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

Immunotherapy may provide an effective adjunct to current treatment modalities for prostate cancer. To achieve this goal, several fundamental immunologic questions need to be addressed. These include optimization of cellular immune responses to candidate tumor antigen and exploration of the extent to which HLA Class I and Class II diversity will restrict application of a candidate prostatic cancer vaccine. PSA was chosen as a candidate antigen for study and dendritic cells (DC) the vehicle for antigen delivery. DC armed with PSA generated predominately HLA Class II mediated CD4+ (helper) responses. However when combined with an anti-PSA monoclonal antibody, CD4+ as well as CD8+ T cell responses were generated. CD8+ T cell responses were at least in part restricted by PSA peptides known to bind the HLA-A*0201 allele. T cell clones were generated from peripheral blood from two patients with disease of the prostate (cancer, prostatitis) and HLA restriction studied. We identified peptide sequences in PSA that were presented by HLA-B*0702 to CD8+ T cells (previously unreported), and an HLA Class II allele (HLA-DR*1501) restricted CD4+ T cell response. The result of these studies contribute to a rational basis for prostate cancer immunotherapy.

14. SUBJECT TERMS
Prostate cancer, tumor antigen, immune response, dendritic cells, Autoimmunity, antigen processing, HLA restriction

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Title: Prostate Tumor Antigen Discovery: Development of a Novel Genetic Approach

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(5) Introduction

The American Cancer Society has estimated that approximately 180,000 new cases of prostate cancer are diagnosed each year, and that 37,000 will die from the disease. New therapeutic modalities will be required to lessen the morbidity and mortality of this disease. Over the past decade, great strides have been made in our understanding of cancer biology and the immune system. This has led to the introduction of immune-based therapies in a number of malignancies. The approach that has been used is to develop vaccines that deliver candidate tumor antigens that may be recognized by the immune system. These are delivered either as whole proteins, component peptides of the proteins, and recombinant DNA/RNA constructs that express the tumor antigen. One of the major hurdles in immune-based therapies in cancer has been the development of adequate delivery systems that will generate effective immune responses to the cancer cell. Over the past several years, it has been demonstrated quite clearly that dendritic cells, the primary antigen presenting cell in the immune system, armed *ex vivo* with tumor antigens or constitutive peptides can be an effective means of delivery of tumor-specific antigens.

Dendritic cells (DCs) are the only antigen presenting cells that can induce a primary immune response. These cells acquire antigeneic materials from the environment and process the antigens through pathways that lead to presentation of derivative peptides by HLA Class I and Class II molecules to T cell receptors on CD8+ T cells (cytotoxic) and CD4+ T cells (helper), respectively. Recent data in mice indicate that both helper and cytotoxic T cell responses are required for effective anti-tumor immunity. While not demonstrated in humans, it is likely that helper responses will be required to generate cytotoxic T cells that are directed at antigenic peptides presented by Class I molecules on the tumor cell surface.

HLA Class I and Class II gene/gene products are highly polymorphic in the general population. To date, several hundred of the Class I and Class II alleles have been identified. These alleles have limited representation in an individual with potentially 6 different Class I and 6 different Class II alleles expressed. The signature of each allele is embodied in the antigen binding cleft or groove that is formed into a mature molecule. These differences dictate the amino acid sequence of the peptide that can be presented by each of the molecules. Given the peptide presenting function of these molecules, this polymorphism may protect the species from infectious pathogens, but creates obstacles for immunotherapy of cancer. The polymorphism of each allele is represented for the most part by amino acid differences in the peptide binding groove that in turn dictates the sequence of the amino acids that can be bound. Thus, targeting individual tumor-specific proteins for immunotherapy may fail if the amino acid sequence (motifs) that are

contained within that protein are such that they cannot be bound recognized by the HLA alleles represented in the individual recipient of that therapy.

The overall objectives of this proposal are to develop methods to test *in vitro* responses to candidate prostate tumor antigens and to determine the extent to which different HLA alleles restrict this response. The specific technical objectives are as follows: 1) stimulation and detection of recalled T cell lymphocyte responses to known prostate tumor antigens; 2) comparison and optimization of antigen delivery by myelomonocytic and non-myelomonocytic cell derived dendritic cells; and 3) development of oligonucleotide derived tumor antigens cDNA library for antigenic screening.

During the first year of the granting period, the following tasks were accomplished: 1) Methods were developed to isolate and mature primary antigen presenting cells (dendritic cells) from precursors in peripheral blood mononuclear cells; 2) We found that dendritic cells present antigens from apoptotic prostate tumor cell lines; 3) CD4+ and CD8+ T cell responses were induced by dendritic cells armed with PSA and PSA/antiPSA antibody complexes; 4) CD4+ lymphocytes from some patients with chronic prostatitis/pelvic pain syndrome responded to PSA suggesting an autoimmune etiology; 5) Paired normal prostate epithelium and primary cancer cells from the same patients were shown to express antigen processing and peptide transport genes that could be upregulated by IFN-γ. These genes were also expressed in 2 or 3 long-term prostate cancer cell lines.

(6) Body

Characterization of T cell responses to PSA and HLA allele specific PSA peptides

Functional primary antigen presenting cells can be derived from myelomonocytic, plasmacytic, or B cell precursors in peripheral blood. The most abundant precursor is the myelomonocytic or DC1 precursor expressing combinations of CD11c, CD14, and HLA-DR. Other DC precursor lineages are found in 1-3% total lymphoid population of peripheral blood mononuclear cells. DCp1were selected for studies because they have been shown by others to preferentially generate Th1 type T cell responses. Mononuclear cells were isolated from whole blood obtained apheresis products from normal donors and from patients with prostate cancer or noninfectious prostatitis. Each donor was typed for HLA Class I and Class II alleles at the DNA level. We investigated and compared several methods for isolation of DC1 precursors: a) sorting by flow cytometry; b) isolation of loosely adherent dendritic cells; and c) negative selection using immunomagnetic beads. The latter was found to be most reproducible with high cell recovery and 85-90% purity. Once isolated, the cells were cultured for 4 days in GM-CFS/IL4, exposed to various antigen preparation, matured with cytokines and tested for induction of CD4 and CD8+ T cell responses on autologous cells. We and others have found that the timing of antigen (proteins, cell products) exposure relative to dendritic cell maturation is critical in order to achieve T cell recognition. We compared the antigen presenting capacity of the dendritic cells that have been matured with CD40L,

TNF-α, IFN-α, LPS, polyIC, and macrophage-conditioned media. Markers for dendritic cell maturation are the cell surface expression of CD80, CD86, and CD83. In general, CD40L was the most reproducible, however, at present it is not approved for human use and therefore would not be usable in a clinical trial. We found low concentrations of combinations of TNF-α and IFN-α were effective in generating DC capable of antigen presentation of proteins and peptides.

There are a number of methods to characterize T cell responses to antigens. These have historically consisted of measuring ^{3}H-thymidine incorporation as a marker of antigen recognition or the expression of HLA Class II and more recently CD69 on the surface of T cells. In order to more specifically characterize and quantitate CD4+ and CD8+ T cell responses that result from recognition of candidate prostate tumor antigens, we developed the ELISPOT and flow cytometry based intracellular cytokine production. These assays have been used to characterize the responses to PSA or PSA derivative peptides. During the first year of the project, we demonstrated that the combinations of PSA with the monoclonal antibody directed against this protein were a very effective means of generating CD4 and CD8+ T cell responses when presented *in vitro* by dendritic cells. Multiple experiments were conducted comparing the different methods of isolation of dendritic cells to determine if the isolation method was responsible for the differences seen between the whole protein and the combination of antigen plus monoclonal antibody.

Figure 1 shows the results of a representative experiment where monocyte derived dendritic cells were used to compare the specificity of CD4 and CD8+ T cell responses to prostate specific antigen, antibodies to PSA, and the PSA/ antiPSA complexes. Dendritic cell precursors were isolated by negative selection as described above, cultured with GM-CSF/IL4 and at day 4 and exposed to 5 μg/ml of PSA, 25 μg/ml of the monoclonal antiPSA antibody or equimolar concentrations of PSA and antiPSA (5μg/ml and 25 μg/ml), respectively. After 3 days, the cells were washed and highly purified and autologous T cells (isolated by negative selection >95% purity) were added and cultured for 7 days in the presence of IL6 (10^{3} U/ml, R&D Systems, Minneapolis, MN) and IL12 (10 ng/ml, R&D Systems, Minneapolis, MN). After 7 days, T cells were restimulated with dendritic cells armed under conditions described above and examined for intracellular production of IFN-γ using a flow cytometry based method. CD4+ T cell responses predominated when PSA alone was presented by DC with low levels of CD8+ T cell responses detected. Restimulation of the PSA exposed T cells by the PSA/antiPSA complexes resulted in both CD4+ and CD8+ T cell responses. There were essentially no differences in responses observed in T cells that had not been initially exposed to any antigen or exposed to anti-PSA and restimulated with any of the antigen or antigen/antibody complexes. The most dramatic responses were observed when PSA/antiPSA complexes were used to arm DC. The cells that had received the primary stimulation by the antigen antibody complexes and restimulated with these same complexes demonstrated the highest levels of CD4 and CD8+ T cell responses.

Additional experiments were conducted to determine if HLA Class I (HLA-A*0201) restricted CD8+ T cells were generated as a result of primary stimulation with

PSA or PSA/antiPSA. In these experiments, autologous T cells were exposed to dendritic cells armed with PSA and/or PSA/antiPSA as described above and restimulated with two HLA-A2 restricted peptides, (FLTPKKLQLV, pep 1) (KLQCVDLHV, pep 2) PSA or PSA/antiPSA (Figure 2). Modest levels of both CD4 and CD8+ T cell responses were seen when T cells that had been exposed to PSA were restimulated with these two peptides. As seen in other experiments, restimulation with PSA effected vigorous CD4+ T cell responses, while both CD4 and CD8+ T cell responses were seen with restimulation with the PSA/αPSA combination. CD8+ T cells response to the peptides was the highest when PSA/antiPSA was used as a primary stimulant. These experiments demonstrate that dendritic cells exposed to the PSA/antiPSA complexes were capable of processing the antigen from the antigen antibody complex through pathways that allowed presentation by and with specific HLA Class I restricted peptides.

