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Award Number DAMD17-98-1-8494

TITLE: Identification of Prostate Specific Antigens for  
Immunotherapy Vaccines

PRINCIPAL INVESTIGATOR: Timothy L. Ratliff, Ph.D.

CONTRACTING ORGANIZATION: University of Iowa  
Iowa City, Iowa 52242

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Fort Detrick, Maryland 21702-5012

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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Iowa Iowa City, Iowa 52242  E-Mail: <a href="mailto:tim-ratliff@uiowa.edu">tim-ratliff@uiowa.edu</a>			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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<b>13. ABSTRACT (Maximum 200 Words)</b> The activation of immune T lymphocytes that recognize and kill prostate cancer cells is an area of intense investigation. Conceptually, the activation of such cells could lead to elimination of metastatic disease. However, in practice the ability to activate appropriately targeted T lymphocytes and sustain the activation state of the T cells in a manner sufficient to eliminate tumors has been problematic. One important issue is the identification of appropriate antigens for targeting the immune response. Studies have been initiated to identify unique prostate antigens using A2 transgenic mice. Initial immunization studies using dendritic cells pulsed with RNA from prostate tumor cell lines shows the utility of the approach. A molecular subtraction technique is underdevelopment that will provide enrichment of prostate antigens prior to immunization. Once T cell lines are isolated, they will be used to identify the antigen.				
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## Introduction

The activation of immune T lymphocytes that recognize and kill prostate cancer cells is an area of intense investigation. Conceptually, the activation of such cells could lead to elimination of metastatic disease. However, in practice the ability to activate appropriately targeted T lymphocytes and sustain the activation state of the T cells in a manner sufficient to eliminate tumors has been problematic. One important issue is the identification of appropriate antigens for targeting the immune response. In this proposal we outline studies aimed at identifying immunodominant antigens with prostate specificity.

## Body of Progress Report

Our effort to identify immunodominant antigens with prostate specificity are proceeding rapidly, but changes in strategies have been initiated because of new scientific developments and our preliminary observations. The modifications to the experimental approach and the reasons for the change are described below. Once validated, we believe that the new approach will provide a basis for efficient identification of antigens from all tissue types including primary prostate cancer from clinical specimens. We outline below the approved tasks and our progress toward accomplishing each.

**Task 1.** To effectively immunize major histocompatibility antigen (MHC) A2 transgenic mice against prostate cancer cells.

The procedure for effective immunization has been accomplished. Our initial objective was to use transgenic mice expressing the human MHC haplotype, A2.1 to identify immunodominant antigens. Since A2.1 is expressed in genetically modified mice and mouse T cells respond to the A2.1 peptide antigen complex, no binding of the auxiliary signaling receptor, CD8, is possible. Our original working hypothesis was that the absence of CD8 signaling would be beneficial, since only strong signals would be recognized by mouse T lymphocytes.<sup>1</sup> Our immunization with LNCaP cells revealed that the A2 transgenic mice did not respond adequately after immunization. We determined that the T cell receptor signal in the absence of CD8 signaling was insufficient for activation of an appropriate spectrum of antigen specific T lymphocytes. Only multiple amplifications in vitro lead to cytotoxic T lymphocyte (CTL) activity and the activity level was minimal. In order to overcome this problem, we obtained another transgenic mouse line that expresses a modified A2.1 protein. The new A2.1 protein has the alpha 3 domain from mice, which allows murine CD8 binding and signaling but does not change the specificity of the peptide:MHC interaction.<sup>2,3</sup> This mouse line has provided the necessary sensitivity for identification of reactive T lymphocytes ( see below).

**Task 1a.** Identify prostate cancer xenografts that express A2 MHC but that share few or no MHC at the B and C loci. (months 1-12)

We have tested MHC expression on prostate cell lines and xenografts available to us as well as other pertinent cell lines. Table 1 below shows the results. These data show that LNCaP and PC3 express A2. Only one xenograft, LUCaP-35, was observed to express A2. We have obtained LUCaP-35 from Dr. Robert Vessella at the University of Washington (**MTA, Appendix 1**). These cells will be used to test reactive T cells identified by LNCaP immunization and at a later date for the identification of unique antigens. We are in the process of transfecting each prostate cell line and cell lines of non-prostate origin with A2 in order to have reagents available to validate the specificity of T cells generated by our immunization process.

