

# Humoral immune response in prostate cancer patients after immunization with gene-based vaccines that encode for a protein that is proteasomally degraded

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**Keywords:** clinical trial, prostate cancer, vaccination, prostate-specific membrane antigen, immunological monitoring, humoral immunity

Prostate-specific membrane antigen (PSMA), whose expression is upregulated in poorly differentiated, metastatic, and hormone refractory prostate cancer, could be targeted by gene-based vaccines. The aim of this study was to characterize the humoral immune response against PSMA in prostate carcinoma patients who have been vaccinated against PSMA with gene-based vaccines. Sera from prostate cancer patients who had been immunized repeatedly with plasmid DNA and a recombinant adenoviral vector, both carrying an expression cassette for human PSMA, and sera from healthy donors were tested for anti-PSMA antibodies by Western blot analysis and immunofluorescence. PSMA-producing LNCaP cells, recombinant PSMA protein, and a specific antibody against PSMA were used as positive controls. Specific anti-PSMA antibodies were detected by both Western blot and immunofluorescence in the sera of patients who had been vaccinated against PSMA with plasmid and recombinant adenoviral vectors. The specificity of the anti-PSMA antibodies was confirmed by preincubation and blocking experiments. Positive reactions were detected in 86% of the vaccinated prostate cancer patients. Anti-PSMA antibodies were not detected either in the patients' sera prior to vaccination or in the sera from healthy men and women. These data demonstrate that PSMA, a specific marker for prostate cancer, is a target for humoral immune response induced by gene-based PSMA vaccination. Detection of anti-PSMA antibodies by immunoblot analysis and by indirect immunofluorescence could be used to monitor the vaccination effect.

## Introduction

Prostate cancer is the leading cancer in men, and mortality from the disease is second only to that from lung cancer (1, 2). There are several biomarkers that are used for screening, diagnosis, and predicting disease progression, such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (3). The most commonly used of these markers is PSA (4, 5), a member of the kallikrein family, which has a Mr of 33 kDa (6, 7, 8).

The identification of new biochemical markers is very important to the diagnosis and monitoring of this disease. One such potential marker is PSMA, which is a highly restricted

prostate epithelial cell membrane glycoprotein of approximately 100 kDa (9, 10, 11, 12, 13). In contrast to PSA, which is a secretory protein, PSMA is a type II integral membrane protein whose expression increases after androgen deprivation therapy (14).

PSMA has been characterized using the mAb 7E11-C5.3, a murine IgG1 derived after immunization with purified cell membrane fractions and isolated from the human prostatic adenocarcinoma cell line, LNCaP. The mAb 7E11 specifically binds the intracellular domain of PSMA (10, 11, 15). The LNCaP cells have been derived from a supraclavicular lymph node of a patient with hormone-refractory prostate cancer (15). LNCaP cells provide the best *in vitro* model for the study of human prostate cancer because of their ability to produce PSMA, an attractive target for both antibody-directed imaging and immunotherapy of prostate cancer (16).

PSMA has been detected in normal prostate, in benign prostate hyperplasia (BPH), and in prostate carcinoma (3, 11, 15), and significant PSMA expression has been detected in tumor-associated neovasculature (17, 18). Additionally, the high PSMA expression level is maintained in poorly differentiated tumors and prostate carcinoma metastasis, and is low in the normal prostatic epithelium (10). Therefore, PSMA is an attractive target for immunotherapy of prostate carcinoma.

This study is part of a study conducted under the Bulgarian National Drug Institute Investigational New Drugs Applications, described in a previous paper (19). According to the design of the study, prostate carcinoma patients were immunized against PSMA with gene-based vaccines (19, 20). The aim of the study was to look for the presence of specific antibodies against PSMA in the sera of prostate carcinoma patients; that is, to look for an immune response following immunization with plasmid or adenoviral vectors against PSMA. At this stage it is not possible to come to any conclusions about the clinical significance of such antibodies, nor to correlate the presence or concentration of antibodies with the development of the disease.

