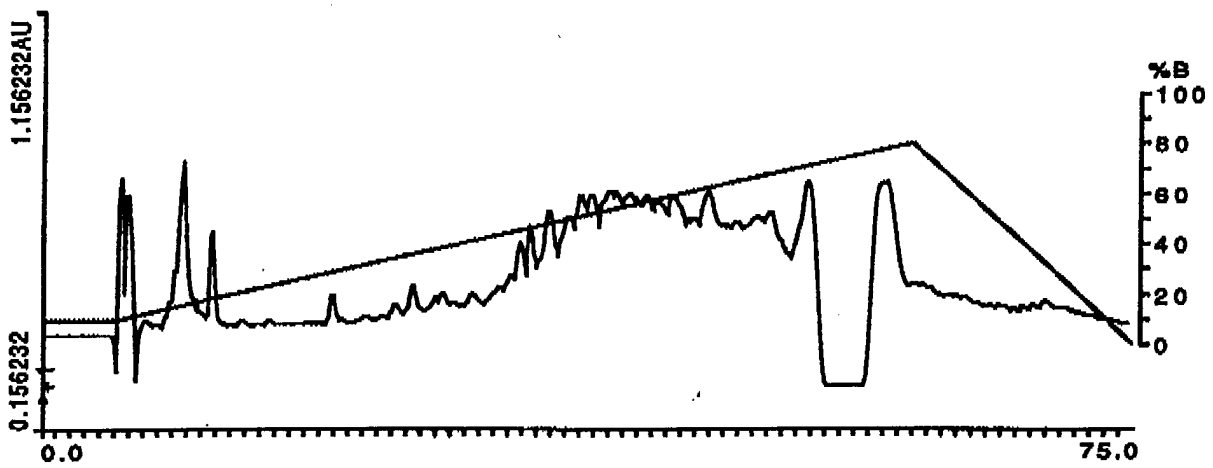

Figure 5: HPLC profiles of protein extracts obtained from the MS tumor. A) Mock run of samples. B & C) Profile of protein extracts obtained from two different runs. Protein extracts were loaded onto an Phenomenex Jupiter C4 column (4.6 x 150 mm, 300 Å, 7 um) and eluted with a 60 minute Acetonitrile/TFA gradient as described in *Experimental Design & Methods.* The gradient is depicted in the figure. Individual fractions were collected at 1 minute intervals. We collected a total of 72 fractions.

The results shown here suggests that there are many proteins in our protein extract, as illustrated by the many peaks in the profile. Furthermore, we were also able to reproduce the profiles of the protein extract on two different runs (Figure 5B & 5C), allowing us to return to specific peaks and obtain more antigen if the need arises. This is very important, since if we can garner reproducible results from our HPLC, we can obtain similar fractions through many runs rather than just one, thus not limiting our source of antigen. This addresses part of Task 4 in my *Statement of Work.*

We have also started priming naïve $CD4^+$ T cells using dendritic cells as APCs and our individual fractions as antigen. We are currently in the 4^{th} week of restimulation with autologous macrophages and antigen, and we have detected visually proliferating T cells in some wells and not others. However, since the cell number is still quite low, we are waiting for the wells to reach higher numbers before starting bioassays to test the specificity of these T cells. This addresses in part, Task 4 in my *Statement of Work.*

ADDRESS TO THE STATEMENT OF WORK

The work presented in this annual report has followed the *Statement of Work* as listed in the DOD grant. We have attempted to address Task 1, Task 2, and Task 4 outlined in the first two technical objectives with reasonable success. In Task 1, we were able to generate large number of MS tumors either in tissue culture or in nude mice, and we were able to isolate the HLA Class I molecules and acid-extract the bound peptides. We also fractionated the peptide extract on a narrowbore column, and individual fractions were used to prime naïve $CD8^+$ T cells. This addresses in part, Task 2 in the *Statement of Work.* We also generated protein extracts via initial fractionation procedures using reverse-phase HPLC. Individual fractions were then used to prime naïve $CD4^+$ T cells. This addresses, in part, Task 4 in the *Statement of Work.* Currently, we are in the process of starting bioassays to test the specificity of these $CD4^+$ and $CD8^+$ T cells we have grown in culture. In addition, upon generating enough MS-A2 tumors in nude mice, we intend to perform Task 1 and Task 2 again and address the peptide antigens in the context of HLA-A2.1. We will also repeat the priming assays using samples from different donors.