Table 1. Cell Lines Expressing MHC Class 1 Allele, A2

Xenograft or Cell Line	A2 Expression
JY	+
Jurkat	+
T24	-
253J	-
DU145	-
PC3	+
LNCaP	+
ALVA-31	-
LUCaP-35	+

**Task 1b.** Identify from among the A2 MHC-expressing prostate cancer xenografts those that also express prostate specific antigen, prostate specific membrane antigen, and human kallikrein 2 (months 1-12)

This task will not be necessary because of the new strategy that will be used to identify novel antigens (see conclusion section below). By using dendritic cells as antigen presenting cells, we can protein pulse dendritic cells and use them as targets for isolated CTL. This is a more efficient approach.

**Task 1c.** Verify that the prostate cancer xenografts are capable of immunizing A2 transgenic mice (months 2-13)

This task has been delayed and will be pursued after completion of the identification of antigens from LNCaP immunization.

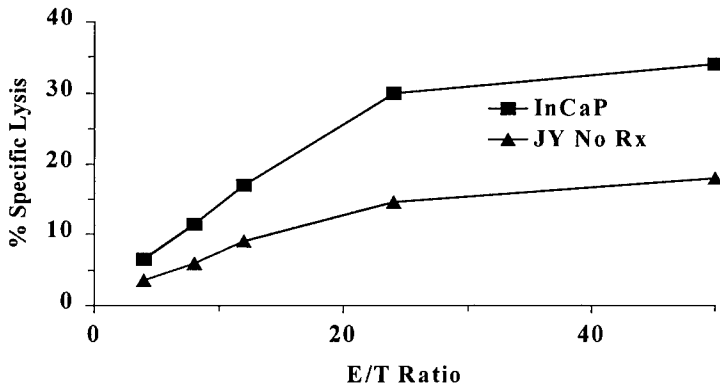
**Task 1d.** Immunize A2 MHC transgenic mice for the isolation of MHC A2 restricted cytotoxic T lymphocytes (months 3-18)

Immunization of A2.1K<sup>b</sup> mice has been accomplished. As indicated above, immunization of A2.1 mice was not successful, but with the addition of the murine CD8 binding domain to the A2.1 molecule, the anticipated immunization profile is obtained. Immunization of A2.1K<sup>b</sup> mice with either a dendritic cell/LNCaP conjugate as described by Celluzzi and Falò<sup>4</sup> or LNCaP cells alone was tested for the ability to activate CTL (Figure 1). 7-10 days later spleen cells were isolated and co-cultured with LNCaP cells in vitro in a standard CTL stimulation assay.<sup>5</sup> Four days after initiation of culture viable lymphocytes were isolated by discontinuous density centrifugation and tested for lytic activity against LNCaP cells and a control non-prostate A2-expressing line JY. The data show that immunization with LNCaP cells was not effective in inducing CTL activity (data not shown). In contrast, the LNCaP/dendritic cell conjugate effectively immunized the A2.1K<sup>b</sup> mice. These data showed that dendritic cells were strong priming agents as had been previously described in other model systems.<sup>4</sup> Evaluation of the reactivity of the activated CTL showed that the predominant antigen was allogenic MHC. Thus we have further modified the protocol to exclude whole cells and are using mRNA from tumor cells. To validate the system, we immunized mice with an adenovirus carrying the gene for ovalbumin, a strong inducer of CTL in C57BL/6 mice. In this study spleen cells were co-cultured with dendritic cells pulsed with antigen. The data show that dendritic cell co-culture with primed spleen cells was an effective means of stimulating the expansion of ovalbumin specific CTL (Figure 2). In the next experiment A2.1K<sup>b</sup> mice

were immunized with an adenovirus carrying the gene for PSA (Figure 3). In these experiments the in vitro co-culture was with cells infected with pox virus carrying PSA as described for other models.<sup>5</sup> The pox virus was used so that only PSA specific antigen recognition was possible. Since adenovirus carrying the PSA gene was used for the priming event, it could not be used to expand the CTL in vitro because of the presence of virus specific CTL. While the background lysis is higher than desired, the data show significant PSA-specific lytic activity.