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## Results

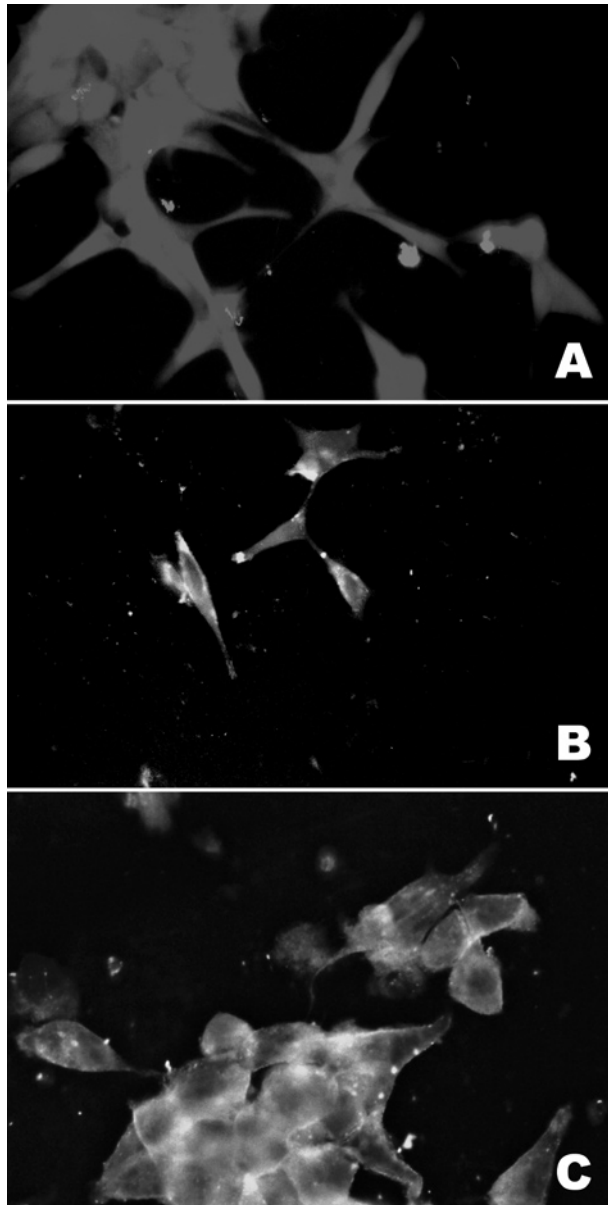
### PSMA immunofluorescent detection

Serum from patients vaccinated with XC-PSMA encoding plasmid DNA were tested by indirect immunofluorescence for the presence of antibodies reacting with LNCaP cells. Serum collected from a prostate carcinoma patient (patient no. 6) prior to the vaccination failed to stain LNCaP cells (Figure 1A), whereas the sample collected from the same patient 3 mo after initiation of immunization reacted against antigens located at the cell surface (Figure 1B). Similar positive staining was observed when serum samples from 10 patients

at different stages of treatment were tested. Bearing in mind the possibility of nonspecific staining due to “sticky” serum components, IgGs from sera from positively reacting patients were purified by Protein G affinity chromatography and tested by immunofluorescence. The staining of LNCaP cells with purified IgGs was similar to that observed after staining with whole serum or to that following staining with rabbit polyclonal anti-PSMA serum (positive control; Figure 1C).

In order to confirm the anti-PSMA specificity of the antibodies in a serum sample, the LNCaP cells were treated with serum samples preincubated with recombinant PSMA or LNCaP lysate for 2 h at room temperature (RT). After centrifugation, the preincubated serum samples were used in immunofluorescent experiments on LNCaP cells. No specific staining was observed in cases where sera from patients which had been vaccinated and which were preincubated with recombinant PSMA protein or LNCaP lysate were used.