During the first year of this proposal, we demonstrated that T cell responses could be generated with dendritic cells armed with apoptotic prostate cancer cell lines. However the specificity of the responses observed were difficult to determine. The LNCaP prostate cancer cell line produces relatively large quantities of PSA while DU-145 (another prostate cancer cell line) does not. We conducted a series of experiments to determine if PSA specific responses were generated with DC armed with a LNCaP cell lysate. To generate the antigenic material, cells were disrupted by freezing and thawing and the product exposed to dendritic cells isolated as described above and matured with combination of TNF-α and IFN-α. After maturation, dendritic cells were combined with T cells at a 1:10 ratio and cultured for 7 days described above. After 7 days the T cells were washed and reexposed to dendritic cells armed with PSA. Table 1 shows the results of these studies. PSA-specific responses were generated by dendritic cells armed with LNCaP, but not with the cells armed with a similar preparation of the prostate tumor cell line DU-145.

Identification of HLA Class I and Class II Restricted Epitopes in PSA

Autoimmunity shares many of the features that might be desired in cancer immunotherapy. In order to generate an effective immune response against products of tumor cells, tolerance to these proteins must be broken and autoreactivity generated. In previous studies, we demonstrated that some patients with chronic prostatitis have circulating T cells recognizing prostate proteins. No such reactivity was found in normal men. To further characterize this response, T cell lines were generated by repeated PSA stimulation using peripheral blood lymphocytes from several patients, one with autoimmune chronic prostatitis and another with prostate cancer. Responses were monitored by testing for IFN-γ and culture fluids by ELISA. T cells from these patients that showed specific responses were cloned by limiting dilution. The HLA type of the cancer patient (PR 97) was HLA-A*0101, B*0702, B*3501, CW*0401, CW*0702, DRβ1*101, DRβ1*1501, DRβ5*0101, and the prostatitis patient (PR 115) HLA-A*0301, A*2402, B*0702, B*1501, CW*0303, CW*0702, DRβ1*04, DRβ1*1501, DRβ4*01, DRβ5*0101. An HLA Class II monomorphic monoclonal antibody blocked PSA stimulation of a CD4+ T cell line (Figure 3). Blocking was also achieved with a HLA-DRB1*1501 antibody and not with an antibody to DRB1*04 (Figure 4). PSA stimulation

of a CD8+ cell line was blocked by a monomorphic anti-HLA Class I antibody and an anti-HLA-B7 monoclonal antibody (Figures 5, 6). HLA-B7 and HLA-DRB1*1501 restricted responses to PSA have not been reported. This result extends the number of Class I alleles that can present PSA to generate CD8+ T cell responses and establishes for the first time an HLA Class II restricted response.

A B cell line was established from the cancer patient and transfected with a recombinant clone expressing PSA. Figure 7 shows the response of one of the clones to the EBV line expressing PSA indicating that these cells can recognize endogenously produced PSA.

HLA-B*0702 binding PSA peptides recognized by a CD8+ T cell line 5H10 were identified. Peptide motifs in the PSA that would potentially bind the HLA-B*0702 molecule were identified using bioinformatic and molecular analysis section databases via the website: http://bimas.dcrt.nih.gov (Parker, K.C., Bednarek, M.A., and Coligan, J.E.). 10 (decamer) and 10 (nanomer) amino acid sequences were identified in PSA that potentially could be bound by the HLA-B*0702 cell and tested for peptide restricted responses. Autologous B cell lines and allogeneic B cell lines that shared the HLA-B*0702 were incubated with peptides, washed, and co-cultured with the T cell line. Cultured supernatants were collected on day 2 and IFN-γ was measured by ELISA. Figure 8 shows the results of these studies. Three peptides were identified in PSA that were recognized by this CD8+ T cell line. Insofar as we know, this is the first HLA-B*0702 restricted peptide that has been identified in PSA.

(7) Key Research Accomplishments

- Dendritic cells isolated from precursors in peripheral blood were matured with cytokines that could be used in a clinical setting.
- CD4+ T cell responses predominated when PSA was presented by DC, while both CD4+ and CD8+ responses were observed when DCs were armed with PSA combined with an anti-PSA antibody.
- CD8+ T cells generated with PSA/antiPSA complexes recognized HLA-A*0201 restricted PSA peptides.
- Dendritic cells armed with lysates of a PSA producing cell line generated PSA-specific responses.
- CD4+ and CD8+ T cell lines and clones were generated from peripheral blood from patients with prostate cancer and granulomatous prostatitis.
- Previously undescribed HLA-B*0702 restricted PSA peptides were identified and as well as an HLA Class II allele restricted PSA response.

(8) Reportable Outcomes

One peer-reviewed manuscripts (appended)
Two abstracts (appended)
One review (appended)

(9) Conclusions

Immunotherapy of prostate cancer may serve as an effective adjunct to current treatment modalities. The overall objective of this project is to identify candidate antigens that will serve as targets for prostate cancer immunotherapy. This objective is based on the hypothesis that products of prostate cancer cells are capable of generating an immune response that will be effective as an adjunct therapy for this malignancy.

Several of the roadblocks to developing a successful cancer vaccine are summarized as follows: Products of tumors that are candidate antigens are likely to be perceived by the immune system as "self" and thus the individual will be tolerant to the potential antigen. Vehicles that will deliver the tumor antigen and generate immunity need to be developed. In addition, the extensive heterogeneity of HLA Class I and HLA Class II molecules that exist in the population potentially restricts the number of determinants in tumor antigens that will generate an immune response in a given individual.

The specific aims of the project were: (1) to develop an *in vitro* technique to isolate primary antigen presenting cells (dendritic cells) and to arm these cells for antigen presentation and generation of CD4 and CD8+ T cell responses; (2) to develop reproducible methods of assessing CD4 and CD8+ T cell responses given their individual function in generating an effective cellular immune response; and (3) determine the extent to which HLA heterogeneity restricts response to these antigens. To achieve these goals, methods for isolating dendritic cells from peripheral blood sources were developed and their capacity to generate *de novo* immune response tested. ELISPOT and intracellular cytokine production (flow cytometry based) assays were used to assess CD4+ and CD8+ cognate antigen recognition. We demonstrated the capacity of DCs to acquire PSA presented in the form of whole proteins or in combination with a monoclonal anti-PSA monoclonal antibody. DC armed with PSA alone generated predominancy for CD4+ T cell responses indicating Class II mediated antigen presentation, while the combination of the antigen plus antibody generated both CD4 and CD8+ T cell response. CD8+ T cell responses are desired for the generation of direct cytotoxicity to the tumor cell. We further demonstrated that the PSA/antiPSA complexes generated HLA restricted CD8+ T cell responses to peptides known to bind to these alleles. We previously showed that apoptotic prostate tumor cell lines could induce immune responses when exposed to and presented by dendritic cells. However, these studies lacked specificity determinations. Using tumor cell lysates from the LNCaP cell line which produces large quantities of PSA and a nonPSA producing cell line (DU-145), we demonstrated that PSA specific responses could be generated with cell lysates from the former but not the latter.

One of the confounding issues in immunotherapy is the extent to which HLA allele differences in the population will restrict the capacity of given individuals to generate effective cellular immunity when a specific protein is administered as the vaccine. Using T cell lines and T cell clones generated from a patient with prostate cancer and a patient with granulomatous prostatitis, peptides were identified that were specifically presented by the HLA-B7 molecule. In addition, we also demonstrated that CD4+ T cell response to PSA that was restricted by the HLA Class II molecule, HLA-DR*1501. This is the first demonstration of a Class II restricted response to PSA and extends the number of alleles that are capable of presenting PSA derived peptides. In summary, the results of these studies are generating a basis for rational immunotherapy of prostate cancer.

(10) References

None

(11) Appendices

Figures 1-8; Table 1; One peer-reviewed manuscripts; Two abstracts; One review.

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Figure 1. T-cell responses to PSA generated with monocyte-derived dendritic cells armed with PSA or PSA-PSA antibody complexes