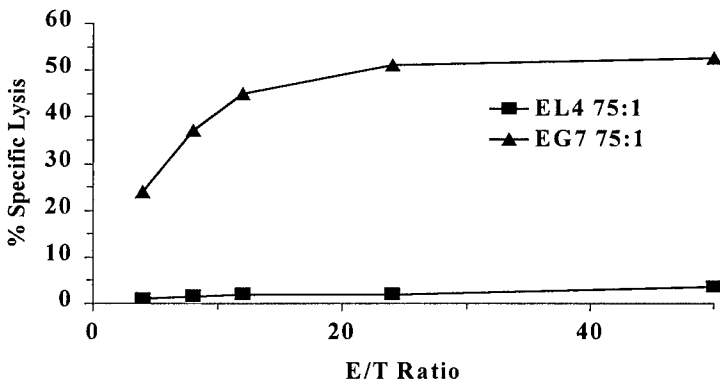
**Figure Legends**

**Day 4 CTL on 2/5/99 (LNCaP/D.C.)**



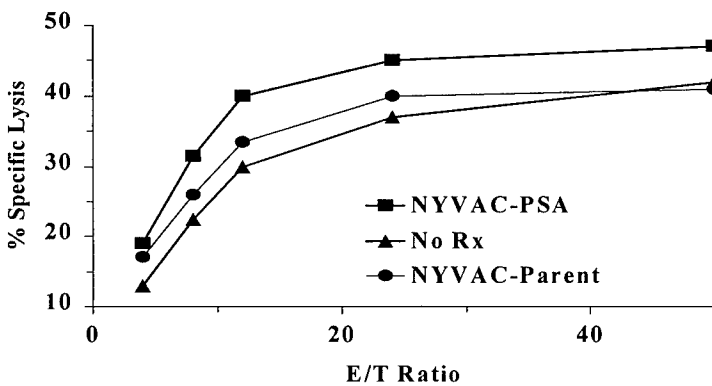
**Figure 1.** Activation of CTL activity using dendritic cell/LNCaP conjugates as priming agents. Immunization with LNCaP alone did not induce lytic activity.

**Day 5 CTL on 8/10/99 75:1**



**Figure 2.** Evaluation of the most effective ratio of dendritic cells for co-culture conditions in secondary in vitro CTL expansion assays. Show is the 75:1 (responder to dendritic cell) ratio. Others tested but not shown are 10:1 and 25:1.

**N-PSA vs. No Rx**



**Figure 3.** Utility of dendritic cells as priming agents for PSA in A2.1K<sup>b</sup> mice. In this study, dendritic cells expressing PSA delivered by a pox virus (NYVAC-PSA) were used in the secondary expansion culture at a 75:1 ratio.

These data show that the use of dendritic cells for the in vivo priming of mice and also for the purpose of expanding CTL in vitro is the preferred method for activating CTL.

**Task 2.** To identify prostate specific MHC A2 restricted cytotoxic T lymphocyte clones

- in vitro expansion and cloning of A2 restricted cytotoxic T lymphocytes (months 3-12)
- characterization of the specificity of the cloned cytotoxic T lymphocyte cell lines (months 6-18)

These tasks have been delayed but should be completed in year two

**Task 3.** To identify and purify MHC A2 binding peptides recognized by the prostate specific cytotoxic T lymphocytes.

- prepare MHC A2 expressing prostate cancers for extraction of A2 binding peptides (months 1-30)
- extract and concentrate MHC A2 binding peptides (months 12-30)
- separate MHC A2 binding peptides by high pressure liquid chromatography and test peptide fractions for recognition by the prostate specific cytotoxic T lymphocytes (months 12-30)
- purify and sequence active peptides by tandem mass spectrometry
- synthesize a panel of potentially active peptides to confirm appropriate amino acid sequence. (months 18-30)
- gene bank search for identification of proteins containing the identified sequence (months 24-30)

The work outlined in Task 3 will begin in year two.

**Task 4.** Biological characterization of the identified peptides.

- verification of the activation of peptide responsive T lymphocytes in MHC A2 transgenic mice (months 24-30)
- testing of the ability of lymphocytes from prostate cancer patients to respond to the identified peptide (months 24-30)
- testing of the ability of cytotoxic T lymphocytes responding to the identified peptide to mediate antitumor activity.(months 24-30)

The work described in task 4 will begin in year three.

### **Key Research Accomplishments**

- Identification of an appropriate immunization method that will allow use of primary prostate tissue for immunization
- Determination that A2.1K<sup>b</sup> mice respond to prostate antigens.

