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13. ABSTRACT ***(Maximum 200 Words)***

The major research focus of this grant is to develop effective treatments for patients with low volume metastatic disease using dendritic cell (DC)-based vaccines loaded with tumor RNA. Toward this goal, during the second year of this grant we have made the following progress: 1) Improved efficiency of RNA transfection into tumor cells using electroporation. 2) Improve methods to isolate and amplify tumor RNA from a small number of tumor cells. 3). Develop methods for isolating breast tumor cells from the blood or bone marrow of breast cancer patients. We have continued to encounter difficulties in identifying patients with sufficient tumor cells to initiate the clinical trial with non-amplified RNA (Specific aim #2). Fortunately, development of the RNA amplification methodology has progressed faster than we anticipated in the grant application and it now appears that we will be poised to carry out the trial using amplified RNA (Specific aim #4), in year 3 rather than year #4 and complete the trial including analysis during the life of this grant. We are very pleased of the prospect of exceeding our time line as shown in the Statement of Work

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INTRODUCTION.

The major research focus of this application is to develop effective treatments for patients with low volume metastatic disease using autologous dendritic cell-based tumor vaccines. The proposed studies are based on our recent discovery that RNA pulsed DC of murine and human origin are highly effective stimulators of T cells and tumor immunity. A key advantage of using tumor RNA as source of antigen is that sufficient antigen can be generated by RNA amplification techniques from small tumor specimens. Ongoing phase I clinical studies with carcinoembryonic (CEA) peptide and CEA RNA transfected DC have demonstrated (so far) the safety of this treatment. Furthermore, preliminary analysis of patients treated with CEA RNA transfected DC show induction of CEA-specific T cell responses in the vaccinated patients. The central hypothesis of this proposal is that vaccination with tumor RNA transfected DC against a broad repertoire of tumor antigens expressed in patients with breast cancer will constitute an effective therapy for metastatic breast cancer.

Therefore, the primary objective of this research proposal is to develop optimal methods for using DC pulsed with tumor RNA as a broadly applicable treatment for patients with metastatic breast cancer. The specific objectives of the proposed study are:

1. To optimize antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with RNA isolated directly from tumor cells.

3. To develop methods to isolate, amplify, and enrich for biologically active mRNA from breast cancer tissue.

4. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with amplified tumor RNA.

BODY OF PROGRESS REPORT

According to the Statement of work the plans for Year 2 are:

1. Optimization of antigen presentation by RNA transfected DC- Enhancing RNA uptake by DC.

2. Develop methods of isolating breast tumor cells from small tumor specimen including developing microdissection techniques to isolate tumor cells from pathology slides.

3. Start phase I clinical trial using directly isolated tumor RNA; Treat 4 patients.

4. Analyze T cell responses in the vaccinated patients using cytotoxicity assay, ELISPOT and FASTIMMUNE assay.

1. Optimization of antigen presentation by RNA transfected DC- Enhancing RNA uptake by DC

As described in the previous progress report, we have shown that treatment of the RNA transfected DC with TNF-alpha or CD40L, two reagents known to cause DC maturation, led to enhanced DC function as measured by CTL induction (1). We have continued to fine tune the conditions with emphasis on CD40L because this reagent may be available from Immunex for clinical use. Discussions are in progress.

We have also begun to explore the use of electroporation to enhance RNA uptake by DC, based on a preliminary report presented in the 1st Dendritic Cell Clinical Trials meeting in Zurich in February 2000. Preliminary data are quite encouraging as it seems that electroporation, in comparison to the current protocol which involves a simple incubation of the "naked" RNA with DC, is significantly more efficient as measured by GFP expression from GFP RNA electroporated DC.

Plans for the next year

1. To determine whether the RNA electroporated DC serve as superior stimulators of CTL responses in vitro, especially when RNA from breast cancer patients is used, namely to compare the in vitro induction of breast tumor CTL using breast tumor electroporated DC compared to the current protocol.

2. Develop methods of isolating breast tumor cells from small tumor specimen including developing microdissection techniques to isolate tumor cells from pathology slides.

As discussed in the previous progress report, we are significantly ahead of our plans: we have obtained preliminary indications that we can not only generate cDNA libraries-the stated objective of year #1-but also microdissect and amplify the cDNA and recover the "amplified"

RNA template (2). In the course of this year we have continued to develop the amplification protocol.