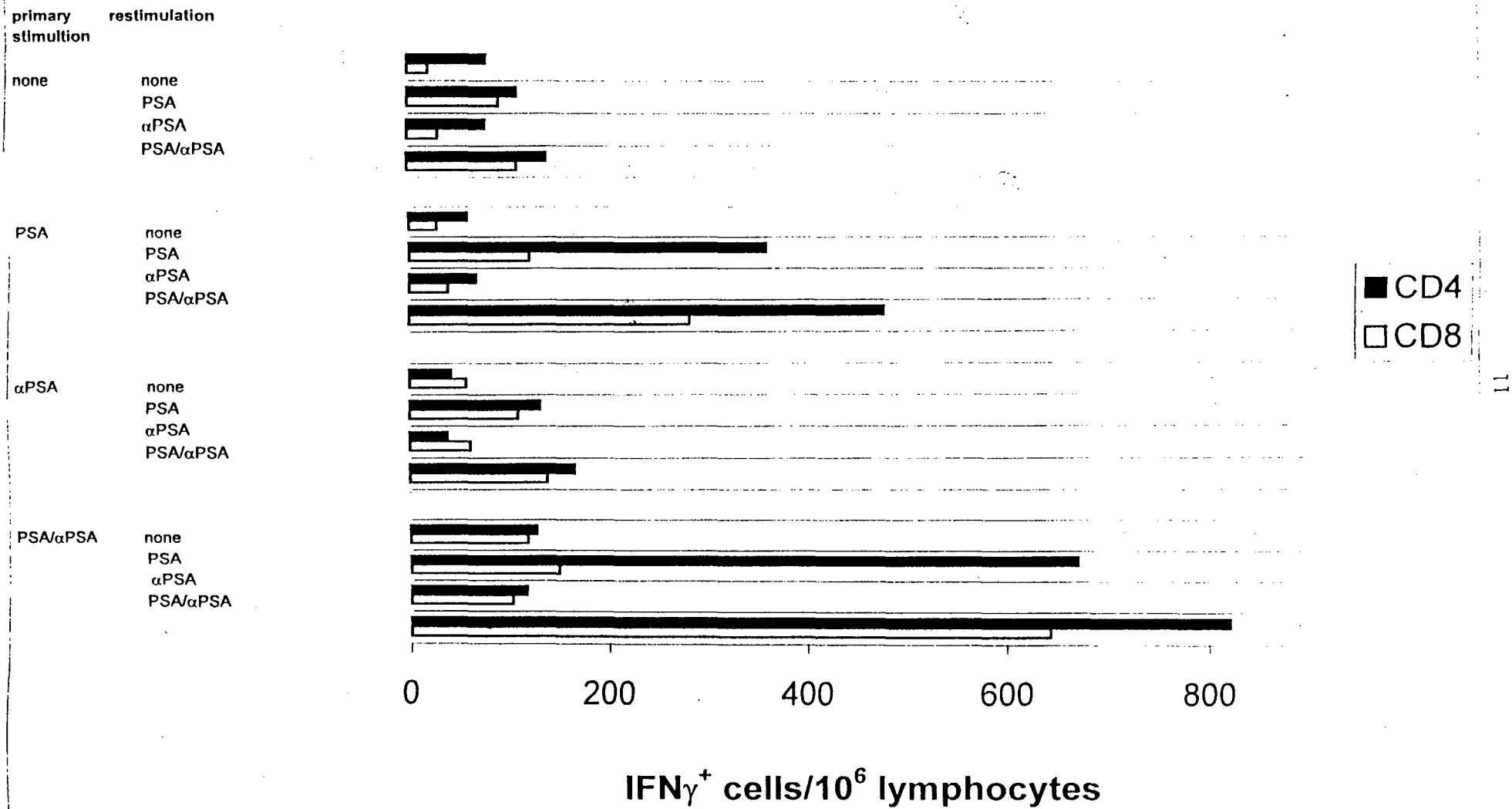
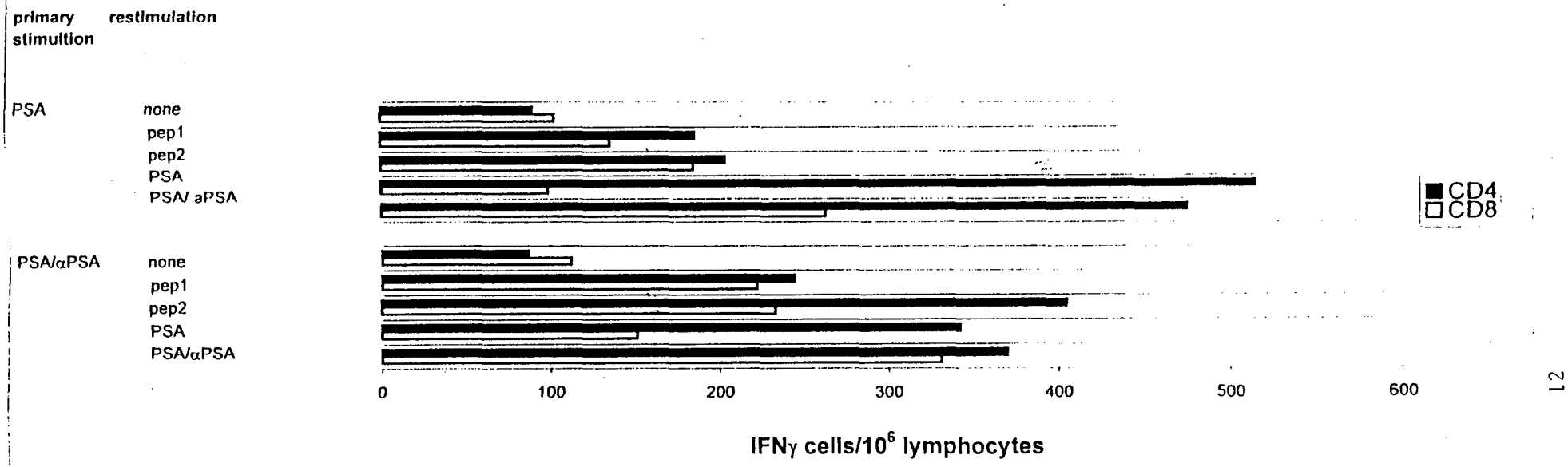


Figure 2. T-cell response to PSA peptides generated by DC armed with PSA and PSA-PSA antibody complex



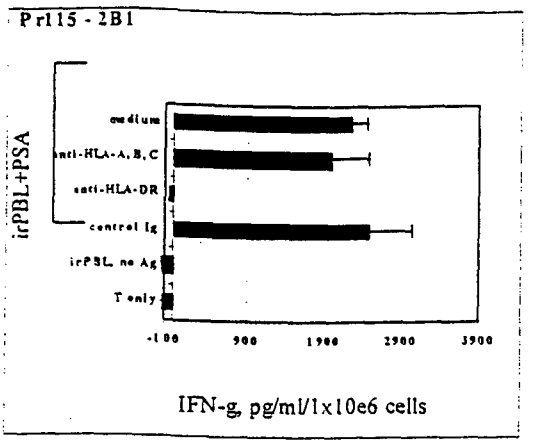


Figure 3. IFN-γ secretion of T cell clone 2B1 in response to PSA is inhibited by anti-HLA-DR mAb.

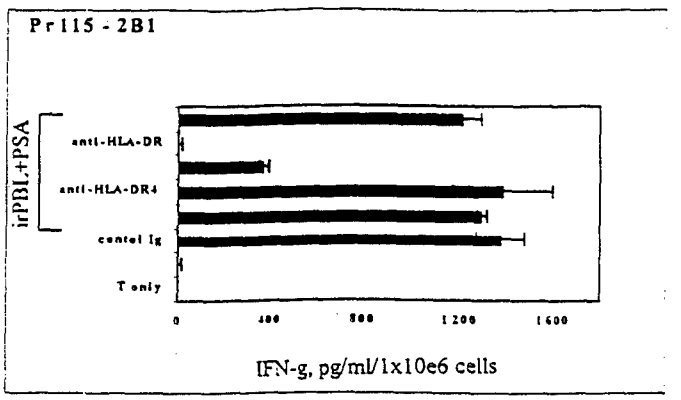


Figure 4. IFN-γ secretion of T cell clone 2B1 in response to PSA is Inhibited by anti-HLA-DR15 mAb.

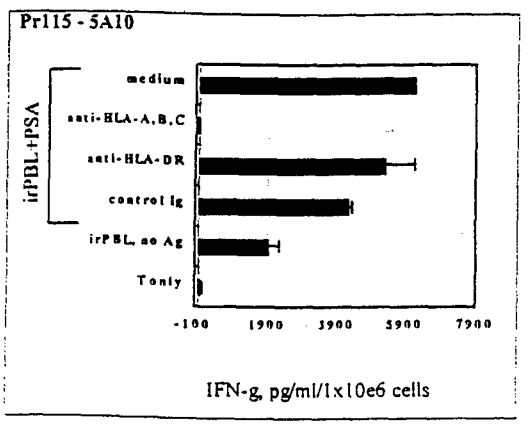


Figure 5. IFN-γ secretion of T cell clone 5A10 in response to PSA is inhibited by anti-HLA-A,B,C mAb.

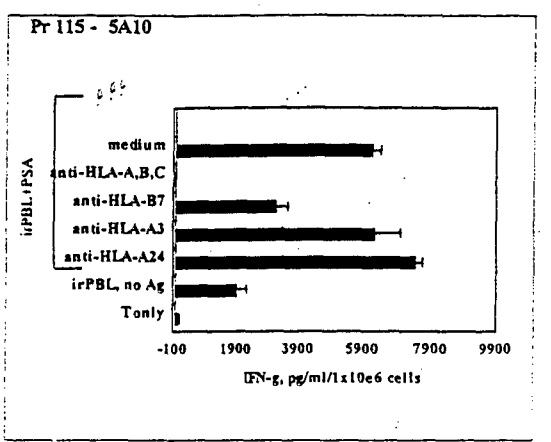


Figure 6. IFN-γ secretion of T cell clone 5A10 in response to PSA is inhibited by anti-HLA-B7 mAb.

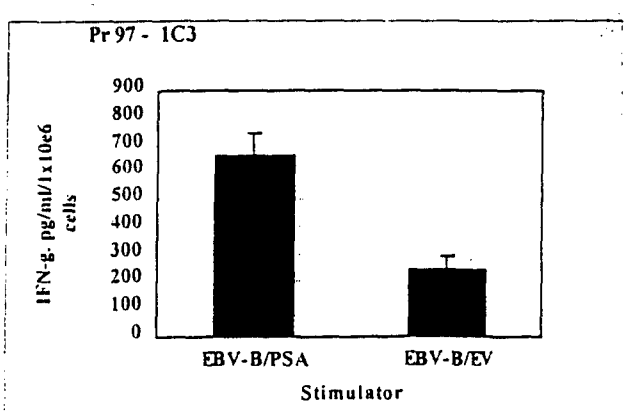
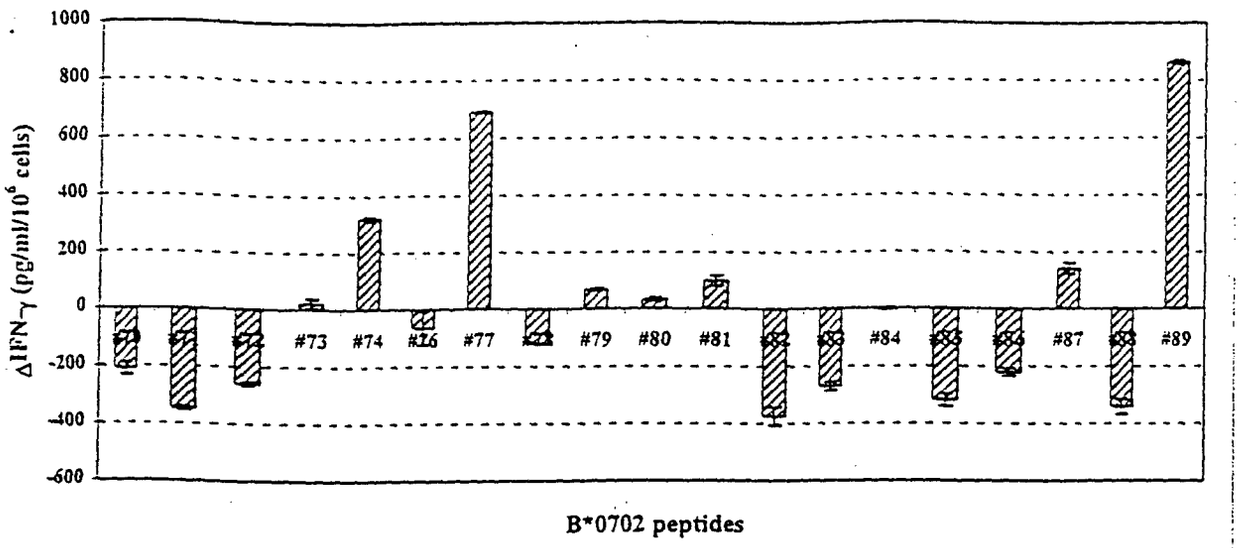


Figure 7. IFN-γ secretion of T cell clone 1C3 in response to autologous EBV-B cell line transduced with PSA.