**Reportable Outcomes:** none to date



## Conclusions

These results show that A2.1K<sup>b</sup> mice can be immunized against prostate specific antigens, which provides the basis for the identification of novel immunodominant proteins from prostate. In order to circumvent the need for extensive cloning of CTL in vitro and the extensive testing required to eliminate clones cross reactivity with other non-prostate cells, we will use a molecular subtraction approach to identify antigens capable of activating prostate specific CTL. This approach will use the Clontech PCR Select cDNA subtraction kit and the Clontech Smart PCR cDNA synthesis kit. The PCR Select system will allow us to enrich for messenger RNA (mRNA) sequences specific for the prostate. However, since the sequences consist of varying portions of each mRNA sequences, we need a mechanism to identify prostate specific full length mRNA sequences. To accomplish this task the sequences from the PCR Select subtraction will be used to hybridize to full length sequences contained in the prostate library developed with the SMART PCR system. The resulting sequences enriched for prostate antigens will be cloned into either a retroviral system or Bluescript. If the retrovirus system is used, the viruses will be used to transduce dendritic cells for immunization. If Bluescript is used, mRNA will be synthesized from Bluescript and used to pulse dendritic cells. At this time we favor the retrovirus system because of the efficiency of gene transfer. Once the CTL are expanded, they will be screened as described in the original proposal. The advantage of the molecular approach described is two fold. First, it allows the subtraction of unwanted sequences (those that are cross reactive with other cell types) prior to immunization, which will increase the frequency of prostate specific CTL after immunization. Secondly, the SMART system can be used on primary tumor or normal prostate taken at surgery. Minimal RNA is needed to develop the library.

## References

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## Appendices

1. Material Transfer Agreement from Dr. Robert Vessella at the University of Washington

# Appendices



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February 18, 1999

Jim Leaven  
The University of Iowa  
Office of the Vice President for Research  
Division of Sponsored Programs  
2 Gilmore Hall  
Iowa City, Iowa 52242-1320

Dear Mr. Leaven:

Enclosed please find one original of a materials transfer agreement covering the transfer of xenograft LuCaP 35 from Dr. Robert Vessella to Dr. Ratliff. This agreement has been signed by both the University of Washington and the University of Iowa. If you have any questions, please give me a call. Thank you.

Sincerely,

Ariadna A. Santander  
Program Operations Coordinator  
telephone 206-616-2011  
fax 206-685-9452  
e mail: [filimus@u.washington.edu](mailto:filimus@u.washington.edu)

AS:jc  
Enclosures

Cc: Robert L. Vessella

THE UNIVERSITY OF IOWA



February 16, 1999

recl'd 2/17/99

VIA FEDERAL EXPRESS

Ms Aridna A. Santander  
Program Operations Coordinator  
Office of Technology Transfer  
University of Washington  
1107 NE 45<sup>th</sup> St., Suite 200  
Seattle, WA 89105

Re: Material Transfer Agreement for Xenograft, Dr. Timothy Ratliff, Receiving Scientist

Dear Ms. Santander:

I have enclosed two fully executed originals of the above-referenced agreement, which have been signed by Dr. Timothy Ratliff and by an authorized representative of the University of Iowa. You will note that I have made one change in the agreement to limit our liability in the unlikely event of gross negligence or willful misconduct on the part of the University of Washington. If this change is agreeable, please have the appropriate person initial one copy of the agreement and return it to me at the below address.

On behalf of Dr. Ratliff and the University, I want to thank the University of Washington for its graciousness in sharing this material for the advancement of research.

If you have any questions, please call me at (319) 335-2428. Thank you for your assistance.

Sincerely,

  
Jim Leaven

Enclosure

cc: Dr. Timothy Ratliff & Dr. Robert L. Vessella (w/attachments)

**XENOGRAFT RESEARCH AND COLLABORATIVE AGREEMENT  
BETWEEN RESEARCH INSTITUTIONS AND  
THE DEPARTMENT OF UROLOGY - UNIVERSITY OF WASHINGTON**

You hereby agree to the following conditions and understandings with regards to the receipt of XENOGRAFT(S) from the Department of Urology, University of Washington Medical Center.

