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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 an epithelial tumor (MS) that does not express the known breast tumor-specific antigens, MUC-1 and Her-2/neu. We have extracted peptides from the HLA Class I molecules of the tumor, fractionated the peptides by reverse-phase HPLC (RP-HPLC), and loaded onto DCs to prime naïve $CD8^+$ T cells. Our results showed that 12 of the 65 individual $CD8^+$ T cell cultures recognized the original tumor. Further analysis of the positive fractions by electrospray mass spectrometry yielded 8 peptide candidates. We are in the process of acquiring the amino acid sequences of these peptides. We have also fractionated protein extracts of the tumor by RP-HPLC and loaded onto DCs to prime naïve $CD4^+$ T cells. Our first attempt yielded an immunostimulatory fraction that contained predominantly two proteins of 17 kD and 19 kD. We have repeated this process, and our results showed 12 of the 52 individual $CD4^+$ T cell cultures recognized the original tumor. We are in the process of characterizing the identity of these positive protein fractions.

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FOREWORD

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Henry Kaw 6/28/99

PI - Signature Date

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INTRODUCTION

The prospect of successful immunotherapy against breast tumors relies on the discovery of tumor-specific antigens and their ability to stimulate immune responses in the host. Previous studies done to identify tumor-specific antigens utilized tumor cells and tumor-reactive T cells isolated from cancer patients to generate secondary responses to the tumor antigens *in vitro*. The problem with this approach was two-fold: one was that it used tumor cells, which were poor antigen presenting cells, and two was that it used T cells from cancer patients, which have been shown to be defective in cancer patients. Thus, it was difficult to generate any notable T cell responses to the tumor, thereby preventing the discovery of new 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. *We hypothesize that by using naïve T cells from healthy donors along with the newly established and powerful dendritic cell-based in vitro priming system, we could circumvent the problem of tumor-induced immunosuppression in vivo and uncover new tumor antigens that have eluded detection in previous studies.*

BODY

I. Technical Objectives

The project continues to follow the original technical objectives:

1. To use human dendritic cells to prime *in vitro* naïve, autologous $CD8^+$ T cells to peptides eluted from HLA Class I molecules of the tumor, MS
2. To use human dendritic cells to prime naïve, autologous $CD4^+$ T cells to fractionated tumor cell lysates of the tumor, MS
3. To clone the genes pertinent to the identified immunogenic tumor peptides and/or proteins

II. Studies & Results

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

In our last annual report, we demonstrated our success in eluting peptides from HLA Class I molecules of the tumor, as well as successfully fractionating the peptides on the HPLC. Here I will describe the conclusion of that first attempt (RUN I) using MS as well as the results from the second attempt (RUN II) done with MS-A2 (MS transfected with HLA-A2).

In our first run, after successfully eluting and fractionating the peptides on the HPLC, we loaded the individual peptide fractions onto dendritic cells and used them to primed autologous naïve $CD8^+$ T cells. The $CD8^+$ T cell cultures were then restimulated every 7-10 days with macrophages and peptides, and by the 3rd restimulation, we switched to using the original tumor (irradiated) as stimulators. Initially, we were concerned that

we would not see differences in the T cell cultures after priming with their individual peptide fractions, due to the enormous priming potential of the dendritic cells. However, we started observing differences among the different $CD8^+$ T cell cultures primed with different fractions. The differences among the different $CD8^+$ T cell cultures became more pronounced as more restimulations were done, and by the 7^{th} restimulation, about ~50% of the $CD8^+$ T cell cultures were dead. This suggested that we were successful in providing antigenic selection to the T cell cultures.

We repeated the same procedure using the MS-A2 tumor (MS transfected with HLA-A2) with an HLA-$A2^+$ LRP (leukocyte research product; our source of DCs and T cells) donor. Briefly, we purified the HLA Class I molecules using the protein A-sepharose –W6/32 immunoaffinity column, eluted the molecules from the column, and acid-extracted the bound peptides. We then concentrated the peptides and fractionated them using RP-HPLC. The procedure to this point has been successfully repeated, allowing us to generate class I –extracted peptides when needed. This confirms our success of *Task 1* as outlined in the *Statement of Work.* The fractionated peptides were then individually loaded onto dendritic cells and used to prime naïve, autologous $CD8^+$ T cells. The T cell cultures were then restimulated every 7-10 days using autologous macrophages and peptides until the 4^{th} week, where we started using irradiated tumor as stimulators. Similar to the previous run, we saw differences among the different T cell cultures over a period of 5-6 weeks. To assess the specificity of these T cell cultures, we tested these T cells on their ability to recognize and kill the original tumor, MS-A2. Our results showed that 12 of the 65 individual $CD8^+$ T cell cultures were able to recognize and kill the original tumor, suggesting that our priming procedure was successful in generating tumor-specific T cell lines. Interestingly, one of these CD8 T cell cultures (one primed with fraction #32 peptides) was also capable of killing an established lung tumor cell line, 201T-A2, suggesting that perhaps the antigen in fraction #32 is a shared tumor antigen. In summary, these results suggests the presence of potential tumor antigens in each of these 12 peptide fractions, of which we attempted to characterize using mass spectrometry (see Technical Objective #3). Our success in priming and generating tumor-specific T cell lines addresses *Task 2* from the *Statement of Work.*