8/4/00

Figure 8 Screening of HLA-B*0702-binding PSA peptides using PSA-specific HLA-B*0702 restricted CD8+ T cell line 5H10



- Positive peptides:
  #74 [EPALGTTCYA]
  #77 [RPSLYTKVVH]
  #89 [GSIEPEE FL]

Table 1. PSA specific T cell responses generated with DC armed with lysates of LNCaP cells.

Primary Stimulation	Restimulation	IFNγ production cells/10^5 total T cells CD4+	CD8+
0	0	22	18
LNCaP	0	41	27
DU-145	0	64	59
0	PSA	25	16
0	LNCaP	38	19
LNCaP	PSA	186	76
LNCaP	LNCaP	206	184
LNCaP	DU-145	49	63
0	DU-145	46	29
DU-145	PSA	33	17
DU-145	LNCaP	56	45
DU-145	DU-145	228	214

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PSA Is a Candidate Self-Antigen in Autoimmune Chronic Prostatitis/Chronic Pelvic Pain Syndrome

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BACKGROUND. Previous studies demonstrated that recognition of seminal plasma antigens can occur in patients with chronic prostatitis/chronic pelvic pain syndrome. This suggests that an autoimmune component may contribute to symptoms in some men. To determine if any of the principal secretory proteins of the prostate could be candidate antigens in autoimmune prostatitis, we examined the recall proliferative response of purified CD4 T cells in patients with chronic prostatitis and in normal volunteers using purified seminal plasma antigens and autologous dendritic cells.

METHODS. Peripheral blood mononuclear cells were harvested from 14 patients with chronic prostatitis and 12 normal volunteers by density gradient centrifugation. The stimulating cells were irradiated autologous dendritic cells produced by culture of monocyte-enriched fractions with IL-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). Purified CD4 T cells were the responding population. Recall proliferation assays were performed, using purified seminal plasma proteins as antigens.

RESULTS. In 14 patients with chronic prostatitis, we detected a greater than 2-fold increase in proliferative response to PSA compared to control in 5 patients (36%). No response to Prostatic Acid Phosphatase (PAP) or β-microseminoprotein was observed in these 14 patients. In 12 normal volunteer donors with no history of genitourinary disease or symptoms, no proliferative response above background was observed for any prostatic antigen.

CONCLUSIONS. The data suggest that some men with symptoms of chronic prostatitis have evidence of a proliferative CD4 T-cell response to PSA. PSA is a candidate antigen in chronic prostatitis/chronic pelvic pain syndrome and may be an appropriate target for immunotherapy for prostatic cancer. *Prostate 44:49–54, 2000.*

KEY WORDS: **prostatitis; prostate-specific antigen; autoimmunity; T lymphocytes; dendritic cells; prostate cancer**

INTRODUCTION

Chronic prostatitis/chronic pelvic pain syndrome is a common diagnosis, but very little is understood about the etiology of the disease. Men with this syndrome present with an episodic and relapsing condition characterized principally by pain in the pelvic region, voiding symptoms, and effects on sexual function [1]. These symptoms cannot be distinguished from those of men with acute bacterial infections of the prostate gland; however, the overwhelming majority of men with chronic symptoms cannot be demonstrated to have bacterial infection [2]. The disease represents a major problem in the US, resulting in 2 million office visits yearly to primary care physicians and urologists [3]. Most of these men are treated with prolonged courses of antimicrobials, with unknown and doubtful benefit.

Grant sponsor: National Institute of Diabetes, Digestive, and Kidney Diseases; Grant number: R01-DK53732; Grant sponsor: DOD Prostate Cancer Research Program; Grant number: DAMD17-98-1-8466; Grant sponsor: US Department of Veterans Affairs.

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We recently reported that some men with chronic prostatitis chronic pelvic pain syndrome have evidence of CD4 T lymphocyte recognition of seminal plasma, derived both from normal men and men with seminal vesicle atresia [4]. We used seminal plasma as the source of antigens in that study because a significant proportion of the volume of the semen is contributed by the prostate. In addition, semen from men with seminal vesicle atresia consists almost entirely of the secretions of the prostate and is characterized by azoospermia, absent fructose, and low volume. These data suggested that some men with chronic prostatitis/chronic pelvic pain syndrome could have an autoimmune component to their disease that could be either a cause or consequence of their symptoms.

To further define the potential antigens contained within the seminal plasma that could be recognized in these patients, we studied patients and normals using purified seminal plasma proteins of prostatic origin. The major secretory proteins of the prostate contained within the seminal plasma are prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and β-microseminoprotein (β-MSP) [5]. These proteins, purified from human seminal plasma, were used as antigens in CD4 T-cell recall proliferation assays in patients with chronic prostatitis/chronic pelvic pain syndrome and normal volunteers. We found that some men with chronic prostatitis/chronic pelvic pain syndrome had a CD4 T-cell proliferative response to PSA and that this was not observed in normal volunteers. This suggests that PSA is a candidate antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome.

MATERIALS AND METHODS

Culture Medium

Culture medium consisted of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% human AB serum (heat-inactivated) (Gemini Bioproducts, Calabasas, CA), 2% L-glutamine, and penicillin/streptomycin (Biofluids, Rockville, MD).

Preparation of Cell Populations

Fifty milliliters of peripheral blood were drawn from each normal volunteer and prostatitis patient into a syringe containing 1,000 units of heparin. The blood samples were centrifuged over a density gradient of Lymphocyte Separation Medium (ICN Biomedicals/Cappel, Aurora, OH) to obtain the peripheral blood mononuclear cell (PBMC) population. The PBMCs were then washed, and resuspended at 5×10^6 cells per ml in a 1:2 dilution of culture medium and PBS. The cells were then centrifuged over a 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient made up in PBS containing 5% human AB serum at 1,000*g* for 25 min at 4°C. This resulted in the separation of the PBMC into two distinct fractions, a cell population at the 40% Percoll gradient interface and a cell pellet at the bottom of the tube. Upon recovery and washing, the two populations appeared to be well-fractionated/separated on the basis of size of the cells, whereby the interface consisted predominantly of large-sized cells (monocytes and macrophages), while the pellet consisted mainly of small, uniform-sized cells (lymphocytes). The cell population from the interface was then incubated with anti-CD2- and anti-CD19-coated Dynabeads (DYNAL, Inc., Lake Success, NY), followed by depletion using the DYNAL-MPC-1 magnet as per the manufacturer's instructions. This technique always resulted in about 80% of the recovered cells postdepletion being positive for the monocyte surface antigen CD14, with less than 2% each of CD3 cells (T lymphocytes) and CD19 cells (B lymphocytes) as determined by FACS analysis. Similarly, the cell population from the pellet fraction was incubated with a cocktail of anti-CD8-, anti-CD14-, and anti-CD19-coated Dynabeads and subjected to the DYNAL magnet depletion technique. The resulting cell population recovered from the pellet fraction postdepletion was always greater than 90% positive for the T lymphocyte antigen CD4, as determined by FACS analysis.

Dendritic Cell Cultures

The highly enriched CD14 monocyte cell population from each patient was cultured in the presence of the cytokines GM-CSF and IL4 (Genzyme Corporation, Cambridge, MA) at a concentration of 10,000 U/ml for each cytokine. The cells were cultured in 6-well plates at $2–3 \times 10^6$ cells per well in 3 ml of Culture Medium (CM). These were incubated at 37°C and 5% CO_2 for a period of 7–8 days. FACS analysis of the cells harvested at the end of the culture period indicated them to be enriched for cells displaying antigens consistent with a dendritic cell (DC) phenotype. These cells were highly positive for the expression of HLA DR, CD80, and CD86, with low expression of CD83, and negative for the expression of CD14 and CD19 antigens.

Proliferation Assay

Recall antigen proliferation assays were performed using irradiated DC (3,000 cGy, ^{137}Cs source) at 1×10^4 cells/well in the absence and presence of 1×10^5 CD4 T cells/well in 96-well U-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ). Purified prepara-

tions of the prostatic proteins PAP, PSA, and β-microseminoprotein PSP94 (Fitzgerald Industries, Inc., Concord, MA) were added to the respective wells at 10 μg/ml, and tetanus toxoid was used at 1:100 dilution of the commercially available product (Connaught Laboratories, Willowdale, Ontario, Canada). Some wells did not have any antigens added to them in order to measure the nonspecific or background stimulation of the CD4 T cells by DC and components of the culture medium. All stimulations were performed in duplicate or triplicate, and the plate was cultured for a period of 5 days at 37°C and 5% CO_2. ^{3}H-thymidine was added at 1 μCi/well on the fourth day of culture, and the plates were harvested on day 5 using a Tomtec cell harvester (Wallac, Inc., Gaithersburg, MD); the counts per minute (CPM) were determined by liquid scintillation, counting with the Betaplate System (Wallac, Inc.). The stimulation index (SI) was calculated as the mean CPM obtained in the presence of antigen divided by the mean CPM obtained in the absence of antigen for all samples.

Statistical Analysis

The stimulation index was calculated for each antigen in prostatitis patients and normal volunteers. A stimulation index greater then 2, meaning that the proliferative response to the three prostatic antigens was greater than twice the CPM response in the absence of antigen, was interpreted as a significant response for that prostatic antigen. The number of individuals with a significant SI response to each prostatic antigen was compared between the normal and prostatitis groups, using Fisher's exact test.

For the response to the control antigen tetanus toxoid (TT), we expected that most individuals would be responsive to this antigen but that the degree of response would be variable, depending on time since last vaccination, Major Histocompatibility Complex (MHC) haplotype, and other variables. Since patients and volunteers were expected to be responsive to TT, we compared the mean proliferative response of the normal and prostatitis groups using the Wilcoxin rank sum test. A P value less than 0.05 was interpreted as excluding the null hypothesis for both tests.

RESULTS

The characteristics of the patients and volunteers are shown in Table I. The median age of the patients was 41 years (range, 24–57), and the median age of the normal volunteers was 34 years (range, 22–65).