CONCLUSIONS

For immunotherapies against breast cancer to be successful, we must identify breast tumor antigens so that we can enhance the immune system's response to the tumor. We have devised a novel tumor antigen discovery system that utilizes dendritic cells to prime naïve T cells against biochemically obtained peptides and proteins from a breast tumor cell line that does not express both of the two known breast tumor-specific antigens to date.

We have isolated HLA Class I molecules from the breast tumor (MS), and acid-extracted the peptides bound to the HLA Class I molecules. These peptide extracts were then fractionated by reverse-phase HPLC, and the individual fractions were given to DCs to prime naïve $CD8^+$ T cells. Our results here show that we have been able to isolate HLA Class I molecules and acid-extract peptides with reasonable success. We are beginning specific bioassays to further analyze positive fractions in our T cell cultures. If a positive result is obtained, the positive peptide fraction will then be submitted to electrospray-ionization tandem mass spectrometry to further determine its sequence. In addition, we have recently succeeded in stable transfection of the HLA-A2.1 gene into the MS tumor, so that future peptides extracted from the MS-A2 tumor will be in the context of HLA-A2.1, which is a very common allele in the Caucasian population. If successful, the identification of a breast tumor antigenic peptide in the context of HLA-A2.1 would be beneficial to many people in the population.

We have also fractionated protein extracts via reverse-phase HPLC, and our HPLC profiles indicate the abundance of proteins in our extracts. Furthermore, we were able to garner reproducible results with our protein fractions, which is very important and useful if we had to return to the original sample to either obtain more antigen for priming, or to further fractionate that fraction. We are currently in the progress of beginning bioassays to characterize the T cells that were visually observed to proliferate in response to the protein fractions. Asides from bioassays, we will also attempt to perform secondary fractionations such as ion-exchange chromatography on some positive fractions. The identification of a T helper epitope is not an easy process, as is evidenced by the scarcity of T helper tumor-specific antigens in the literature. However, the identification of a CD4 T helper tumor-specific antigen, if successful, would be of

significant advantage and usefulness in boosting the immune system and combating breast cancer.

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APPENDICES

Technical Objective #1: To use human dendritic cells to prime in vitro naïve autologous CD8+ T cells to peptides eluted from HLA Class I molecules from the tumor cell line, MS

Tasks:

Mass generation of MS tumor cells in nude mice
($> 10^9$-10^{10} cells)

↓

Acid-extraction of peptides eluted from HLA Class I molecules

↓

Reverse-phase High Performance Liquid Chromatography fractionation of eluted peptides (2^{nd} dimension fractionations will be done if necessary)

↓

Stimulation of CD8+ T cells with dendritic cells loaded with tumor-peptide fractions

↓

Mass spectrometrical analysis of positive peptide fractions

↓

Tumor Antigen (s) Discovered

↓

Search database to determine if gene is known; if not known, then degenerate probes will be generated to isolate the gene
(Technical Objective #3)

<u>Technical Objective #2:</u> To use human dendritic cells to prime in vitro naïve, autologous CD4+ T cells to fractionated tumor lysates

Tasks:

Crude cell extract obtained after running through HLA Class I & Class II columns

↓

Initial fractionation of crude cell extract via hydrophobic interaction chromatography and/or reverse phase chromatography

↓

Stimulation of naïve CD4+ T cells with dendritic cells loaded with tumor protein fractions

↓

Biochemical fractionation of proteins stimulatory for CD4+ T cells depending on the nature of the identified fraction (i.e. hydrophilic or hydrophobic)

↓

Purified proteins involved in T cell activation will be subjected to N-terminal amino acid analysis

↓

<u>Tumor Antigen (s) Discovered</u>

↓

Search database to determine if gene is known; if not known, then degenerate probes will be generated to isolate the gene
(<u>Technical Objective #3</u>)