**Figure 1**



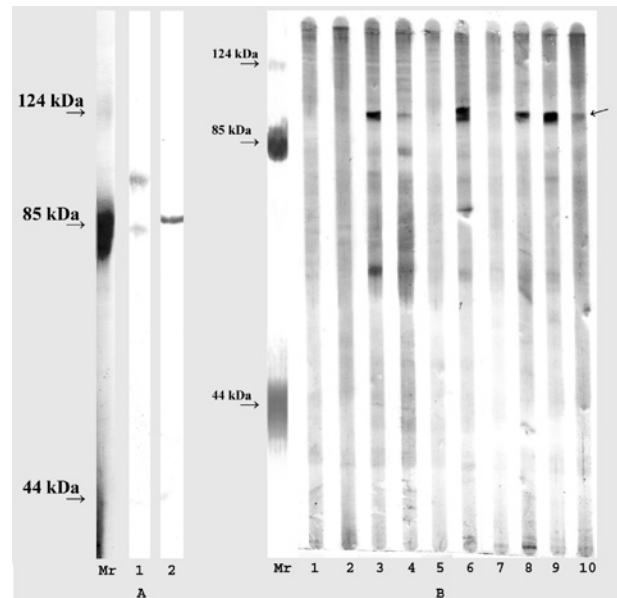
**Indirect immunofluorescence.** LNCaP cells fixed in 4% paraformaldehyde were treated with (A) a serum sample from patient no. 6, collected before the start of vaccination; (B) a serum sample from the same patient collected 3 mo after the start of vaccination; and (C) with specific polyclonal anti-PSMA antibodies.

### Immunoblot detection of antibodies against PSMA

Using polyclonal rabbit anti-PSMA serum, PSMA in lysates from the LNCaP prostate cancer cell line is detected as two bands, one with a Mr of 100 kDa and the other with a Mr of 80 kDa (Figure 2A, lane 1), while the recombinant PSMA migrates as a single band with an Mr of 85 kDa (Figure 2A, lane 2). In a preliminary experiment in which sera from vaccinated patients were tested under the same experimental conditions, 6/10 sera stained a protein band with an Mr of 100 kDa, corresponding to the same band stained by the specific antibodies (Figure 2B, lanes 1-10).

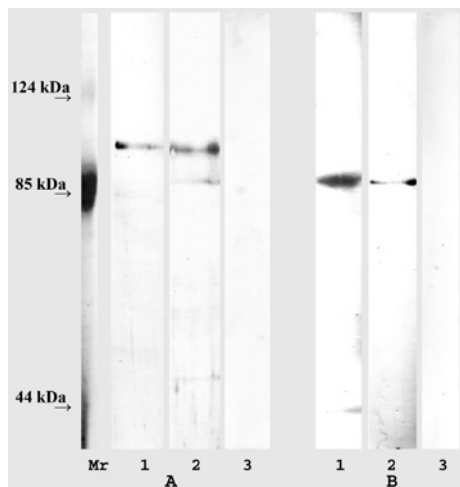
The anti-PSMA specificity of antibodies present in the vaccinated patients' sera was confirmed by Western blotting as follows. The blots were treated with either positive serum from

**Figure 2**



**Immunoblot analysis of the reactions of sera from patients vaccinated with gene-based PSMA vaccine against LNCaP lysate.** (A) Mr, molecular weight markers; lane 1, LNCaP lysate; lane 2, recombinant PSMA. The blots were incubated with monospecific anti-PSMA serum and subsequently with antirabbit IgG labeled with alkaline phosphatase (AP). (B) Mr, molecular weight markers; lanes 1-10, LNCaP lysates treated with sera from different patients vaccinated with gene-based PSMA vaccine and subsequently incubated with antihuman IgG labeled with AP.