The results of the recall proliferation assays using prostatic antigens are shown in Figure 1. In 5 of the 14 prostatitis patients, a proliferation index greater than 2 was observed when PSA was used as the antigen. No response was observed among prostatitis patients to PAP or β-MSP. The stimulation index for normal volunteers did not exceed 2 for any of the three prostatic

TABLE I. Demographics of Prostatitis Patients and Normal Volunteers

Subject number	Age	Race
Prostatitis patients		
Pr85	56	White
Pr86	57	White
Pr87	32	Hispanic
Pr88	54	White
Pr90	49	White
Pr92	24	Black
Pr94	34	White
Pr98	25	White
Pr101	42	White
Pr102	33	White
Pr103	43	White
Pr104	57	White
Pr105	41	White
Pr106	36	White
Normal volunteers		
ND12	39	Asian
ND13	40	Black
ND14	28	White
ND15	38	White
ND16	65	White
ND17	24	Asian
ND18	22	White
ND20	23	White
ND21	26	White
ND22	31	White
ND23	58	White
ND24	42	White

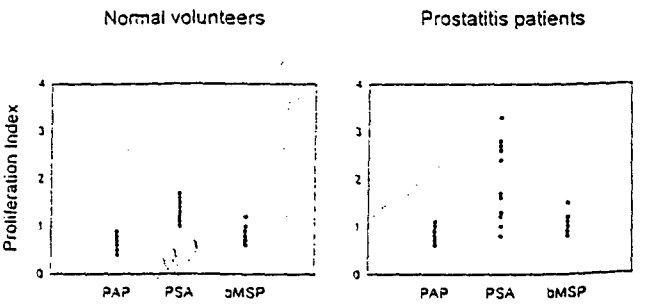

Fig. 1. Purified CD4 T lymphocytes from normal volunteers (left) or prostatitis patients (right) were stimulated with irradiated autologous dendritic cells pulsed with purified prostatic antigens, as shown. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a 5-day coculture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by the CPM in the absence of antigen. PAP, prostatic acid phosphatase; PSA, prostate-specific antigen; bMSP, beta-microseminoprotein.

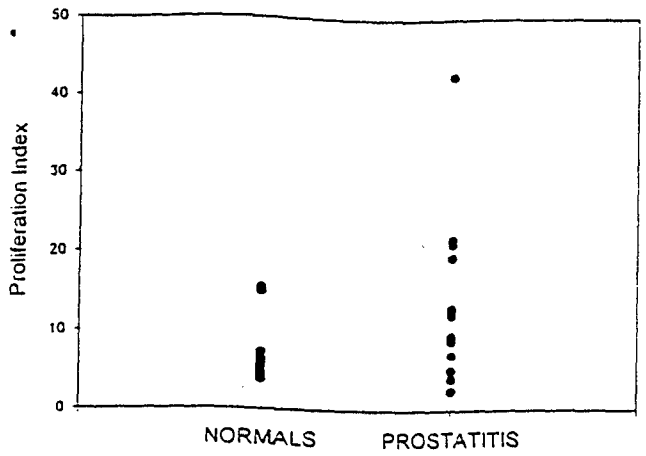

Fig. 2. Purified CD4 T lymphocytes from normal volunteers or prostatitis patients were stimulated with irradiated autologous dendritic cells pulsed with tetanus toxoid. Proliferation of the responder CD4 T cells was determined by the uptake of ^{3}HTdR following a 5-day coculture. Proliferation index is the ratio of the counts per minute (CPM) in the presence of antigen divided by the CPM in the absence of antigen.

protein antigens tested. The difference between the response to PSA of prostatitis patients was significantly different from that of normal volunteers (P = 0.03, Fisher's exact test). There was no difference between the stimulation indices for PAP or β-MSP when comparing normals to prostatitis patients.

CD4 lymphocytes from normal volunteers and prostatitis patients were responsive to the recall antigen TT, as shown in Figure 2. The mean (SEM) proliferation index was 7.0 (4.0) for normals and 13.5 (10.3) for prostatitis patients. Hence, CD4 T lymphocytes from both groups had comparable positive proliferative responses to TT.

DISCUSSION

We previously demonstrated that men with chronic prostatitis/chronic pelvic pain syndrome have evidence of CD4 T-cell reactivity with seminal plasma and that the antigen being recognized is derived from the prostate [4]. To determine if any of the principal secretory products of the prostate that are secreted into the seminal plasma might be recognized by T cells, we examined CD4 T lymphocytes from men with chronic prostatitis/chronic pelvic pain syndrome for a proliferative response to purified prostatic proteins obtained from the seminal plasma. We found that CD4 T cells from some men with chronic prostatitis/chronic pelvic pain syndrome manifested a proliferative response to PSA that was not present in normal male volunteers. This suggests that PSA is being recognized by the immune system in some men with chronic prostatitis/chronic pelvic pain syndrome, providing evidence that this disorder represents an autoimmune disease in some patients.

The cause of chronic prostatitis/chronic pelvic pain syndrome is unknown. Infection has long been viewed as the etiology for this problem. Certainly patients with bacterial infection of the prostate do exist. Patients with bacterial infections of the prostate typically respond promptly to therapy with antimicrobial agents. However, most patients with chronic symptoms fail to achieve a durable and lasting remission of symptoms with antimicrobial therapy. In addition, a large body of literature to date has failed to provide convincing evidence that some fastidious organism is responsible for symptoms in a significant proportion of patients. Alternate explanations for chronic prostatitis deserve further study.

The hypothesis that chronic prostatitis/chronic pelvic pain syndrome represents an autoimmune disease in some patients is supported by several observations. First, the chronic, relapsing, and episodic nature of symptoms is consistent with an autoimmune etiology. Second, the prostate is commonly found to contain inflammatory infiltrates when prostate tissue is removed for any reason, typically for prostatic cancer or benign prostatic hypertrophy [6]. The reasons for this inflammation and the implications of its presence are completely unknown. Third, we presented evidence that CD4 T lymphocytes from some men with chronic prostatitis/chronic pelvic pain syndrome manifest a recall proliferative response to seminal plasma [4]. Lastly, we have also shown that the proinflammatory cytokines TNFα and IL-1β are elevated in the semen of some men with chronic prostatitis/chronic pelvic pain syndrome, but not in asymptomatic normal men [7]. These proximal, proinflammatory cytokines are also elevated in the joint fluid in rheumatoid arthritis patients, and the inhibition of these cytokines in patients with rheumatoid arthritis has clearly improved symptoms [8]. These data are consistent with but do not prove that prostatitis is an autoimmune or autoinflammatory condition in some men.

The hallmark of autoimmunity is the demonstration of an immune response against a normal self-antigen. In our study, by choosing to measure the proliferation of T lymphocytes as an indicator of the presence of an immune response in subjects, we were able to demonstrate immunity to PSA in only 5 of the prostatitis patients. It is possible that the use of or inclusion of other immunological assays, such as the detection and measurement of certain cytokines in the lymphocyte cultures stimulated with prostatic antigens, may increase our ability to find evidence of an immune response in a larger number of these patients. Alternatively, the possibility exists that the remaining 9 patients whose cells did not register a proliferative

response may truly have a different etiology for their condition. This is a strong possibility, since the rather loose characterization of pelvic pain as a defining and prerequisite symptom for being considered a patient with prostatitis tends to result in the inclusion of a broad group of individuals who may have other factors contributing to their condition.

The study of antigens in the prostate that could be the target of a T lymphocyte response has principally occurred as a result of investigations in prostate cancer immunotherapy. The central question of cancer immunotherapy is whether antigens exist in human cancers that can be recognized by the immune system, and whether this recognition can be therapeutic in patients with cancer. The description of many such tumor antigens in the past few years, principally in melanoma, led to the surprising finding that many melanoma antigens were derived from normal proteins of melanocyte lineage cells (reviewed in Houghton [9]). This suggests that cancer recognition by the immune system is self-recognition, and that many of the features of a successful cancer immunotherapy will resemble autoimmunity. Hence, the search for immunotherapies for prostatic cancer has included a search for normal self-antigens in the prostate. The goal of prostate cancer immunotherapy has therefore included the goal of inducing autoimmune prostatitis. Thus, our observation that this phenomenon may be occurring in some men with chronic prostatitis is interesting both in the hopes of explaining a baffling chronic condition as well as providing potential targets for prostate cancer immunotherapy.

Several investigators have provided evidence that prostatic antigens can be recognized by T cells. Liu et al. [10] demonstrated in rats that vaccination with a syngeneic prostate homogenate could induce a T-cell immune response to prostate steroid-binding protein (PSBP). This protein, like PSA in humans, is a major secretory product of the rat prostate, but no homologue in humans exists. Vaccination of rats with purified PBSP induced a vigorous antibody and T-cell response and induced inflammatory infiltrates destructive to the prostatic epithelium in some animals. Fong et al. [11] immunized rats with a vaccinia virus expressing human PAP and demonstrated that an inflammatory prostatitis in animals could be engendered. A vaccinia construct containing rat PAP could not induce prostatitis in vaccinated animals. This suggests that a T-cell response to self prostatic antigens can be induced in rats.

In human studies, the culture of T cells demonstrating clear specificity for prostatic antigens has been very difficult. All human tumor antigens recognized by T cells have been identified by the production of such specific T-cell lines, either directly from human tumors or from the peripheral blood of cancer patients, and by the subsequent identification of the antigen being recognized using a variety of techniques. Since prostate specific T-cell lines have not been reported by these methods, investigators have identified known proteins that are specifically expressed by the prostate and have attempted to prove that these proteins or peptides derived therefrom are potential antigens for T lymphocytes. It is possible to induce specific T-cell lines from normal volunteers by in vitro stimulation of lymphocytes with synthetic peptides and interleukin-2 to expand reactive T cells recognizing the peptide [12]. Using such an approach, PSA has been identified as a potential antigen for T cells, principally in normal volunteers of defined Human Leukocyte Antigen (HLA) haplotypes [13–16]. We examined a group of HLA-A2 prostate cancer patients and found similar reactivity with one PSA peptide in only 1 of 7 patients [17].

