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13. ABSTRACT (Maximum 200 Words) Our research is focused towards the development of an immunotherapy for prostate cancer that specifically targets the expressed prostate specific antigen (PSA) of prostate tumor cells. With over forty thousand deaths a year and the near lack of curative treatments, an effective therapy would greatly benefit society. Our research to date has suggested that PSA can serve as a tumor rejection marker in our PSA transgenic mice whose prostates express human PSA. Vaccination with a tumor cell that expresses PSA elicited a specific anti-PSA response that prevented the outgrowth of a tumor challenge of PSA expressing cells. After indicating the pertinence of PSA as a target of an immunotherapy, we attempted to identify PSA derived peptides that are immunogenic in the HLA-A*0201 haplotype. Of nine PSA peptides selected for our study based on binding studies, two have proven to be immunogenic in the HLA-A*0201/Dd transgenic mouse model after vaccination of peptide pulsed dendritic cells. These results indicate that not only can we use PSA as a plausible rejection marker but we can also elicit a CTL response directed against the human PSA peptides in a HLA-A*0201/Dd transgenic mouse. These results allow us to experiment with our vaccination strategies for the development of an efficacious anti-prostate cancer immunotherapy.				
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FOREWORD

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~~N/A~~ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

~~NA~~ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



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Date

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Introduction

Prostate cancer is a relatively common disease of men that carries a lifetime risk of about 10% and is reported to be present in 80% of eighty year olds. Although the majority of the individuals who contract the disease are not affected by it, the widespread occurrence of the cancer enables it to account for around forty thousand deaths a year in the United States of America. Surgical resection can cure early disease but is often avoided due to the usual slow growth of the tumors and complications of impotence and incontinence that can result from the operation. In the absence of a curative treatment for metastatic disease, a potent immunotherapy that would eradicate the prostate cancer cells would greatly benefit those individuals suffering from the malignancy. We have initiated experiments to develop a vaccine to elicit an anti-prostate specific antigen (PSA) immunity capable of destroying existing tumor cells that express PSA. Our research has focused on creating an immunotherapy restricted to the human HLA-A*0201 haplotype and capable of eliminating pre-existing tumors. Should we be successful, the techniques of our vaccine could be applied to all the human haplotypes upon the determination of immunogenic peptides relevant for each individual haplotype.

Body

This annual report covers the time period allotted for the completion of technical objectives one and two, TO1 and TO2, respectively, as stated in our Statement of Work. TO1 was slated to occur in months one to six and detailed the experiments to prove that PSA could serve as a relevant tumor rejection marker in a system where it is regarded as a self protein. This would be accomplished via either vaccination with the PSA protein or DNA construct and subsequent challenge with a tumorigenic PSA expressing cancer cell line. TO2 was scheduled to occur in the first twelve months and was supposed to identify which HLA-A*0201 restricted PSA peptides would be immunogenic in the HLA-A*0201/Dd transgenic mouse. This objective was to be achieved through vaccination of the mice with either free peptides emulsified in adjuvant or the intramuscular injection of DNA constructs encoding each minimal peptide. Both technical objectives were completed as described in the grant application within the first year as scheduled with limited results. We have modified our research approach and have now mostly accomplished our stated goals using different vaccination methods that will be detailed below.

TO1 consisted of three tasks to establish PSA as an appropriate tumor rejection marker. Task 1 entailed the transfection of a syngeneic tumor cell line with a PSA construct and its subsequent injection into mice for evaluation of its tumorigenicity. Task 2 involved the vaccination of the PSA transgenic mice with either the whole PSA protein emulsified in incomplete Freund's adjuvant (IFA) or a DNA construct containing the PSA gene. Task 3 consisted of the measurement of any induced anti-PSA immunity due to the vaccinations through tumor challenges and assays for cytotoxic T lymphocyte (CTL) responses.