Our emphasis to date has been on the generation of tumor-specific T cell clones using dendritic cells as our priming system. This has been worked on and addressed in *Tasks 1& 2* of the *Statement of Work* in this year and last year's annual report. Since we have been successful in priming $CD8^+$ T cells, and have identified antigenic peptide fractions, we believe our priority is to continue the pursuit of the antigen discovery process and identify new tumor antigens. Thus, we will place a hold on the priming studies in which we propose to use the tumors as antigen-presenting cells as listed in *Task 3* from the *Statement of Work.*

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

In our last annual report, we demonstrated our success in fractionating protein extracts via RP-HPLC. Here I will describe the conclusion of that first attempt (RUN I) using MS as well as the results from the second attempt (RUN II).

In our first run, after successfully fractionating the protein extracts by HPLC, we loaded the individual protein fractions onto dendritic cells and used them to primed autologous naïve $CD4^+$ T cells. By the 4^{th} restimulation, we were able to see differences among the individual $CD4^+$ T cell cultures. Similar to the $CD8^+$ T cell cultures, the differences among the $CD4^+$ T cell cultures became more pronounced as more restimulations were done. By the 9^{th} restimulation, most of the $CD4^+$ T cell cultures died because of mycoplasma contamination, except for the $CD4^+$ T cell cultures that were primed with protein fraction #2 and fraction #44. The $CD4^+$ T cells primed with protein fraction #2 died a few weeks later, so we focused our attention to the $CD4^+$ T cells primed with protein fraction #44, which were still alive after 16 restimulations.

To further analyze the content and immunostimulatory capacity of protein fraction #44, we subfractionated #44 using a shallow gradient of acetonitrile on a C4 column and collected 10 sub-fractions (#44.1-#44.10). Silver staining of the sub-fractions showed 7 visible bands in fraction #44.6 (predominantly ~17 and 19 kD, with faint bands at ~27, 29, 34, 26, and 51 kD), correlating with immunostimulatory activity as detected in a proliferation assay using fraction #44-primed T cells. We are in the process of further separating these protein bands and correlating with immunostimulatory activity. Our eventual goal is to identify and sequence the protein(s).

For the next run, we repeated the same procedure as above with a different donor LRP. The fractionated protein extracts were then individually loaded onto dendritic cells and used to prime naïve, autologous $CD4^+$ T cells. The T cell cultures were then restimulated every 10-12 days using autologous macrophages and protein fractions. Similar to the previous run, we saw differences among the different T cell cultures over a period of 5-6 weeks. To assess the specificity of these T cell cultures, we tested these T cells on their ability to recognize the original tumor in a proliferation assay. Our results showed that 12 of the 52 individual $CD4^+$ T cell cultures were able to recognize and proliferate in response to the original tumor, suggesting that we were successful in generating tumor-specific $CD4^+$ T cell lines. Further characterization of the T cell lines showed that ~ 93 – 96 % of the cells were CD4 positive, indicating that we were indeed maintaining CD4 T cell cultures. In contrast, the T cell cultures that did not recognize the original tumor consisted of only 50-70% $CD4^+$ T cells. In summary, these results suggest the presence of potential protein tumor antigens in each of these 12 protein fractions. We are currently in the process of characterizing these protein fractions. Our success in priming and generating tumor-specific T cell lines addresses *Task 4* of the *Statement of Work.*

Similar to the priming of $CD8^+$ T cells, our emphasis to date has been on the generation of $CD4^+$ tumor-specific T cell clones using dendritic cells as our priming system. This has been worked on and addressed in *Tasks 4* of the *Statement of Work* in this year and last year's annual report. Since we have been successful in priming $CD4^+$ T cells, and have identified antigenic protein fractions, we believe our priority should be to continue the pursuit of the antigen discovery process and identify new tumor antigens. Thus, we will place a hold on the priming studies in which we propose to use the tumors as antigen-presenting cells as listed in *Task 5* from the *Statement of Work.*

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

In our last annual report, we did not address this technical objective because we had to establish the in vitro priming system first before progressing to this part of the project. Here I will describe our attempts to characterize the peptides identified in Technical Objective #1 and deduce its amino acid sequences.