Figure 3

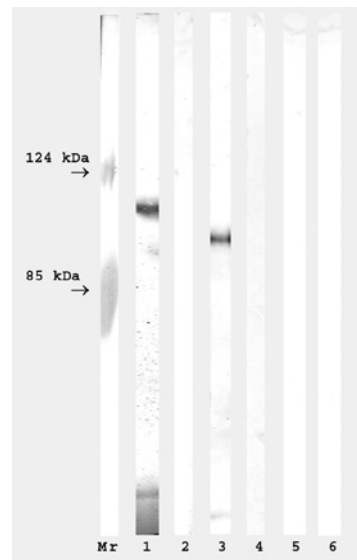


**Immunoblot analysis of the reaction of sera from vaccinated patients against PSMA.** LNCaP lysates (A) or recombinant PSMA (B) were run on 12% polyacrylamide gel, transferred onto nitrocellulose, and treated with serum from a healthy male donor or from immunized patients who had or had not developed antibodies against the target antigen. (A) Lane 1, LNCaP lysate pretreated with serum from a healthy male donor diluted 1:50; lane 2, LNCaP lysate pretreated with a negative patient's serum diluted 1:50; lane 3, LNCaP lysates pretreated with a positive patient's serum diluted 1:50. (B) Lane 1, recombinant PSMA pretreated with serum from a healthy male donor diluted 1:50; lane 2, recombinant PSMA pretreated with a negative patient's serum diluted 1:50; lane 3, recombinant PSMA pretreated with a positive patient's serum diluted 1:50. After pretreatment all blots were treated consecutively with specific rabbit anti-PSMA antibodies and antirabbit IgG serum labeled with AP.

a vaccinated patient or with a negatively reacting preimmune serum. Both blots were consecutively treated with rabbit anti-PSMA serum and then with antirabbit IgG alkaline phosphatase (AP) conjugate. Under these conditions two bands, one of 100 kDa and the other of 80 kDa, were detected in LNCaP lysates pretreated either with serum from a healthy male donor or with a negatively reacting patient's serum (Figure 3A, lanes 1 and 2). The same sera did not block the binding of the polyclonal rabbit anti-PSMA serum to recombinant PSMA (Figure 3B, lanes 1 and 2). On the other hand, no specific bands were detected when the blots of LNCaP lysates were pretreated with a positively reacting patient's serum and subsequently incubated with rabbit anti-PSMA serum (Figure 3A, lane 3). The same result was obtained with recombinant PSMA (Figure 3B, lane 3). These results clearly show that antibodies present in the positively-reacting patient's serum block the binding of the specific rabbit anti-PSMA antibodies to the corresponding antigen (Figure 3B, lane 3); that is, that there is a competition for binding to PSMA between antibodies in the patients' sera and the polyclonal rabbit anti-PSMA serum.

Later, the specific binding of anti-PSMA antibodies present in the positively reacting serum to nondenatured PSMA was confirmed when the serum samples were preincubated with recombinant PSMA for 2 h at RT. After centrifugation, the preincubated serum samples were used in immunoblotting experiments on LNCaP lysate and recombinant PSMA. The specificity of the reaction was confirmed by the fact that when lysates from H295R cells were tested by Western blot, no specific bands were observed when the membranes were treated with

Figure 4



**Immunoblot analysis of the specificity of the reaction of patients' sera against PSMA.** LNCaP lysate or recombinant PSMA were run on SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membrane, and incubated either with a positive patient's serum or with the same serum after it had been preincubated with recombinant PSMA (120 µg/ml). Mr, molecular weight markers; lane 1, LNCaP lysate treated with positive serum (dilution 1:50); lane 2, LNCaP lysate treated with the same serum (dilution 1:50) after preincubation with recombinant PSMA (120 µg/ml); lane 3, recombinant PSMA treated with positive serum (dilution 1:50); lane 4, recombinant PSMA reacted with the same serum (dilution 1:50) after preincubation with recombinant PSMA (120 µg/ml); lane 5, LNCaP lysate treated with serum from a healthy male donor, diluted 1:50; lane 6, recombinant PSMA treated with serum from a healthy male donor, diluted 1:50. Lastly all blots were treated with antihuman IgG labeled with AP.

sera from vaccinated patients. Additionally, PSMA blots were treated with patients' sera preincubated with H295R lysates, and this preincubation did not block the binding of antibodies to PSMA, again demonstrating the specificity of the reaction of vaccinated patients' sera against PSMA.