TO1 task 1 was accomplished through the transfection of the pREP4/PSA construct into the syngeneic, spontaneous mastocytoma cell line, OM-2, via electroporation. Cells were grown in media containing the selection agent, hygromycin, and tested for PSA expression via reverse transcription/polymerase chain reaction (RT-PCR). Positive cell lines were subcloned at 0.1 cells per well to establish a monoclonal tumor cell line which was also tested for PSA expression by RT-PCR. One positive clone was then chosen and injected into PSA transgenic mice for determination of the appropriate concentration of tumor cells that gave 100% tumor take (**see figure 1**). Unfortunately the cell line turned out to spontaneously regress after about two weeks, but it was decided that it would still constitute an acceptable cell line for our studies since it did produce palpable tumors in mice. We chose 10 million PSA/OM-2 cells per mouse as the concentration of tumor cells to inject in future experiments.

Tasks 2 and 3 were accomplished in the PSA transgenic mice but with no positive results. For the protein vaccination, PSA transgenic mice were vaccinated with 100 micrograms of PSA per mouse emulsified in IFA or IFA alone. Two weeks after the vaccination, all mice were injected with the PSA/OM-2 cells and followed for tumor growth. As shown in **figure 2**, this vaccination did not provide a protective anti-PSA immunity to the mice. Additionally, T cells from cohort animals vaccinated identically failed to recognize or kill PSA/OM-2 cells in a standard ⁵¹chromium release assay that

tests CTL function (data not shown). Thus vaccination of the mice with whole protein proved to be an ineffective strategy for immunization.

We next attempted to vaccinate the mice with four intramuscular vaccinations of either pZeoSV2/PSA or control pZeoSV2 DNA vectors. As above, mice were challenged with PSA/OM-2 cells and followed for tumor growth. As seen in **figure 3**, DNA vaccination also failed to elicit protective anti-PSA T cells in the mice. A ⁵¹chromium release assay on similarly vaccinated mice also failed to show any anti-PSA activity from the isolated T cells (data not shown). Based on these data, we concluded that either vaccination with whole PSA protein or the PSA DNA construct were ineffective immunization strategies and thus we turned our efforts to other immunization methods.

Two other vaccination strategies were utilized in our attempts to induce a protective anti-PSA immunity including immunization with PSA pulsed dendritic cells and PSA expressing tumor cells. Dendritic cells were cultured with PSA protein for five hours and injected intravenously into the tail vein of PSA transgenic mice. Two weeks after the fourth vaccination, mice were challenged with PSA/OM-2 cells and followed for tumor growth. As seen in **figure 4**, this vaccine also failed to elicit a protective anti-PSA immunity. Analysis of CTL function via ⁵¹chromium release assays also failed to show any anti-PSA immunity (data not shown).

Our last vaccination strategy utilized four injections of the human prostate cancer cell line, LNCaP, that has high expression of PSA. Two weeks after the last vaccination, mice were challenged with either the PSA/OM-2 cell line of above or a newly created vector transfected OM-2 cell line, pREP4/OM-2. Mice challenged with the PSA/OM-2 cell line did not grow palpable tumors like the mice who received the pREP4/OM-2 cell line (see **figure 5**). These experiments suggested that PSA could serve as a tumor rejection marker in a system where PSA existed as a self protein which is an important finding.

Although we detailed our desire to test the autoimmune status of the mice following each vaccination, we have had to alter our approach. We initially stated that we could weigh the mice before and after the vaccinations to determine if runting, a characteristic loss of weight in the autoimmune state, was present. This, however, would require the mice to be sedated to get accurate measurements on the scale (mobile mice alter the scales read out with every move). We felt it best to forgo the sedation and concentrate on the immunohistochemistry of sections of each mouse's prostate since the sedative may have an effect on the mouse's immune system and mortality. The immunohistochemistry of the mouse's prostate is pending at this time point on the optimization of the protocol. Our lab has had difficulty in producing consistent slides of the prostate and thus we have delayed the analysis until the protocol has been optimized, a process on which we are currently working.

Although the LNCaP vaccination protocol produced the desired results, we wanted to develop a tumor cell line for use in the PSA transgenic mice that would progress and not regress. We injected mice with the OM-2 cell line and waited after the original tumor regressed for the outgrowth of a progressor tumor cell line. In several cases, tumors did grow out, were resected and introduced back into tissue culture. These cells were then transfected with the PSA construct, subcloned to create a monoclonal tumor line, and reinjected into mice to determine if they retained the ability to

progressively grow in the mice. Of twenty subclones tested, one line called PSA/OM-95 2A did retain the progressor phenotype (see **figure 6**) and will replace the PSA/OM-2 cell line in future experiments.

