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

The goal of this project is to characterize T cell tolerance to prostate tumor antigens and identify the role of costimulatory receptors in overcoming this tolerance. Identification of these processes will assist in the development of novel therapeutic approaches for treating prostate cancer. We currently use the TRAMP model which is a transgenic mouse line that develops primary prostatic tumors due to expression of the SV40 T antigen (TAg) under the transcriptional control of a prostate-specific promoter. In this summary, we report that TRAMP mice express TAg in the thymus which indicates that both central and peripheral tolerance exist. We have begun initial studies to characterize tolerance to TAg in TRAMP mice by generating vaccines that express TAg and elicit anti-TAg responses in no-transgenic mice. We have also begun studying the trafficking and activation of TAg-specific T cells transferred into tumor-bearing TRAMP mice. Results from these preliminary studies suggest that naïve, tumor antigen-specific T cells undergo initial stages of activation and home to the prostate, where the antigen is expressed. Our on-going studies are aimed at understanding the activation state of these T cells after they encounter their cognate antigen in the tumor as well as the effects of androgen ablation on tumor-specific T cell activation.

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Introduction

It is well-appreciated that growing tumors suppress the anti-tumor response by at least 2 mechanisms-generalized immunosuppression and antigen-induced tolerance. The goal of this research project is to test the hypothesis that modulating costimulatory receptors expressed by T cells can reverse tolerance to prostate tumor antigens and elicit a more potent anti-tumor immune response. We use a transgenic mouse model of human prostate cancer, the **TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate (TRAMP) model, to study T cell responses to prostatic tumors. In TRAMP mice, primary tumors develop as a consequence of prostate-specific expression of a transforming antigen, the SV40 T antigen (TAg). We use TAg as a surrogate tumor antigen. We also have two other mouse lines which bear T cell receptor transgenes that encode either MHC class I-restricted (TcR-I) or class II-restricted (TcR-II) antigen receptors. Our goal is to use these murine lines to understand how T cells develop tolerance to tumor antigens and to test whether modulation of costimulatory receptors is sufficient to overcome tolerance to tumors by understanding these basic immunologic processes.

Body

Task 1: *To determine the Developmental Stage at Which TRAMP Mice Become Tolerant to TAg:*

Previous studies by others suggest that TRAMP mice develop tolerance to TAg during development (1). However, those studies suggested that TAg was not expressed in the thymus and therefore peripheral, not central, tolerance was the sole mechanism by which tolerance to this tumor-associated antigen (TAA) occurs. Because previous studies have suggested that tissues-specific promoters may exhibit leakiness in the thymus (2), we undertook a series of experiments to determine whether TAg expression is truly absent in the thymus. As shown in Figures 1 and 2, we detected TAg mRNA expression in the thymus as early as 7 weeks of age. Interestingly, TAg expression was detected in the prostate at 1 week of age, the earliest time point at which we can reliably dissect the prostatic tissues. This is in contrast to earlier reports (3) suggesting that transgene expression by the androgen-dependent probasin promoter was not detected until 5-7 weeks of age. To date, we have not detected Tag mRNA expression in any other tissues.

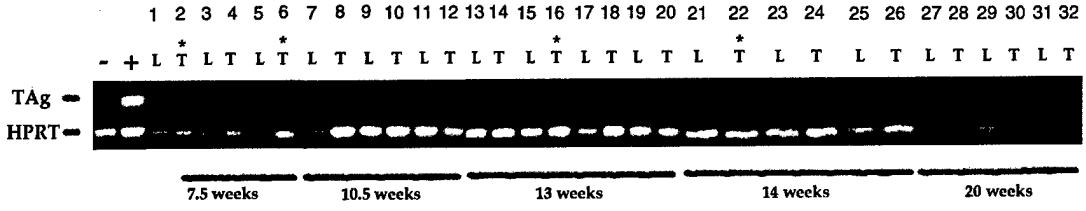

Figure 1. Detection of SV40 TAg expression by RT-PCR. 100ng of total RNA from lung (L), thymus (T) or prostate (+) was reverse-transcribed and amplified with SV40 Tag and HPRT specific primers. Lanes 1-6, tissue from three, 3.5 week-old mice; lanes 7-12, tissue from three, 10.5 week-old mice; lanes 13-20, tissue from four, 13 week-old mice; lanes 21-26, tissue from three, 14 week-old mice; lanes 27-32, tissue from three 20 week-old mice. * indicates presence of Tag band.