1. The University of Washington (hereinafter the UNIVERSITY) will provide to your institution (hereinafter INSTITUTION) the xenograft(s) LvCap 35, \_\_\_\_\_, \_\_\_\_\_, and \_\_\_\_\_ for research purposes only, as a service to the research community. The xenografts are available to you on a nonexclusive basis.
2. None of the xenografts provided nor their biological constituents (e.g. cells, genetic material, secreted products) may be used for any commercial development directly or indirectly unless a license granting the same is executed between UNIVERSITY and INSTITUTION through the UNIVERSITY'S Office of Technology Transfer.
3. You agree that the xenografts or their biological constituents will not be distributed to any third party for any reason without prior written consent from the UNIVERSITY.
4. You agree that any publication arising from research performed with the xenografts will acknowledge their receipt from the Department of Urology, University of Washington, and a reprint will be send to Robert Vessella, Ph.D. When studies are a collaborative nature, co-authorship on the manuscript/abstract may also be appropriate.
5. You agree that under no circumstances will the name of the xenograft be altered in any presentation, verbal or written.
6. YOU AGREE THAT THE XENOGRAFTS ARE PROVIDED WITHOUT WARRANTY OR MERCHANTABILITY OF FITNESS FOR A PARTICULAR PURPOSE OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. ALTHOUGH THESE HUMAN TUMOR XENOGRAFTS HAVE BEEN SUBJECTED TO TESTS AND OBSERVATIONS WHICH INDICATE THE ABSENCE OF DELETERIOUS PROPERTIES, THE UNIVERSITY ACCEPTS NO RESPONSIBILITY FOR ANY INJURY (INCLUDING INJURY RESULTING IN DEATH), DAMAGE OR LOSS THAT MAY ARISE FROM THE USE OF THE XENOGRAFTS. YOU ALSO UNDERSTAND THE IMPORTANCE OF QUARANTEENING ANY RODENTS RECEIVED FROM OUTSIDE YOUR FACILITY OR THOSE WHICH ARE INITIALLY IMPLANTED WITH THE XENOGRAFTS AND IN CONSULTING WITH YOUR ANIMAL CARE COMMITTEE IN ASSURING THAT THE MODELS ARE "CLEAN" AND "SAFE" PRIOR TO INTEGRATION INTO YOUR ANIMAL FACILITY. 2/17/99  
A.D.  
BX
7. You agree to assume all risks and responsibility in connection with the receipt, handling, storage, and use of the xenografts. Furthermore, at the conclusion of your studies, any stored xenografts or viable xenografts will be destroyed. 2/16/99
8. You and your INSTITUTION agree that any person within the INSTITUTION utilizing the xenografts will be advised of, and is subject to, the conditions of this Agreement. misconduct of UNIVERSITY.
9. The UNIVERSITY scientific contact investigator is: Robert L. Vessella, Ph.D.
10. The UNIVERSITY IRB approval number is: 27-011-E

If the forgoing terms are acceptable, please have a representative of the INSTITUTION sign in the space indicated for signature. Please return an original of this Agreement to the Office of Technology Transfer and a copy to Robert L. Vessella (addresses provided on page 2). Once we have received this signed Agreement, we will provide you with the xenografts.

University of Iowa  
\_\_\_\_\_  
Type Name of Your Institution

Timothy L. Ratliff, Ph.D.  
Type Name of Principal Investigator

*Timothy L. Ratliff*  
Signature of Principal Investigator

2-11-99  
Date

Brian Harvey  
Type Name of Authorized Representative

*Brian Harvey* 2/16/99  
Signature of Authorized Representative

February 16, 1999  
Date

# XENOGRAFT RESEARCH AND COLLABORATIVE AGREEMENT

(Page 2)

Provide Complete Mailing Address For Xenograft Shipment:

University of Iowa  
\_\_\_\_\_  
Animal Care Unit  
\_\_\_\_\_  
2-669 BSB  
\_\_\_\_\_  
Iowa City, IA 52242  
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Return Signed Original Agreement To:

Aridna A. Santander  
Program Operations Coordinator  
Office of Technology Transfer  
University of Washington  
1107 NE 45<sup>th</sup> St; Suite 200  
Seattle, WA 98105

Return Copy of Signed Agreement To:

Robert L. Vessella, Ph.D.  
Department of Urology, #356510  
University of Washington  
Seattle, WA 98195

Phone: (206) 543-1461  
FAX: (206) 543-1146



DEPARTMENT OF THE ARMY  
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REPLY TO  
ATTENTION OF:

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1 Apr 03

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

  
PHYLIS M. RINEHART  
Deputy Chief of Staff for  
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