After identifying the 12 positive peptide fractions (as defined by T cell reactivity to the original tumor), we decided to analyze the samples via electrospray ionization mass spectrometry. Since the peptides were extracted from the cleft of HLA class I molecules, we expected peptides of 8-12 amino acids, corresponding to a m/z ratio of between 700-1300 Daltons. In addition, another criteria we used in identifying peptides for sequencing was based on the amount of the peptide available in the sample, which had to be enough for sequencing (at least 1×10^5 ion current). Based on the above two criteria, we identified 8 peptide species from the 12 positive fractions. The fact that we were able to detect peptides in our mass spectrometric profiles was further confirmation that our acid-extraction procedure of peptides from HLA Class I molecules was successful. The next step was to determine the sequence of these peptides.

Our attempts to sequence the peptides by tandem mass spectrometry have been moderately successful, since the amount of peptides we had was on the low end of the mass spectrometer detection level. We are excited about obtaining a peptide sequence for m/z 717 of fraction #30. The sequence identified was too short (7-mer) for adequate protein database search. We have synthesized the peptides, and now we are in the process of confirming whether this peptide is our tumor antigen by seeing whether the fraction #30-primed $CD8^+$ T cells recognizes the peptide or not. In addition, we are in the process of sequencing the remaining peptides by tandem mass spectrometry. The work described here in part, addresses *Task 7* from the *Statement of Work*.

III. Address to the Statement of Work

The work presented here in this annual report has followed the Statement of Work as listed in the DOD grant. We have attempted Tasks 1, 2, and 4 with reasonable success, as well as repeated the above tasks using different LRP donors. We are confident of our in vitro priming system, and we have started addressing Task 7 of the last technical objective of this project.

APPENDICES

1. Key Research Accomplishments:

- Acid-extraction of peptides bound to HLA Class I molecules
- Reversed-phase separation of peptides by HPLC
- Successful priming of $CD8^+$ T cells using dendritic cell-loaded peptides
- Identification of 12 primed-$CD8^+$ T cell cultures capable of recognizing the original tumor
- Mass spectrometric analysis of peptides
- Reversed-phase separation of protein extracts by HPLC
- Successful priming of $CD4^+$ T cells using dendritic cell-loaded proteins
- Identification of 12 primed-$CD4^+$ T cell cultures capable of recognizing the original tumor

2. List of Reportable Outcomes:

- Oral Presentation at the UPCI (University of Pittsburgh Cancer Institute) noon seminar, Sept. 10, 1998, titled "Dendritic cell-based tumor antigen discovery system: Work in Progress"
- Poster presentation at Experimental Biology '99, April 17-21, in Washington, D.C., titled "Priming $CD4^+$ and $CD8^+$ T cells against epithelial proteins and peptides using a dendritic cell-based tumor antigen discovery system"

ABSTRACT FOR EXPERIMENTAL BIOLOGY '99:

PRIMING $CD4^+$ AND $CD8^+$ T CELLS AGAINST EPITHELIAL PROTEINS AND PEPTIDES USING A DENDRITIC CELL-BASED TUMOR ANTIGEN DISCOVERY SYSTEM

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The prospect of successful immunotherapy against tumors relies on the discovery of new tumor-specific antigens capable of stimulating immune responses in the host. We have tested 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 an epithelial tumor cell line (MS) that does not express the other known epithelial tumor antigens, MUC-1 and Her-2/neu. We isolated HLA Class I molecules from the tumor, and acid-extracted the bound peptides. The peptides were then fractionated by reverse-phase HPLC, and individual fractions were collected and loaded onto DCs to prime naïve $CD8^+$ T cells. Our preliminary results show that we have been able to prime $CD8^+$ T cells to specific peptide fractions. We have also fractionated protein extracts from the tumor using reverse-phase HPLC, and loaded the individual fractions onto dendritic cells to prime naïve $CD4^+$ T cells. We have identified an immunostimulatory protein fraction containing predominantly two proteins of 17 kD and 19 kD. Our results so far validate the usefulness of this new dendritic cell-based tumor antigen discovery system. (Supported by DOD grant DAMD 17-9-1-7057 to H.K. and NIH grant 1PO1CA 73743 to O.J.F.)