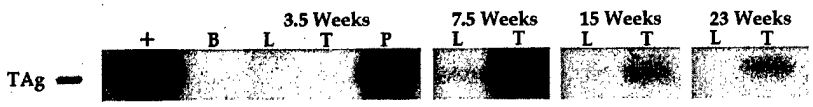

Figure 2. SV40 TAg is expressed in the thymus by 7.5 weeks of age. RT-PCR products were analyzed by gel electrophoresis and transferred to nitrocellulose. Expression was detected with a ^{32}P-labeled TAg-specific probe. (+), positive control; B, blank lane; L, lung tissue; T, thymus tissue; P, prostate tissue.

We are currently trying to confirm these findings by assessing TAg protein expression in the thymus. We are using two different approaches. The first is to identify TAg protein using Western blot and immunohistochemistry. We have purified a monoclonal antibody that reacts with TAg-expressing tumor cells (see below) and will use that antibody to assess TAg protein expression in the thymus. The second approach we will use is to determine whether expression of TAg in the thymus has a functional consequence for T cells. To determine whether cells of the TRAMP thymus express immunologically-relevant TAg epitopes, lysate from TAg thymi will be used to stimulate TcR-I or TcR-II cells and proliferation and cytokine expression will be tested.

Two alternative approaches for testing thymic expression of TAg are possible. First, TRAMP mice could be back-crossed onto the TcR-I or –II background. Alternatively, bone marrow chimeras could be prepared consisting of TRAMP mice reconstituted with bone marrow from either of the TcR transgenic lines. In either case, the thymic and peripheral repertoire of T cells would be compared to reconstituted wild-type mice. If TAg protein is expressed in the thymus, we would expect that the TAg-specific cells would be deleted and alter the peripheral repertoire.

To determine when functional T cell tolerance to TAg is induced in TRAMP mice, we proposed two approaches. The first used synthetic peptides as vaccines. We purchased the peptides corresponding to the epitopes recognized by TcR-I and TcR-II and sensitized wild-type C3H mice. We were unable to detect any significant responses *in vitro* following sensitization to the peptide in complete Freund's adjuvant (CFA). We further tested a prime-boost regimen using incomplete Freund's adjuvant (IFA), but still could not detect a significant response. As a final test, we used known helper-epitope from Hepatitis B virus but we could still not detect a T cell response *in vitro*.

Therefore, we have resolved to use the alternative approach we originally proposed that employs a non-prostatic, syngeneic tumor transduced to express TAg. TRAMP mice will be vaccinated with the cell-based vaccine and peripheral T cells will be tested for TAg-reactivity. Our preliminary results suggest that this approach may be more feasible for assessing tolerance to TAg. We first generated a vaccine by transfecting the C3H-derived K1735 melanoma with the full-length TAg gene and derived clones by limiting dilution (K-TAg, Figure 3). We generated other transfectants that either co-expressed the GM-CSF gene to provide adjuvant effects or were transfected with a TAg mini-gene that encoded only the TcR-I and –II epitopes to focus antigenicity. Immunohistochemistry confirmed that these transfectants express the TAg protein.

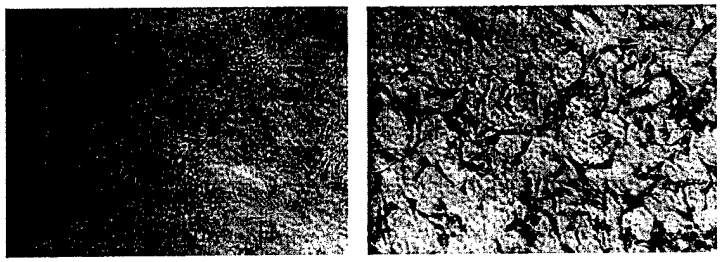

Figure 3: K-TAg cell lines express TAg protein. K1735 tumor cells were transfected with the full-length TAg cDNA by electroporation, cloned by limiting dilution, and cultured in selection medium. Selected clones were immunostained with a monoclonal antibody directed against the amino terminus and the reaction visualized with diaminobenizidine (dark precipitate).