In conclusion of TO1, we performed the protein and DNA vaccination experiments listed in the grant application but did not receive positive results. We thus moved to other immunization strategies and found that vaccination with the human, PSA expressing prostate cancer cell line, LNCaP, elicited a protective anti-PSA immunity. This confirmed the ability of the PSA protein to serve as a pertinent tumor rejection marker and allowed us to continue our studies. With the completion of the immunohistochemical evaluation of prostate sections taken from the LNCaP vaccinated mice, we will consider TO1 to be completed.

TO2 was designed to identify peptides of the PSA protein that are both immunogenic and endogenously presented in the HLA-A*0201 system. This technical objective consisted of three tasks designed to accomplish this task. Task 1 involved the creation of DNA constructs containing the minimal gene encoding each peptide's sequence. Tasks 2 and 3 consisted of the vaccination of the HLA-A*0201/Dd transgenic mice with either free peptide emulsified in IFA or the DNA constructs encoding each peptide and the subsequent measurement of the induced anti-PSA immunity. As with TO1, our initial plans were altered due to the lack of positive results. The new strategies will be detailed below and have produced good results.

Task 1 of TO2 was abandoned before it was initiated due to other research from this laboratory. In separate studies using the E6 and E7 proteins of the human papilloma virus type 16 as targets of immune attack, we initially failed to generate an induced CTL immunity. Thus we focused our efforts on using the free peptides emulsified in IFA since previous experience with this method was fruitful. Although our lab has recently received positive results in vaccination with DNA constructs encoding peptides, we will not attempt this part of our proposal due to the greater success of a dendritic cell vaccination method that will be discussed below.

For tasks 2 and 3, we only performed the experiments with respect to the immunization of HLA-A*0201 transgenic mice with free peptide emulsified in IFA. All of our attempts to vaccinate these mice failed to produce any results in standard ⁵¹chromium release assays (data not shown). Since our positive control, vaccination with a known immunogenic HPV peptide, also failed to give us positive results, we concluded that the lack of results may be due to some defect in our ⁵¹chromium release assay and not a true indication of the immunogenicity of our peptides. Thus we have spent great amounts of time in troubleshooting the assay and have finally optimized the protocol to where we can trust the results. We found that we need to vaccinate our mice with peptide pulsed dendritic cells in order to achieve an immunity against the peptides. This being so, we will not complete TO2 as we originally reported.

Instead of vaccinating the mice with either free peptide emulsified in IFA or DNA constructs encoding each peptide, we infuse 500,000 bone marrow derived dendritic cells pulsed for several hours with each peptide. To do this, we had to establish the protocol for generating the bone marrow derived dendritic cells in our laboratory. Upon completion of this, we have been vaccinating HLA-A*0201/Dd transgenic mice with the peptide pulsed dendritic cells and assessing their immunity in the standard ⁵¹chromium

release assay that was also optimized. Utilizing these new methods, we have currently identified two of our peptides as immunogenic in the HLA-A*0201/Dd transgenic mice (see **Figures 7 and 8**). Currently, we are testing the remainder of our peptides in the HLA-A*0201/Dd transgenic mouse.

We will consider TO2 to be complete when we have tested all of our peptides using the peptide pulsed dendritic cell vaccination strategy. With this knowledge we will use the immunogenic peptides in TO3 to determine if vaccination with these peptides pulsed on dendritic cells will protect against PSA expressing HLA-A*0201/Kb positive tumor cells as mentioned in the grant application. We will utilize the dendritic cell vaccination approach since it has proven effective at generating an anti-peptide immunity. We do not foresee changing any other parts of TO3 in the next year unless demanded by negative results.

This annual report has detailed our experiments to date. We were forced to deviate from our stated methods but felt it necessary to achieve the desired results. In TO1, we feel we proved the pertinence of PSA as a tumor rejection marker and will continue to work on the immunohistochemistry to complete the first technical objective. In the second technical objective, we unfortunately needed to optimize our protocols to get reliable results and have done so. The use of these newly established protocols has identified two PSA peptides as immunogenic in the HLA-A*0201/Dd transgenic mouse model. We experienced a set back in regard to time for TO2 but feel that we should complete it within the next four months using the newly established protocols. Work has already begun in the laboratory on TO3 for its eventual achievement.