Prior to preincubation with PSMA, a positively reacting patient's serum stained a protein band of 100 kDa in LNCaP lysate and an 85 kDa band in a recombinant PSMA sample (Figure 4, lanes 1 and 3). After preincubation of the same serum with recombinant PSMA, it did not stain any bands in blots of LNCaP lysate or of recombinant PSMA (Figure 4, lanes 2 and 4). The controls, serum from a healthy male donor or the secondary antibody (antihuman IgG), were completely negative. These results demonstrate that gene-based anti-PSMA vaccination leads to a specific anti-PSMA humoral immune response.

#### Frequency of humoral immune response after immunization with gene-based vaccines that encode for protein that is proteasomally degraded

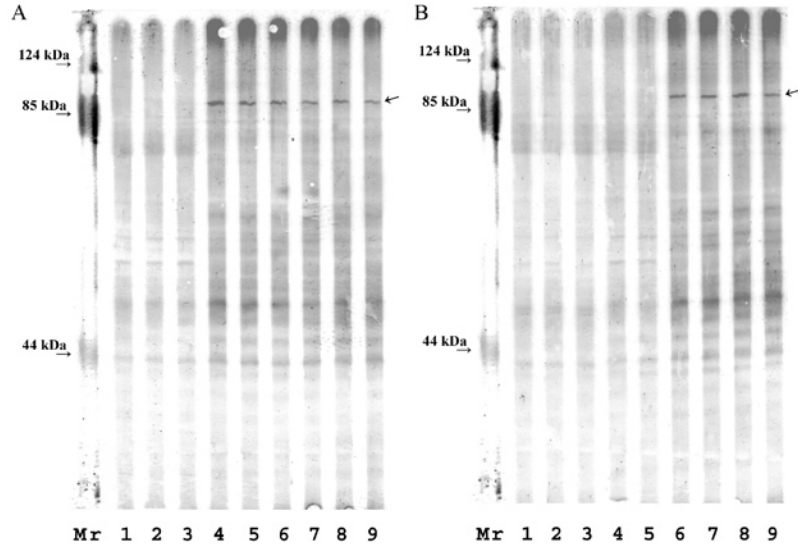
Western blot screening of all patients revealed positive reactions in 86% (36/42) of the prostate cancer patients who underwent gene-based vaccination. When the data for all patients were pooled and analyzed, it became obvious that the appearance and the presence of anti-PSMA antibodies in vaccinated patients varied among different individuals. In some patients, anti-PSMA antibodies could be detected as early as 9 mo after the start of vaccination (Figure 5A), whereas in other patients, the specific anti-PSMA antibodies gave a positive reaction 15 mo after starting vaccination (Figure 5B). In both patients, the antibodies

persisted throughout the observation period (24 mo). However, analysis of the pooled data revealed that the humoral immune response induced after injection of gene-based vaccines seemed to fluctuate. When the patients were followed-up for 36 mo after beginning treatment, it was revealed that the percentage of positive cases changed during that period (Figure 6). The highest proportion of positive cases was recorded during the first 3 mo following the start of vaccination (38-48% of patients) and at the end of the observation period (38-48% of patients).