We readily identified one problem. The current amplification protocol employs a cap switch oligo to ensure the generation of full-length cDNAs and to provide a 5' primer sequence for amplification. As shown in Figure 1, in the current protocol a significant portion of spurious DNA is generated, most likely due to internal priming by the cap switch oligo on the RNA template. We have designed a new cap switch oligo with a blocked 3' end which has apparently solved this problem.

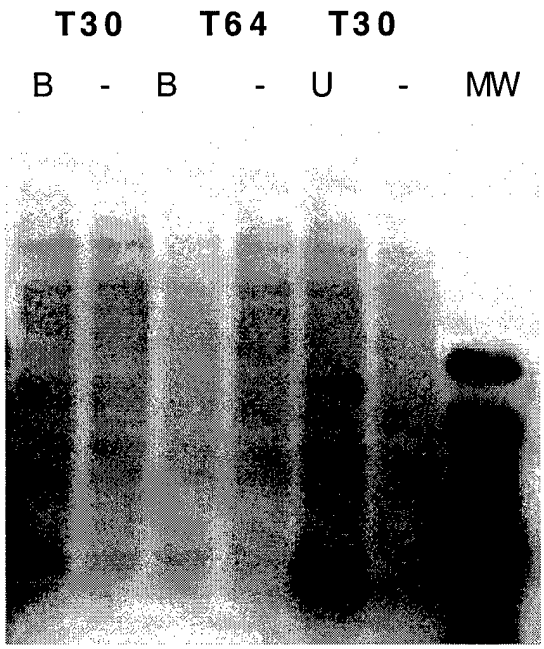

Figure 1: Evidence for internal priming by the cap switch oligo. SW403 RNA (SW403 is a colorectal tumor cell line) was placed in an RT reaction with 32P-dCTP: and the indicated RT primers (T30-30 Ts; T64-64 Ts) and either the unblocked (U), blocked (B) or no (-) cap switch oligo. The presence of additional bands in the presence of unblocked, but not blocked cap switch oligo are clear evidence of internal priming by the unblocked oligo.

Figure 2 shows the result of an amplification reaction using tumor RNA isolated from a breast cancer patient using either the blocked or unblocked oligo.

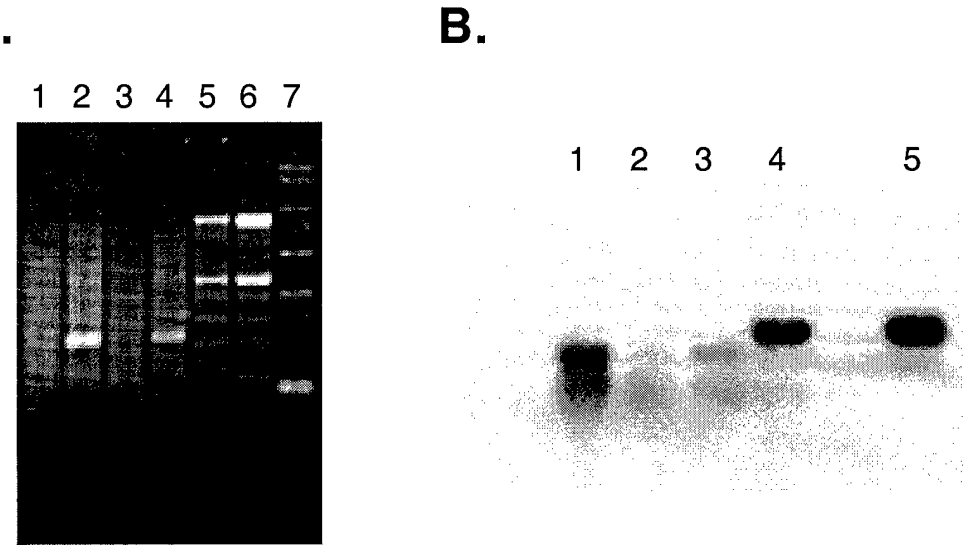

Figure 2: Amplification of RNA from tumor cells isolated from a breast cancer patient (BL). A. Ethidium bromide staining. **B**. Blotting with an actin probe. Lanes1and 2-using the T30 RT primer and blocked (lane 1) or unblocked (lane 2) cap switch oligo. Lanes 3 and 4 using the T64 RT primer and blocked (lane3) and unblocked cap switch primer (lane 4). Lanes 5 and 6, total RNA from patient BL and colorectal cell line SW403, respectively. Lane 7, MW markers.