The TAg-transfected cell lines and their appropriate vector-transfected control lines were recently used as cell-based vaccines to sensitize wild-type C3H mice. Results from a preliminary experiment are presented in Figure 4.

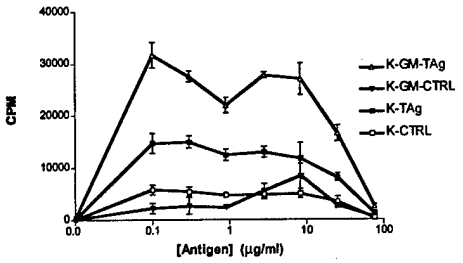

Figure 4: K1735 tranfectants elicit an anti-TAg response. Wild-type, C3H (H-2^k) mice were vaccinated with the indicated vaccines: K1735 cells transfected with TAg (K-TAg), GM-CSF and TAg (K-GM-TAg), or control, empty vector (CTRL). 10 days later, splenocytes were prepared and tested for proliferative response to an immunodominant H-2^k epitope, $TAg_{560-568}$.

TAg-transfected cells (K-TAg and KGM-TAg) elicited an anti-TAg response whereas control-transfected cells did not. It has been our observation that the GM-CSF-expressing vaccines elicit a slightly stronger response. However, we have recently had difficulties obtaining consistent differences between control- and K-TAg-vaccinated mice. We have therefore undertaken more stringent characterization of the K-TAg cell lines and hope to begin sensitizing TRAMP mice (back-crossed one generation onto the C3H background) in the coming months. In the interim, we have begun studying the adoptive transfer of TcR-I cells into TRAMP mice as described below. We hope that as these two models develop, we will be able to concurrently assess the role of costimulatory interactions in developing T cell tolerance to TAA's.

Task 3: *To use an adoptive transfer system where transgenic T cells that recognize MHC class I- and class II-restricted TAg epitopes can be monitored to test the hypothesis that a developing prostatic tumor can tolerize naïve TAA-specific T cells.*

Many studies suggest that as a tumor develops, T cell tolerance to TAA's ensues (4, 5). Most of these studies have employed transplantable tumor lines that express xenogeneic antigens that are thus highly stimulatory to the immune system. The TRAMP model presents a novel model where primary tumors develop under the developmentally regulated expression of a tissue-restricted promoter. We have proposed to study T cell tolerance using the TcR-I and TcR-II transgenic lines which bear transgenes encoding TcR gene that recognize MHC class I- and class II-restricted epitopes of TAg, respectively. Due to difficulties with our cell-based vaccines for Aim 1, we have recently undertaken some preliminary studies for Aim 3.

The TcR-I mice were bred to homozygosity on the C3H background. Lymph node cells (LNCs) from these mice were used as donor cells for transfer in TRAMP x C3H (F_1) mice. Similar transfers were performed using wild-type C57BL/6 x C3H (WT) as recipients or using WT C3H cells as donor cells. Donor LNC were labeled with CFSE, a fluorescent dye that distributes evenly among daughter cells as the cells divide and therefore a linear reduction of fluorescence is observed. As shown in Figure 5, transfer of TcR-I cells into TRAMP mice results in selective expansion and some trafficking to the prostate. In contrast, transfer of WT cells into TRAMP mice did not result in proliferation but rather quiescence in the lymph nodes.