### Discussion

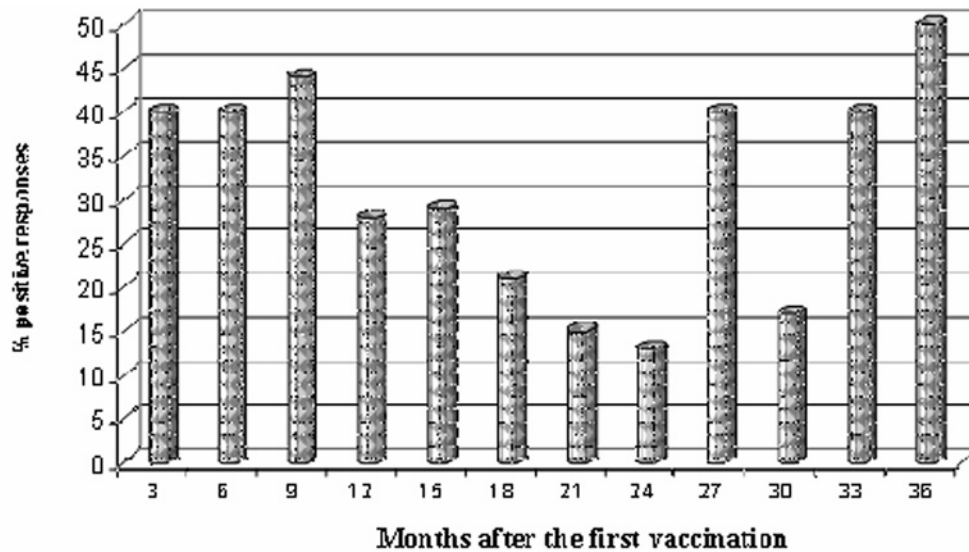
The prostate-restricted nature of PSMA, as well as the association between the level of PSMA expression and the aggressiveness of the disease (21), implies a potentially important role for PSMA in prostate carcinoma biology. PSMA seems to be one of the best targets for immune attack because of its prostate-specific expression (14) and its 10-fold upregulation in most prostate cancers (15, 16, 17). PSMA has been detected in tumor-associated neovasculature but not in normal vasculature and, therefore,

Figure 5



Immunoblot analysis to detect anti-PSMA antibodies in patients' sera at different stages after the onset of vaccination. LNCaP lysate was run on SDS-PAGE, transferred onto PVDF membrane, and treated with (A) serum from patient no. 1 and (B) serum from patient no. 3. Lane 1, serum sample before the start of vaccination; lane 2, serum sample at 3 mo after starting vaccination; lane 3, serum sample at 6 mo; lane 4, serum sample at 9 mo; lane 5, serum at 12 mo; lane 6, serum at 15 mo; lane 7, serum at 18 mo; lane 8, serum at 21 mo; lane 9, serum at 24 mo after the start of vaccination.

Figure 6



Pooled data showing the dynamics of the presence of anti-PSMA antibodies in the blood plasma of patients injected with gene-based PSMA vaccines.

targeting it could be used to inhibit tumor neoangiogenesis (17, 18). Induction of humoral responses directed against PSMA has been shown after vaccination of BALB/c mice with “naked” DNA. Several anti-PSMA antibodies have been reported to date (18). Vaccination with NIH3T3 cells expressing the extracellular portion of human PSMA (PSMc) also induced antibodies to PSMA that strongly and selectively stained PSMA-positive cells. Moreover, the development of LNCaP tumors in athymic mice was significantly inhibited by serum taken from NIH3T3 PSMc vaccinated mice (22), a finding supporting the therapeutic potential of PSMA vaccines. A variety of immunogenic agents and injection sites have been tested for immunotherapy (23, 24), but the most commonly used agents are gene-based vaccines due to their safety, low cost, convenience, and effectiveness (25, 26).

Our experiments are the first in which anti-PSMA antibodies were detected in sera of patients who had been immunized with gene-based vaccines encoding for products that are proteasomally degraded (27). Our data reveal that the humoral immune response induced in prostate cancer patients varies within the studied population. Most of the patients (86%) develop a humoral immune response against PSMA. In the majority of patients, a humoral immune response can be detected as early as 3 mo after the first vaccination. On the other hand, some patients develop an immune response much later. A high proportion of the vaccinated patients show a fluctuating anti-PSMA response. A small number of patients (21%) have a positive-to-negative anti-PSMA response ratio (more than one-half of the serum samples collected during the course of their treatment and follow-up are positive) greater than 1.0. Anti-PSMA antibodies were detected in their serum samples not only at the beginning, but also at the end of the study.

The antibodies seem to react with the native form of the antigen since LNCaP cells stain positively and the activity of the antibodies is “exhausted” by preincubation with recombinant PSMA (Figure 4). It will be interesting to see whether the specificity is directed against a carbohydrate moiety (PSMA is a heavily glycosylated protein) or the protein core (the protein expressed following immunization with our gene-based vaccines is not glycosylated) (27), and experiments are currently underway in our laboratory to explore this.