Other prostatic protein antigens have been explored as potential targets for T cells. Peshwa et al. described CD8 T-cell recognition of peptides derived from PAP [18]. Prostate specific membrane antigen (PSMA), a transmembrane prostate-specific protein, also has been demonstrated to contain antigenic sequences that can be used to generate peptide-specific T cells [19]. These preclinical studies have been performed to support various trials of prostatic cancer immunotherapy with antigen-pulsed dendritic cells [20,21] or vaccination with a recombinant vaccinia virus expressing human PSA [22]. These early clinical trials are designed to induce a prostate-specific immune response presumably directed against metastatic prostate cancer deposits as well as the intact prostate gland. Our data support the notion that PSA may be a target of a human immune response in some men with chronic prostatitis/chronic pelvic pain syndrome, suggesting that attempts to target this antigen in prostate cancer may be successful.

In summary, the data are consistent with the interpretation that some men with chronic prostatitis/chronic pelvic pain syndrome have an autoimmune component to their disease and that PSA may be a normal self-antigen against which this immune response may be directed. In order to establish this definitively will require the culture of T-cell lines specific for PSA, identification of the PSA peptide epitopes being recognized and the HLA haplotypes used to present the peptides, and the determination of precursor frequency of peptide-specific T cells in normals and patients through the course of the disease.

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This paper is dedicated to Donald S. Coffey, Ph.D., on the occasion of celebrating his 40 years of scientific

contributions and his contribution to the lives of all of us fortunate enough to know him.

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Abstract Title: **Generation of prostate specific antigen (PSA)-reactive T cell lines - implications for prostate cancer immunotherapy**

Abstract Author(s): E.N.Klyushnenkova, S. Ponniah, D.L.Mann, and R.B.Alexander

Abstract Institution(s): University of Maryland, Baltimore, MD

Abstract Body: One of the approaches to cancer immunotherapy is the induction of autoreactivity to differentiation antigens shared by tumor cells and normal cells of the same lineage. We have previously demonstrated that some patients with chronic prostatitis may have an autoimmune component to their disease because they have circulating T cells recognizing normal prostatic proteins and such cells were not found in normal men. To characterize this phenomenon further, we stimulated PBMC from such patients with purified PSA obtained from seminal fluid. After 14 days of culture, the cells were re-stimulated with PSA in the presence of irradiated autologous PBMC and IFN-γ secretion in the culture supernatants was measured by ELISA. T cells from 3 of 5 patients tested showed a specific and significant increase in IFN-γ secretion in response to PSA in secondary cultures. Several T cell clones derived from these cultures by limiting dilution retained their PSA reactivity as measured by increase in IFN-S\gamma$ secretion. The PSA-specific responses of 4 of these clones were blocked specifically by anti-HLA-DR mAb while 3 other clones showed specific inhibition by anti-HLA-ABC mAb. Currently we are undertaking the characterization of fine specificity and MHC restriction of these clones. The data demonstrate that PSA-reactive CD4+ and CD8+ T cells can be derived from some patients with chronic prostatitis consistent with an autoimmune etiology of the disease. Since most prostate cancer cells continue to express PSA, immunization with PSA-derived peptides that are recognized by autoreactive T cells can be used as potential vaccines to induce anti-tumor reactivity in prostate cancer patients of appropriate HLA type.

Society for Basic Urologic Research
Fall Symposium
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November 9 – 12, 2000

Abstract Deadline: August 15, 2000

GENERATION OF HUMAN CD4 AND CD8 T LYMPHOCYTE LINES THAT RECOGNIZE PROSTATE SPECIFIC ANTIGEN (PSA) FROM PERIPHERAL BLOOD OF A PATIENT WITH GRANULOMATOUS PROSTATITIS. E. N. Klyushnenkova, S. Ponniah, A. Rodriguez, J. Kodak, D. L. Mann, and R. B. Alexander. University of Maryland, School of Medicine, Baltimore, MD

We have previously demonstrated that some patients with chronic prostatitis may have an autoimmune component to their disease because they showed a specific recall T cell proliferative response to PSA that was absent in normal donors. In order to demonstrate directly that PSA is a T cell antigen we generated long-term CD4+ and CD8+ T cell lines from peripheral blood mononuclear cells (PBMC) of a patient with granulomatous prostatitis using purified PSA as an antigen and recombinant IL-2 and IL-7. The HLA type of the patient was HLA-A*0301, A*2402, B*0702, B*1501, Cw*0303, Cw*0702, DRβ1*04, DRβ1*1501, DRβ4*01, DRβ5*0101. Several T cell clones derived by limiting dilution were specific for PSA as measured by at least a five-fold increase in IFN-γ secretion in response to PSA presented by irradiated autologous PBMC compared to unpulsed PBMC. Three clones were predominantly CD4+ whereas the other three clones were CD8+ as determined by flow cytometry. The PSA-specific responses of CD4+ clones were blocked by anti-HLA-DR mAb while CD8+ clones showed specific inhibition by anti-HLA-ABC mAb. IFN-γ secretion in response to PSA by all CD4+ T cell lines was blocked by antibody against HLA-DR15, but not HLA-DR4. The response of all CD8+ T cell lines was blocked by antibody against HLA-B7, but not HLA-A3 or HLA-A24. To confirm the specificity and HLA-restriction of PSA-specific T cell lines, we prepared EBV-B cell lines from HLA-matching donors that expressed recombinant PSA endogenously. EBV-B cell lines were transduced with a retroviral vector containing the PSA gene or were infected with a recombinant PSA-expressing vaccinia virus, and the expression of PSA was monitored by ELISA. CD4+ T cell lines secreted IFN-γ in response to HLA-DR15+ EBV-B cell lines that expressed PSA, whereas CD8+ T cell lines responded to HLA-B7+ PSA-expressing lines. HLA-matched targets transduced by control vectors as well as HLA-mismatched PSA-expressing targets did not induce the responses. Our data demonstrate that PSA-reactive CD4+ and CD8+ T cells can be derived from a patient with granulomatous prostatitis consistent with an autoimmune etiology of the disease. Our results extend the number of class I and class II alleles that can present PSA to generate CD8+ and CD4+ T cell responses. Since most prostate cancer cells continue to express PSA, immunization with PSA-derived peptides that are recognized by autoreactive T cells can be used as potential vaccines to induce anti-tumor reactivity in prostate cancer patients of appropriate HLA type.

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THERAPEUTIC ANTIBODIES FOR PROSTATE CANCER

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ABSTRACT

Twenty-five years ago, monoclonal antibodies were envisioned as magic bullets capable of targeting radioisotopes, toxins or cytotoxic drugs to the tumor site. It was soon realized that the potential of therapeutic antibodies far exceeded their use as carrier molecules and that native antibodies could also act as effector molecules capable of triggering a wide variety of anti-tumor responses. Today, we recognize that the utility and versatility of antibody-based products are unlimited; at the same time we have also learned that many obstacles need to be addressed to make antibody therapy an effective treatment modality. Past experiences from clinical trials and new development in the field of antibody technology have paved the way towards the creation of new strategies capable of circumventing or minimize these difficulties. Antibody-therapy is currently being tested in multiple trials for a variety of cancers including prostate cancer. This review discusses the different antibody-based strategies currently under investigation for prostate cancer.

INTRODUCTION

Prostate cancer is the most common invasive malignancy and second leading cause of cancer deaths in United States males [1]. Although locally confined disease is curable, 20% to 40% of patients develop recurrent disease after surgery or radiation therapy. Metastatic prostate cancer responds initially to androgen withdrawal therapy, but hormone resistance always develops and the majority of patients inevitably progress to

incurable, androgen-independent disease [2]. According to the American Cancer Society, an estimated 179,300 new cases will be diagnosed in 1999 and 37,000 men will die from the disease each year. Given the profound medical impact of prostate cancer and the lack of adequate therapies, there is a need to develop new modalities of treatment.

Our understanding of prostate cancer biology has grown tremendously over the last decade. This increase in knowledge has been accompanied by an impressive proliferation of new therapies ranging from new combinations of traditional therapeutic agents to novel agents intended to interfere with multiple aspects of prostate cancer progression. For example, novel approaches being tested in early clinical trials include immunotherapy, anti-angiogenesis therapy, differentiation therapy, and gene therapy [3]. This review will focus only on novel therapies employing antibodies as therapeutic agents. We do not intend to provide a complete inventory of the different strategies being studied, but rather to provide a general overview of the approaches under development.

MONOCLONAL ANTIBODIES FOR CANCER THERAPY

Monoclonal antibodies (mab) with their potential for diagnosis and therapy have marked a new era in the management of cancer. The transition of mab from laboratory reagents to clinical diagnostic agents was rapid and led to the development of invaluable assays for the detection and monitoring of many types of cancer. The development of therapeutic antibodies was, as expected, more challenging and their transition to the clinic much slower. The recent approval by the Food and Drug Administration (FDA) of two

recombinant mab for the treatment of cancer has generated considerable enthusiasm for antibody therapy. The first of these, Rituxan (IDEC Pharmaceuticals Inc.) approved in 1997 for non-Hodgkin's lymphoma, is a chimeric antibody directed against the CD20 antigen present on normal and malignant B-lymphocytes [4]. Trastuzumab or Herceptin (Genentech) was approved in 1998 for breast cancer. This unconjugated mab is directed against the product of the proto-oncogene HER2/neu that is overexpressed by one-third of breast cancer patients [5]. In addition to cancer therapy, mab have also been approved for cancer imaging and in other clinical settings such as transplantation (to abrogate rejection), and cardiovascular disease (to prevent clotting of vascular stents). Today more than 100 mab are being developed around the world as potential anti-cancer agents. More details on antibody therapy can be found in the following recent reviews [6, 7].

Despite the tremendous progress made in the field of immunology and cancer biology, we still know little about how mab operate *in vivo*. There are essentially 2 types of therapeutic antibodies used for cancer treatment: conjugated mab that are used as a vehicle for the delivery of cytotoxic agents, and mab that are themselves therapeutically active. The use of mab to target cytotoxic drugs to tumor cells was for many years the most popular approach to antibody-based therapy. In this context, antibodies that bind antigens on tumor cell surfaces have been linked to an agent which is therapeutically active such as toxins, drugs or radioisotopes. Use of unconjugated mab on the other hand, depends on the ability of the mab itself either to kill cells directly (*e.g.*, deliver an apoptotic signal) or to elicit an anti-tumor biological response. For example therapeutic antibodies can be designed to trigger an anti-tumor immune response, suppress tumor blood supply, or directly act on molecules necessary for tumor growth.