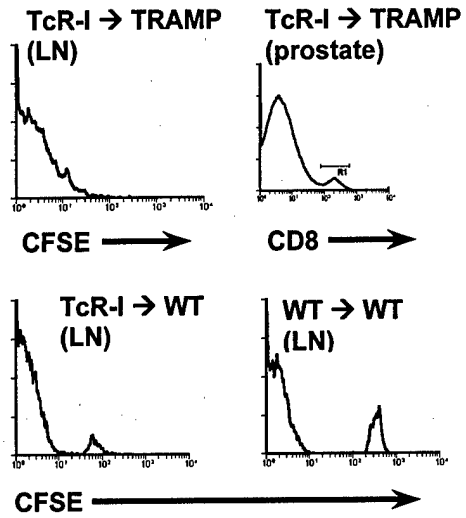

Figure 5: Adoptive transfer of prostate TAA-specific T cells results in expansion and homing to the prostate. TcR-I or WT LN cells were labeled with CFSE and transferred into TRAMP or WT mice. Draining LN cells were prepared 4 days later and stained for CD8 and analyzed by flow cytometry. $CD8^+$ cells were analyzed for CFSE. Prostatic tissues were digested using collagenase and analyzed similarly. Interestingly, $CD8^+$ cells in the prostate were CFSE-negative, suggesting they had previously divided. At earlier time points, no $CD8^+$ cells were detected in the prostate.

These findings suggest that prostate TAA-specific T cells that encounter their cognate antigen undergo proliferative expansion and eventually traffic to the site of antigen expression. It is not clear whether these cells have undergone an abortive proliferation that results in tolerance or whether

these cells are activated and primed for anti-tumor activity. Future experiments will assess their state of activation by determining phenotypic expression of activation marker such as CD25, CD44, and CD62L. In addition, we will test the functionality of these adoptively transferred cells using proliferation assays and ELISPOT analysis of cytokine expression. We will also characterize the trafficking and activation status of TcR-II cells.

We are also interested in determining the effects of androgen ablation on T cell trafficking and activation. Androgen ablation is one of the most common treatments for prostate cancer and in both man and mouse, initiates an immediate inflammatory response in the prostate (E. Kwon and A. Hurwitz, manuscript in preparation). In the TRAMP model, androgen ablation results in reduced prostatic disease progression, although androgen-independent disease does arise. We have begun studies examining whether post-castration inflammation is sufficient to initiate priming of prostate-specific T cells. TRAMP mice were castrated and adoptively-transferred with CFSE-labeled TcR-I cells. Our very preliminary data suggest that only antigen-specific T cells (TcR-I) home to the prostate following androgen ablation. On-going studies will determine the functionality of T cells that traffic to the prostate as well as their impact on prostatic tumor progression as well as characterize the trafficking and activation of TcR-II cells.

Key Research Accomplishments

- Identification of TAg expression in the thymus
- Characterization of TAg Expression during development
- Preparation of TAg-expressing vaccines/Initial characterization
- Initial characterization of adoptive transfer model system

Reportable Outcomes:

No peer-reviewed manuscripts have been published. We do hope to report our findings on the expression of TAg in the thymus of TRAMP mice. We are awaiting functional data that identifies TAg protein expression in the thymus prior to submission.

This work was presented at the Association for the Cure of Prostate Cancer (CaP CURE) Annual Retreats in 2001 and 2002. This work will be presented by Mr. Michael Anderson, a graduate student in the laboratory, at the Upstate New York Immunology Retreat in November, 2002.

Conclusions:

Our long-term goal is to understand the role of costimulatory receptors in regulating T cell tolerance to tumor antigens. Our early data suggest that TRAMP mice may exhibit both central and peripheral tolerance to TAg, a surrogate tumor antigen. This implies that TAg functions as a true self- and tumor antigen. Due to difficulties in eliciting responses to peptide antigens, we are now beginning to characterize tolerance to TAg in TRAMP mice using cell-based vaccines.

Concurrently, we have begun to study the fate of TAA-specific T cells in the TRAMP model. Adoptive transfer of TAg-specific TcR-I cells into TRAMP mice results in clonal expansion and homing to the prostate. Preliminary data suggest that androgen ablation may enhance this effect, but maintain

antigen-specificity. Future studies will characterize the phenotype and functionality of these prostate TAA-specific T cells.

"So what?"
These are preliminary data that justify the use of the TRAMP model. It is clear that we have not been able to meet the initial goals of this proposal. This is, in part, due to technical difficulties with generating reliable TAg vaccines. This is also due to personnel problems. Mr. Anderson, the graduate student dedicated to this project has become more efficient and is being directly supervised by Dr. Hurwitz. In addition, a postdoctoral fellow joined the laboratory this summer and will assist Mr. Anderson.

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