Key Research Accomplishments

1. Establishment of two PSA expressing tumor cell lines that give tumors in the PSA transgenic mouse for use as our tumor model, PSA/OM-2 and PSA/OM-952A.
2. Use of vaccination with a PSA expressing tumor cell line for protection against the outgrowth of a different PSA expressing tumor cell, PSA/OM-2, thus identifying PSA as a candidate rejection marker.
3. Identification of two of the nine selected PSA peptides as immunogenic in the HLA-A*0201/Dd transgenic mouse model.
4. Establishment of a PSA and HLA-A*0201/Kb expressing tumor cell line that gives progressor tumors in the HLA-A*0201/Dd transgenic mouse for use as our tumor model, PSA/EL4A2Kb.

Reportable Outcomes

Manuscripts, abstracts, presentations:

Two Abstracts

1. "Use of Different Vaccination Strategies to Induce Protective Anti-PSA Immunity in PSA Transgenic Mice" Gregory E. Holt, Gilbert Jay, W. Martin Kast, IBC's Sixth Annual Conference on Vaccine Technologies. Arlington, Va., March 25-26, 1999.
2. "Induction of Anti-PSA Cellular Immunity in PSA Transgenic and HLA-A*0201/Dd Transgenic Mice" Gregory E. Holt, Michael P. Rudolf, W. Martin Kast, 2000 Keynote Symposia on Cellular Immunity and Immunotherapy of Cancer, Santa Fe, NM, January 21-27, 2000.

Patents and licenses applied for and/or issued:

None.

Degrees obtained that are supported by this award:

None.

Development of cell lines, tissue, or serum repositories:

Four cell lines:

1. PSA/OM-2
2. pREP4/OM-2
3. PSA/OM-95 2A
4. PSA/EL4A2Kb

Informatics such as databases and animal models, etc:

None.

Funding applied for based on work supported by this award:

Applied for a Fellowship from the American Foundation for Urologic Disease and American Urological Association. Title of the project, "Cancer Prevention and Immunotherapy in a Chronic Prostate Cancer Model."

Employment or research opportunities applied for and/or received on experiences/ training supported by this award:

None.

Conclusions

We are attempting to develop an immunotherapy for the treatment of prostate cancer that specifically targets the prostate specific antigen (PSA) found in the tumor cells. Our research uses two transgenic mouse models to best simulate the human condition for the evaluation of our therapeutic strategies. First, the PSA transgenic mouse model in which the mice express human PSA in their prostates simulates the human condition where PSA exists as a self protein. We now provide preliminary evidence that PSA can serve as a tumor rejection marker in a situation where it is considered a self antigen using the PSA transgenic mouse. Four vaccinations with a PSA positive human prostate cancer cell line protected mice from a tumor challenge of the PSA transfected mouse tumor cell line, PSA/OM-2, but not the vector transfected control tumor cells, pREP4/OM-2. The second transgenic model, HLA-A*0201/Dd, contains a chimeric class I MHC molecule with the ability to present human peptides and interact with mouse T lymphocytes. Using this model, we have identified two peptides derived from PSA as immunogenic for CTLs after intravenous injection of autologous dendritic cells pulsed with the peptides. With the development of a tumor cell line with both the chimeric HLA-A*0201/Kb MHC molecule and PSA, we can now test these two immunogenic peptides for their ability to protect the mice from tumor outgrowth. These two mouse models will allow us to test our vaccines for their efficacy in treating mouse tumors that express PSA in the HLA-A*0201 setting. Should we be successful in delineating an vaccination protocol that eradicates preexisting tumors that express PSA through the direct targeting of the PSA molecule, a transfer of this approach to the human situation would be feasible.

Appendix

Figure 1: Tumor challenge of PSA transgenic mice with the regressor cell line, PSA/OM-2.