With advances in molecular biology technology, it is possible today to construct recombinant antibodies with wide variations in size, configuration, valence, and effector capabilities. As a result, antibodies with multiple effector functions can be generated allowing the development of novel treatment strategies. Such techniques also permit the production of therapeutic antibodies with minimal immunogenicity [8].

If we consider the clinical characteristics of cancer as one of the important criteria in the creation of effective strategies, several aspects of prostate cancer support the development of antibody-based therapies. In many cases, therapeutic approaches are frequently limited by expression of the targeted antigen on normal as well as malignant cells. Prostate cancer expresses two of the most specific tumor markers known in cancer biology: prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA). Antibodies with high affinity and specificity can specifically target these antigens. Other aspects in favor of antibody therapy for prostate cancer are the small volume of metastatic lesions and their accessibility to circulating antibodies. For instance, prostate cancer metastasizes predominantly to bone marrow and lymph nodes; these sites have been effectively treated by antibodies in other settings (*e.g.*, breast cancer and lymphoma). Finally, the maintenance of the quality of life of patients is an important point to consider, especially for slowly progressing diseases such as prostate cancer. Results from clinical trials have demonstrated that the injection of unconjugated antibodies is safe, with only minimal side effects.

PROSTATE TUMOR ANTIGENS FOR ANTIBODY THERAPY

Prostate Specific Antigen (PSA)

PSA (or hK3) is a 33 kDa serine protease belonging to the kallikrein family. This protein is primarily produced by the prostatic epithelium and is secreted into the seminal plasma where it can be found in high concentration. PSA is also detected in low levels in the sera of healthy males without clinical evidence of prostate cancer. However, with prostate malignancy, circulating levels of this antigen increase markedly, correlating with the clinical stage of the disease. PSA is now the most widely used marker for prostate cancer and is today regarded as the best tumor marker available. Virtually all primary (87-100%) and metastatic (94-100%) prostatic carcinomas stained positively with anti-PSA antibodies [9]. However, the intensity of the staining decreases in poorly differentiated primary tumors and in metastases [10]. PSA is neither tumor nor organ specific and has been found to be present in a number of female tissues and body fluids. For example, ultrasensitive assays for measuring PSA protein and mRNA have demonstrated that this protein is present in breast tissues and breast secretions, endometrium, amniotic fluid; sweat, periurethral and anal glands; and tumors of colon, lung, ovary, liver, kidney, adrenal, and salivary glands [11].

Prostate Specific Membrane Antigen (PSMA)

PSMA is a 100 kDa type II transmembrane protein produced primarily by the prostate gland. A high proportion of primary (90-100%) and metastatic (50-98%) prostate cancer expresses PSMA [12-14] and the cellular expression of this protein is increased in high-grade and hormone insensitive prostate cancer [15, 16]. In addition to the prostatic

epithelium, endothelial cells lining the capillary bed of a variety of tumors show positive immunoreactivity for PSMA [13, 17]. This observation suggests that PSMA could also be used for targeting the tumor vasculature. Although PSMA is considered as an excellent marker for prostate cancer, the expression of this antigen is not restricted to the prostate gland. Small but significant levels of PSMA have been detected in salivary glands, brain, duodenal mucosa and proximal renal tubules [13, 18, 19]. Moreover, PSMA-like proteins (PSMA' or PSM') have recently been identified [20, 21]. The extraprostatic expression of PSMA-like proteins and the cross reactivity of PSMA antibodies with PSMA-like proteins is of concern for PSMA-antibody therapy. However slight structural differences between PSMA and PSMA-like proteins should allow the production of antibodies that specifically target PSMA.

TAG-72

TAG-72 is a high molecular weight glycoprotein related to the sialylated Tn antigen expressed on a range of human carcinomas including colorectal, gastric, pancreatic, ovarian, endometrial, breast, non-small cell lung, and prostate. The expression of TAG-72 was demonstrated in both primary and metastatic prostate cancer as well as in androgen dependent- and independent tumors. In general, the proportion of cells staining with the antibody and the intensity of staining are higher in the primary (80%) than metastatic lesions (17-50%) and higher for hormone naïve (84%) than for androgen-independent tumors (50%). High level of TAG-72 expression is also found in healthy tissues such as colon, stomach, pancreas, ovary and testis [22, 23].

Epidermal growth factor receptor (EGFr)

Epidermal Growth Factor Receptor (EGFr or C-erbB) is a transmembrane glycoprotein with specificity for either EGF or TGF-alpha. The EGF receptor system is important in normal cell proliferation, migration and differentiation, and deregulation of this system is commonly observed in human cancers. Overexpression of EGFr has been reported in a wide variety of tumors including breast, ovarian, lung, and squamous cell carcinomas, and has been associated with less favorable prognosis and inferior disease-free survival. Similarly, for prostate cancer, a correlation between overexpression of the EGFr and poor clinical prognosis has been suggested. EGFr has been detected in both primary and metastatic prostate cancer, however, the level of expression seems much lower compared to other type of cancers [24-26].

HER-2/neu (ErbB2)

Her-2/neu proto-oncogene is the second member of the EGFr family. This gene encodes a 185 kDa transmembrane glycoprotein receptor ($p185^{HER2}$) normally expressed at a low level in a number of secretory epithelial cells. Her-2/neu amplification has been found in many tumors, including breast, ovarian, lung, gastric, and oral cancers [27]. Despite numerous studies, the overexpression of Her-2/neu in prostate cancer remains controversial [23, 28-32]. *In vitro* studies with different prostate tumor cell lines provide strong evidence supporting the role of Her-2/neu in the progression of prostate cancer to the androgen-independent state [33, 34].

THERAPEUTIC ANTIBODIES FOR PROSTATE CANCER

Anti-PSA antibodies

In addition to its utility as a marker for diagnosis and monitoring of prostate cancer, PSA is also recognized as an excellent target for immunotherapeutic strategies. An important point to consider for antibody-based therapies directed against PSA is the fact that this antigen is a secreted protein; such strategies can consequently not rely on an ADCC (antibody-dependent cell cytotoxicity) or CDC (complement-dependent cytotoxicity) mechanisms. We believe that this fact does not detract from the development of effective therapeutic products. Indeed past experience from clinical trials indicates that in most cases the amount of antibody targeted to the tumor site is not sufficient to elicit an ADCC or CDC cytolytic activity that could be translated into therapeutic effect. In addition, with the progress made in the field of immunology, we begin to understand that antibodies act through multiple mechanisms of action and that the observed therapeutic effect may be independent of an ADCC or CDC mechanism.

In collaboration with the biopharmaceutical company AltaRex Corp., we have developed a new treatment modality for prostate cancer that uses as a therapeutic agent a murine monoclonal antibody directed against PSA (ProstaRex^{TM}). AltaRex proprietary antibody-based immunotherapy technology is designed to enhance the ability of the human immune system to produce its own anti-tumor response [35-40]. In 3 different animal models, we have demonstrated that this antibody can inhibit the growth of PSA expressing tumors (Dr. Leveugle, personal communication). Results from *in vivo* and *in vitro* experiments highlight the importance of immune complex formation and suggest

that the capture of PSA / ProstaRex™ complexes by antigen presenting cells (APCs) plays a major role in the induction of an autoimmune response against PSA-expressing cells. It is today well established that the capture of immune complexes by Fc-receptors on APCs results in efficient priming of T-cell responses both *in vitro* and *in vivo* [41]. The enhanced processing of antigens into peptides presented by MHC class II molecules and the efficient stimulation of $CD4^+$-cells has been demonstrated in several systems [42-44]. Of particular interest is the recent demonstration that immune complexes can also promote the maturation of dendritic cells and efficient MHC class I presentation of peptides from exogenous IgG-complexed antigens [45]. In accord with these observations, we observed a significant stimulation of both $CD4^+$ and $CD8^+$ responses against PSA after presentation of PSA / ProstaRex™ complexes to human dendritic cells (Dr. Mann, personal communication). The region recognized by ProstaRex™ resides between the amino acids 139-163 of the PSA molecule (Dr. Leveugle, personal communication). This region has elicited much attention since it contains several motifs for HLA class I molecules, and is implicated in the generation of cytotoxic T-lymphocytes capable to lyse prostate tumor cells [46-48]. We believe that the specificity of ProstaRex™ for the region 139-163 of PSA is of particular importance since the binding of ProstaRex™ to PSA may protect this domain from extensive (inappropriate) proteolytic cleavage and favor the generation of HLA-I peptides essential for the activation of cytotoxic T-lymphocytes. For instance, a clipped form of PSA at residues 145-146 has been described indicating the sensitivity of this site to proteolytic degradation [49]. The proteolysis of PSA between amino acids 145-146 will preclude the

generation of HLA-peptide 141-150 and therefore the stimulation of cytotoxic lymphocytes specific for this sequence.

In a different aspect, the binding of ProstaRex™ to the region 139-163 of the PSA antigen may also be beneficial by inhibiting the enzymatic activity of PSA. PSA is a serine protease with chymotrypsin-like activity. A complete inhibition of this activity by ProstaRex™ was observed *in vitro* (Dr. Leveugle, personal communication). The enzymatic activity of PSA has been implicated in the proteolytic cascade during prostate cancer invasion and metastasis. Blocking PSA proteolytic activity with PSA-specific mab resulted in a dose-dependent decrease in the invasion of LNCaP cells in a matrigel assay [50].

Finally we have demonstrated that the immunization of mice with ProstaRex™ induces the production of anti-anti-idiotypic antibodies (Ab3) [51]. Such antibodies are produced by the host itself in response to the immunization and have by definition the same specificity than the injected antibody (i.e. ProstaRex™) [52]. Because Ab3 antibodies share the same specificity with ProstaRex™ , it is expected that they will have the same anti-tumoral activity. The advantage of Ab3 antibodies relies in their very long circulating half-life (up to several months) and consequently their prolonged anti-tumoral effect. For example Ab3 antibodies may be of considerable help in priming T-cells through antigen-antibody complexes since an efficient induction of T-cell response requires a constant stimulation for a period of 2 to 4 weeks by activated APCs. Similarly, Ab3 antibodies may play a role in the maintenance of the inhibition of the PSA enzymatic activity and consequently in the inhibition of cancer invasion and metastasis. Ab3 antibodies as well as the injected ProstaRex™ could also accumulate at the tumor

site and form immune complexes. Tissue-deposited immune complexes crosslinking Fc-receptors on infiltrating immune effector cells (neutrophils and macrophages) may in turn cause the release of inflammatory cytokines, proteolytic enzymes and other toxic molecules involved in the induction of auto-immune responses [53].