We are also developing improved methods to directly amplify RNA from microscopic amounts of tumor tissue circumventing the need to first isolate RNA. Two methods, using either heating in the presence of RNase inhibitors or freeze-thawing, are illustrated in Figure 3.

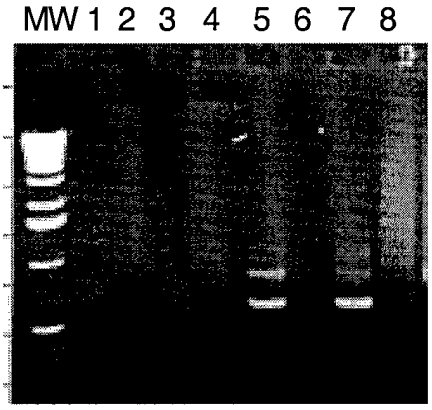

Figure 3: Direct amplification of RNA from tumor cells. 2000 F10.9-3.1 or F10.9-OVA cells, were lysed by heating to 75 oC in the presence of SUPERasin (Ambion), a broad spectrum heat-resistant RNase inhibitor, or by freeze-thawing in the presence of placental ribonuclease inhibitor. 250 cell equivalents were put into an RT reaction +/- the addition of reverse transcriptase. The cDNA was amplified for 30 cycles and 1/10 of the PCR products were

analyzed. Lanes 1-MW markers; lanes 2 to 5-SUPERasin protocol; lanes 6 to 9 freeze-thaw protocol; lanes 2,3,6,7-F10.9-3.1 cells; lanes 4,5,8,9-F10.9-OVA cells; lanes 2,4,6,8-with RT; lanes 3,5,7,9-without RT.

We have not yet focused on the development of laser capture microdissection techniques. The reason is two-fold. First, another laboratory in the department headed by Dr. Johannes Vieweg, has developed this technique for isolation of tumor cells from needle biopsies obtained from patients with prostate cancer and we have access to the technology when needed. Second, we are considering the possibility of isolating tumor cells from the breast cancer patient's blood (see below) which will obviate the need for microdissection.

Plans for next year:

1. Continue optimize amplification protocol-demonstrating the functionality of the amplified RNA in CTL assays and optimizing the faithfulness of the protocol, namely the generation of full length transcripts..

2. Carry out functional assay with amplified breast tumor RNA-in vitro induction of tumor specific CTL.

3. Start phase I clinical trial using directly isolated tumor RNA; Treat 4 patients.

This year we spent considerable time and effort attempting to find patients that would meet our entry criteria. We meet weekly to discuss patients, and asked Dr. Mike Morse and Dr. Jim Vredenberg to work with us, without compensation, to identify patients who were potential candidates. Both Drs. Morse and Vredenberg are active clinical oncologist with special interests in immunotherapy. Our research nurse, Shubi Khan would answer calls from patients and from referring physician, and she sent a large amount of material to potential candidates. We also established a Web site within the cancer center Web site to allow interested subjects access to our clinical activities during this period. In addition, we had to maintain the IRB and FDA files for this protocol and for the manufacturing of the autologous dendritic cell product.

Despite these concerted efforts, we were not able to find any patients who meet our entry criteria for entry into this study. Therefore, we did not treat any patients. This year Duke University underwent review for possible violations in the institutional IRB policy and IRB documentation. During this review, the clinical research activities of Duke investigators were suspended. In addition, the Duke IRB requested an audit and re-review of the hundreds to thousands of open protocols at Duke. This created a tremendous backlog of administrative work, and a backlog of protocols awaited review. Our protocol was reviewed and ultimately approved. Due to this review, we could not enroll patients for about 6 months.

Scientifically, we were not able to find many patients with metastatic breast cancer who were undergoing a medically needed surgical excision for removal of their tumor, and for the preparation of an autologous tumor RNA loaded dendritic cell vaccine. We had no allocated

funds to support the professional and hospital fees required to perform elective surgery on patients with metastatic breast cancer for tumor removal solely for the purpose of isolating tumor RNA for the cancer vaccines. In addition, we had not modified our clinical protocol to allow for elective surgery solely for tumor harvesting and tumor RNA extraction.