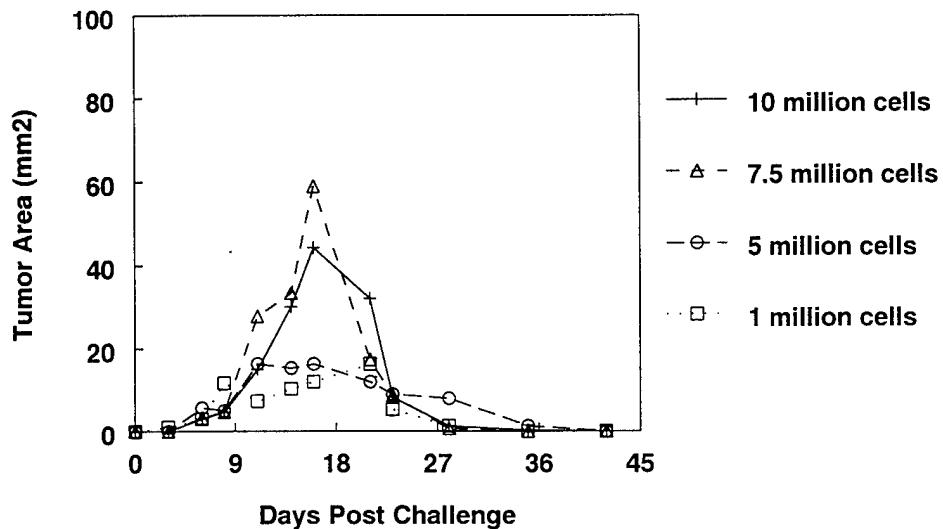
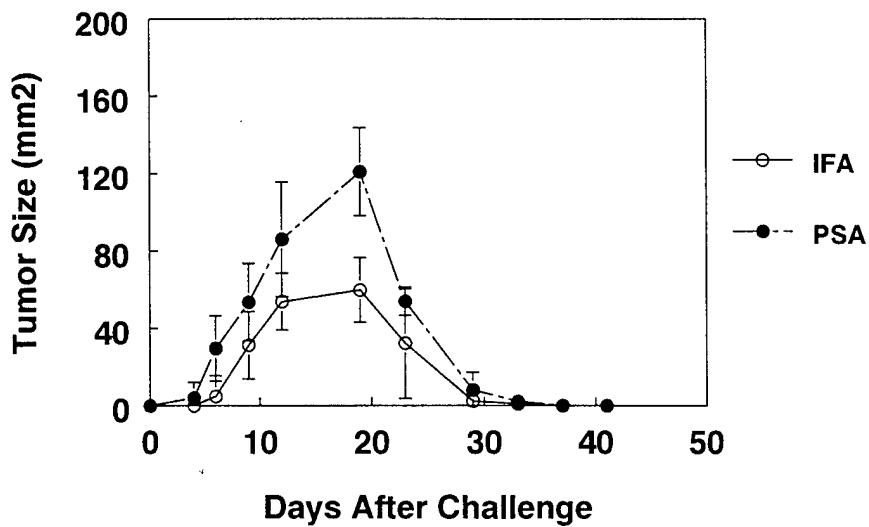


Figure 2: Vaccination with whole PSA protein in IFA fails to protect PSA transgenic mice from PSA/OM-2 challenge.



Appendix

Figure 3: Vaccination with the PSA gene fails to protect PSA transgenic mice from PSA/OM-2 challenge.