Anti-PSA antibodies have also been used as carrier proteins for conjugated chemotherapeutic drugs. The intravenous injection of anti-PSA IgG conjugated to 5-fluoro-2-deoxyuridine could selectively inhibit cell proliferation and induce the death of LnCAP protstate tumor cells grown in nude mice. [54, 55].

Anti-PSMA antibodies

PSMA is highly expressed in prostate cancer and is currently the target of a number of diagnostic and therapeutic strategies. Moreover, as mentioned earlier, PSMA is also expressed by the tumor neovasculature [17] suggesting that anti-PSMA mab may act as a double-edged sword by simultaneously targeting tumor cells and newly formed blood vessels required for tumor growth.

Clinical trials have demonstrated that radioimmunoscintigraphy with anti-PSMA mab is particularly sensitive for detecting soft-tissue metastases and recently the FDA approved the ^{111}In-labeled anti-PSMA mab, capromab pendetide (7E11-C5.3, CYT-356, or ProstaScint; Cytogen Corp.) as an imaging agent for prostate cancer [56, 57]. This antibody binds to an intracellular epitope of PSMA. Therefore, it has been postulated that efficient imaging is due to the binding of the radiolabeled antibody to dead cells and/or

cellular debris in metastatic lesions. Based on the success obtained with this antibody as an imaging agent, clinical trials testing the therapeutic efficacy of the ^{90}Y-CYT-356 have been initiated [58]. In these trials myelosupression was the dose-limiting toxicity and more recent protocols have now included EDTA, a chelating agent that removes free Yttrium from the circulation [59]. Because CYT-356 binds only to an intracellular site of the PSMA molecule, new anti-PSMA mab, directed against the extracellular domain of PSMA have been produced. One of these murine antibodies. ^{131}I-muJ591, is currently under clinical evaluation in a phase I study for patients with hormone independent prostate cancer [60].

A fully human bispecific antibody that targets PSMA and the Fc-receptor for IgA (FcαR1, CD89) expressed by cytotoxic effector cells has been produced by chemical conjugation of the Fab fragments of anti-PSMA '8C12' and anti-CD89 '14.1' respectively (Medarex Inc.). Preclinical studies show that this bispecific antibody can induce antibody-dependent cell cytotoxicity and monocyte-derived macrophage phagocytosis of PSMA-expressing cells [61].

Anti-TAG-72 antibodies

The anti-TAG-72 mab CC49 is being tested in clinical trials for diagnosis and therapy of a variety of carcinomas. In patients with androgen-independent or metastatic prostate cancer, interferon-γ or interferon-α were used in combination with ^{131}I-CC49 mab to enhance TAG-72 expression by tumor cells. Although the addition of interferon enhances the tumor uptake of radiolabeled CC49, only modest anti-tumor effects were observed in phase II studies [62, 63]. The murine origin of CC49 and the rapid development of a

human anti-mouse antibody response (HAMA) precluded multiple injections of CC49. Humanized and CH2-deleted derivatives of CC49 have recently been produced. Those constructs with their expected low immunogenicity may show promise for improved CC49 mab therapy [64-67].

Antibodies directed against growth factors (Anti-EGFr and anti-Her-2/neu antibodies)

Epidermal growth factor receptors are important mediators of cell growth, differentiation and survival. Two members of this family, the EGF receptor and Her-2/neu have been extensively studied as potential target for antibody based therapy. Results from animal experiments have demonstrated the importance of EGFr and Her-2/neu in the progression of prostate cancer, suggesting that antibodies directed against these growth factor receptors may be beneficial for the treatment of metastatic prostate cancer [24, 25, 34]. Antibodies directed against EGF receptor (C225 and ABX-EGF) and Her-2/neu (Trastuzumab) are currently being evaluated in phase I or I/II trials for prostate cancer. Both the chimeric mab C225 (Cetuximab, Imclone) and the human IgG2 mab ABX-EGF (Abgenix Inc.) block ligand binding and receptor activation *in vitro*, and demonstrate pronounced anti-tumor activity in animal models [68, 69]. The humanized antibody Trastuzumab (Herceptin, Genentech) has already proven its clinical utility in treating breast cancer patients. In a prostate tumor animal models Trastuzumab slowed the growth of androgen-dependent tumors. This effect was more pronounced if Trastuzumab was used in combination with paclitaxel and was observed in both androgen-dependent and independent tumors [70].

The recombinant immunotoxin AR209 is composed of an anti-Her-2/neu single chain antibody coupled to a portion of the *Pseudomonas* exotoxin-A. The evaluation of AR209 therapeutic efficacy on human prostate xenografts in nude mice shows that the immunotoxin could slow the progression of small tumors (<200 mm^3) [71].

MDX-H210 (Medarex) is a bispecific antibody which targets the Her-2/neu tumor antigen and FcγR1 (CD64) on neutrophils and mononuclear phagocytes. MDX-H210 is comprised of the F(ab)' of anti-Her-2/neu murine mab '520C9' chemically conjugated to the F(ab)' of anti-CD64 humanized antibody 'H22'. In Phase II clinical studies for late stage prostate cancer patients, MDX-H210 has shown quality-of-life improvements and reductions of PSA levels in some patients. In this clinical trial subcutaneous GM-CSF is combined with intravenous MDX-H210 in an attempt to improve the immunologic and anti-cancer activity of the antibody [72].

Antibody mediated tumor necrosis therapy

Cotara™ (Techniclone Corp.) is a chimeric mab which binds to DNA or DNA associated proteins and targets dead/decaying cells found in the core of solid tumors. The ^{131}I-labeled mab is used to irradiate the living cells of the tumor 'from the inside'; this approach is known as Tumor Necrosis Therapy (TNT). Cotara™ is presently in Phase II clinical trials in the U.S. for malignant glioma and Phase a I II clinical trial in Mexico City for treatment of pancreatic, prostrate and liver cancers. Antibodies have poor tissue penetration and the route of administration of Cotara™ may be crucial for efficient anti-tumor effect. Both intratumoral and intravenous injections will be performed in the phase I/II trial.

Anti-VEGF antibodies

Tumor growth and metastasis are critically dependent on angiogenesis and therapies designed to inhibit the process of new blood vessel formation represents a promising new modality for the treatment of solid tumors. The importance of vascular endothelial growth factor (VEGF) in the angiogenic process is well established and antibodies capable of inhibiting VEGF from binding to its receptors have demonstrated their potential therapeutic utility in animal models bearing different types of tumors. For example in a prostate tumor model, VEGF-neutralizing antibodies were shown to be efficient in slowing the growth and metastatic dissemination of the DU-145 prostate tumor cells [73, 74]. A humanized monoclonal antibody directed against VEGF (RhuMAB, Genentech) is currently under evaluation in a phase II clinical trial for hormone refractory prostate cancer patients.

Anti-CTLA-4 antibodies

In addition to their use as targeting agents for tumor cells and tumor vasculature, therapeutic antibodies have also been used as a powerful tool to target immune cells and potentate anti-tumor immune responses. The CTLA-4 receptor is a critical inhibitory regulator of T-cell functions, and antibody-mediated blockade of CTLA-4 prevents T-cell downregulation and enhances T-cell-responses. Administration of an anti-CTLA-4 antibody to mice injected with the TRAMPC1 prostate tumor cell line, showed significant inhibition of tumor growth to complete rejection of the induced prostate cancer [75]. Adjunct CTLA-4 blockade could also considerably reduce metastatic relapse if administrated immediately after resection of an established TRAMPC2 prostate tumor [76]. When used in combination with a tumor cell vaccine, CTLA-4 blockade was also

effective in reducing tumor incidence in the TRAMP transgenic murine model [77]. A phase I clinical trial of anti-CTLA-4 antibody in patients with advanced prostate cancer is in progress.

CONCLUSION

A number of antibody-based products are currently being tested as potential therapeutic agents for prostate cancer and many of them have already reached phase I and/or II clinical trials. Several antibodies under investigation such as those directed against TAG-72, EGFr and Her-2/neu have already been tested in more advanced clinical settings for other types of cancers [5, 63, 78, 79]. The advanced stage in the clinic as well as the promising results obtained in other types of cancers represent definite advantages for these antibodies. The therapeutic efficacy of such antibodies in prostate cancer will however be dependent of the level of expression of the targeted antigens by prostate cancer cells, an issue that remains controversial at this time.

Other approaches under investigation employ anti-PSA or PSMA antibodies and take advantage of the very high specificity of these antigens for the prostatic tissue. The restricted expression of a targeted antigen to the tumor cells is expected to greatly limit toxicity to healthy tissues and for this reason, the search for "highly specific" tumor antigens has been a challenge taken by many scientists and a major effort of genomic program. PSA is often described as "the best tumor antigen discovered so far", and the more recent discovery of PSMA has also generated much enthusiasm for the

development of prostate cancer targeted therapies. However, antibody-targeted therapies have to deal with the non-specific uptake of a large amount of the injected antibody by immune cells through the Fc-receptors. When antibodies are used as carrier molecules and conjugated to cytotoxic agents, unwanted side effects such as immunosuppression may occur. This effect could be overcome by using single chain antibodies or Fab fragments. On the other hand strategies have been designed to take advantage of the recognition of the Fc-portion of the antibodies by immune cells [53]. Fc-directed therapy has the objective to induce an anti-tumoral immune response by targeting tumor antigens to specialized immune cells and/or by attracting immune cells to the tumor site. Fc-directed therapy can use native antibodies, and can be optimized with the production of recombinant antibodies with improved Fc-binding capacity, or bifunctional antibodies with specificity for Fc-receptor.

Poor tumor penetration by the injected antibodies has also been for long time a major limitation for tumor-targeted therapies. Novel strategies circumvent this problem by targeting the tumor vasculature (anti-VEGF mab, anti-PSMA mab) or specific populations of immune cells (anti-CTLA-4 mab, and in some instances Fc-receptor directed therapies) rather than the tumor itself.

Progress over the last two decades has been substantial, and antibody-based therapy has now shown its promise for patients with breast cancer and B-cell lymphoma. We may expect that in the near future antibody therapy will emerge as a viable treatment option for prostate cancer.

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REPLY TO
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