Since the number of patients who were likely to meet our eligibility criteria seems to be smaller than we had anticipated, we sought alternatives. We were very fortunate to have attracted Ms. Mimi Phan, a Duke medical student, to work in our lab for the year. Ms. Phan's research project was designed to provide and alternative source of tumor cells for research studies and for the extraction of tumor RNA. The cells would be isolated from peripheral blood, or from bone marrow of patients. Because it would be possible to sample peripheral blood or bone marrow for patients, the possible use of the circulating or bone marrow associated malignant cells would make a much larger population of breast cancer patients eligible for these studies.

Therefore, Ms. Phan attempted to isolate tumor cells from the circulation of breast cancer patients. Because the numbers of cells in the circulation would be low, we entered into a research collaboration with Dr. Amy of Nexell Therapeutics (see previous progress report). Dr. Amy has considerable expertise in immunoisolation of malignant cells from peripheral blood and bone marrow using specific antibodies, and immunomagnetic beads. Ms. Phan was successful in isolating tumor cells from bone marrow aspirates of patients with metastatic breast cancer, and these results will be presented at the San Antonio Breast Cancer Meeting this year.

Despite the ability to isolate breast cancer cells from blood or bone marrow, the total numbers of cells that we could isolate was small. Therefore in addition to Ms. Phan's work-and based on the rapid progress of amplifying tumor RNA as described above-we have prepared an amendment to our existing IND application that would allow us to use mRNA that was amplified from the small numbers of cells that could be obtained. This IND amendment was discussed by teleconference with the FDA who requested more pre-clinical data attesting to the stringency and reliability of the amplification process. Specifically, they asked for evidence that specific messages for know genes, such as CEA and other genes, could be amplified, and maintain the relative abundance in the amplified product compared to the non-amplified product. In addition, the FDA requested data that this amplification with high fidelity could be repeated 5 times with similar data. We are currently awaiting the laboratory results that will allow us to satisfy the comments from the FDA and hope that this new data will allow them to grant approval for our request to use amplified mRNA.

In addition, we have written and received approval for a companion study to isolate tumor cells from blood and bone marrow of patients with operable breast cancer. This trial will allow us the opportunity to immunoisolate tumor cells from patients at high risk of recurrence, which may be a better population of breast cancer patients to immunize.

Plans for next year:

1. Complete the IND for using amplified RNA transfected DC in breast cancer patients.

2. Apply the amplification methodology for isolating and amplifying RNA from circulating tumor cells from blood and/or bone marrow

3. Initiate a phase I clinical trial with amplified tumor RNA transfected DC in patients with breast cancer. This corresponds Specific Aim #4 of this grant application and was slated to start in year 4 of this grant (see Statement of Work).

KEY RESEARCH ACCOMPLISHMENTS

- Improve efficiency of RNA transfection into tumor cells using electroporation.
- Progress in developing methods to isolate and amplify tumor RNA from a small number of tumor cells.
- Develop methods for isolating breast tumor cells from the blood or bone marrow of breast cancer patients.

REPORTABLE OUTCOMES

N/A

CONCLUSIONS

1. We have developed a greatly improved method for loading DC with RNA encoded antigen which consist of a) using electroporation to enhance RNA uptake by DC and b) mature DC in the presence of TNF-alpha or CD40L. These improvements will be incorporated into the design of the clinical studies.

2. We have improved the methodology of RNA amplification-specifically we have reduced a background "noise" generated by non-specific priming-yielding higher quality amplified RNA (pending functional demonstration). While this methodology will suffice for the planned clinical studies, additional optimizations of the amplification protocols-along the lines discussed in the grant proposal-will continue.

3. We continue to encounter difficulties in identifying patients with sufficient tumor cells to initiate the clinical trial with non-amplified RNA (Specific aim #2). Fortunately, development of the RNA amplification methodology has progressed faster than we anticipated in the grant application (see this and previous progress report) and it now appears that we will be poised to carry out the trial using amplified RNA (Specific aim #4), in year 3 rather than year #4 and complete the trial including analysis during the life of this grant. We are very pleased of the prospect of exceeding our time line as shown in the Statement of Work.

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1. Morse, M.A., Zhou, L-J., Tedder, T.F., Lyerly, K. & Smith, C. Generation of dendritic cells *in vitro* from peripheral blood mononuclear cells with GM-CSF, IL-4, and TNF-α for use in cancer immunotherapy. *Annals of Surgery* **226**, 6-16 (1997).

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APPENDICES- NONE