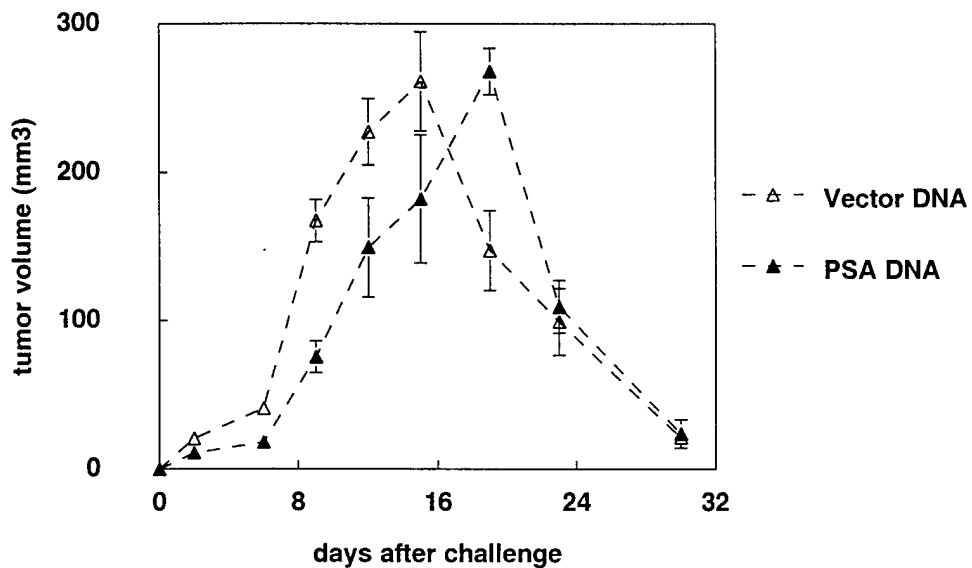
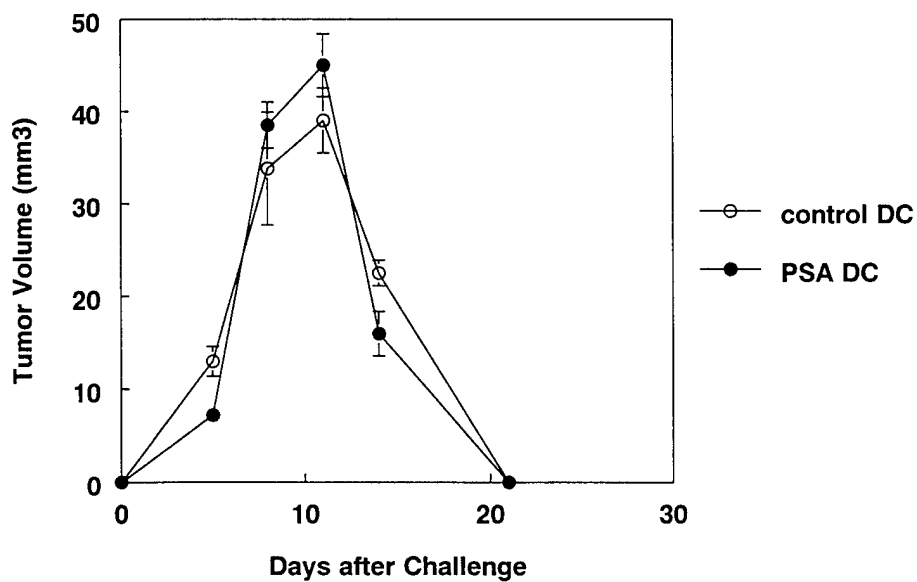


Figure 4: Vaccination with PSA protein loaded dendritic cells fails to protect PSA transgenic mice from a PSA/OM-2 challenge.



Appendix

Figure 5: LNCaP vaccination protects PSA transgenic mice against challenge with PSA/OM-2 tumor cells.

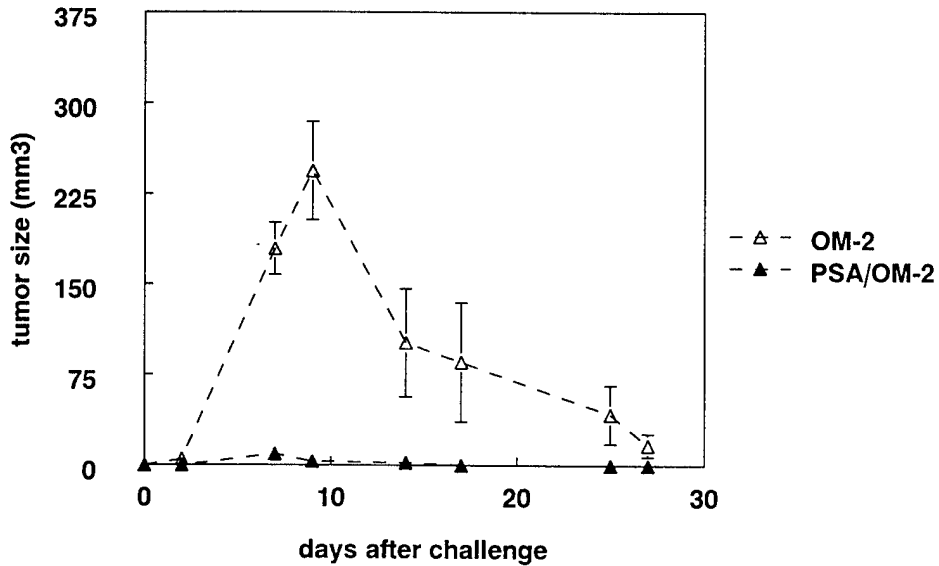
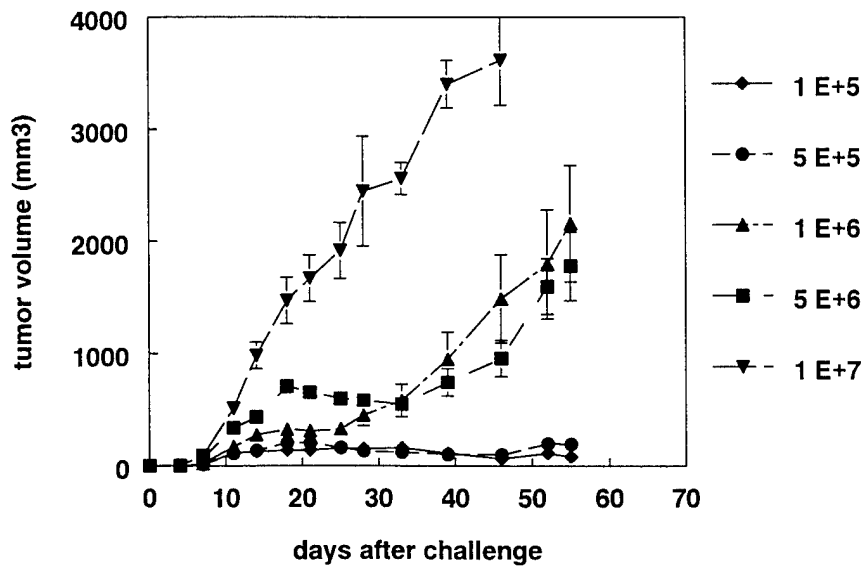


Figure 6: Tumor challenge of the progressor cell line, PSA/OM-95 2A in PSA transgenic mice.



Appendix

Figure 7: Specific Lysis data for PSA TWO. Closed symbol are peptide loaded targets, open symbols are unloaded targets.

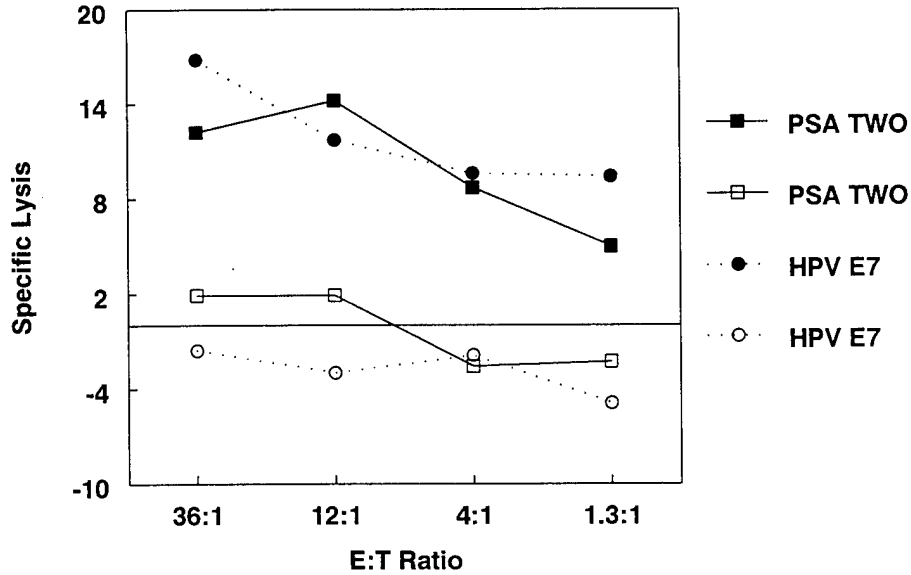


Figure 8: Specific lysis data for PSA SEVEN. Closed symbols are peptide loaded targets, open symbols are unloaded targets.