The aim of this study was to investigate the development of humoral immune responses against a target antigen when immunization is performed with gene-based vaccines encoding for products that are proteasomally degraded (27). Due to patient heterogeneity and various concomitant additional treatments, no correlation between disease course, the presence of anti-PSMA antibodies and the effectiveness of immunotherapy can be made at this time. Additional experimentation and data analysis are currently in progress to address this issue.

Our data clearly demonstrate that PSMA, a specific marker of prostate carcinoma, is a target for humoral immune response induced by multiple gene-based vaccinations. Detecting anti-PSMA antibodies by Western blot and immunofluorescent assays may be a way to monitor the effect of vaccination, but further studies are needed.

## Abbreviations

AP, alkaline phosphatase; PSMA, protein-specific membrane antigen; RT, room temperature

## Acknowledgements

This study was supported in part by a grant from the Ministry of Education and Sciences, Sofia, Bulgaria, and in part by Grant N00014-00-1-0787 from the United States Office of Naval Research. We wish to acknowledge the generous financial support provided by the American Foundation for Biological Research and by the Bulgarian Foundation for Biomedical Research. We would also like to express our appreciation for discussions with Prof. I. Kehayov (IBIR, BAS) and Dr. S. Hayrabedyan (IBIR, BAS), and for the technical assistance of M. Tchakarova, N. Daneva, A. Sapundzhieva, and R. Moisoava.

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## Materials and methods

### Vectors

The cDNA encoding the extracellular portion (amino acids 44-750) of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) after RT-PCR of total mRNA from the human prostate cancer cell line LNCaP (CRL 1740, American Type Culture Collection, Manassas, VA, USA). The forward primer introduced a *NotI* cloning site and a Kozak sequence with a Met codon (GCCACCATG) into the 5'-end of XC-PSMA. The hPSMAT plasmid for the transfection experiments was obtained by *NotI-XhoI* subcloning of XC-PSMA into a pVAX1 mammalian expression vector (Invitrogen, Carlsbad, CA, USA) (27).

The PSMA/CD86 plasmid vector used in the immunization studies contains two expression cassettes. The first one includes DNA encoding for the XC-PSMA under the regulation of the immediate-early CMV promoter/enhancer and a bovine growth hormone polyadenylation signal. The second expression cassette includes DNA that encodes human CD86 under the regulation of a separate immediate-early CMV promoter/enhancer and a separate bovine growth hormone polyadenylation signal. For the construction of the recombinant virus (Ad5PSMA), the expression cassette containing DNA encoding for the XC-PSMA was inserted in a replication deficient (E1, E3 deletions) adenovirus (Ad5). The recombinant adenoviral vector does not contain an expression cassette for human CD86.

### Patients

The prostate cancer study in Bulgaria was a Phase I/II and was approved by the Bulgarian Drug Agency (19, 20). Beginning in May 1998, the study was initiated with a series of two immunizations with a PSMA/CD86 plasmid/GM-CSF cocktail consisting of 200 µg of plasmid DNA and 80,000 IU of soluble GM-CSF protein, brought to a final volume of 200 µl by the

addition of sterile saline. All injections were intradermal, and care was taken to see that a blister formed and that the infusate was retained at the epidermal-dermal junction. Six and twenty-four hours after each immunization, the patient received an additional 80,000 IU of GM-CSF protein intradermally at the site of the original injection. The two immunizations were performed 3 wk apart. Three weeks after the second plasmid DNA immunization, patients received intradermal inoculation with  $5 \times 10^8$  PFUs of adenovirus Ad5-PSMA. After priming, patients received booster inoculations every 12 wk, beginning at week 21. Booster inoculations alternated between PSMA/CD86 plasmid/GM-CSF cocktail and Ad5-PSMA, administered as described previously. No 6- or 24-h GM-CSF was given following the plasmid booster injections. Booster inoculations continued until each patient had received a total of 15 inoculations.

Fifty-five patients with evidence of active disease were entered in our study on a random basis without regard to their disease status. Three patients with advanced cancer died early in the study from overwhelming disease. No adverse effects were seen among the remaining 52 patients. Forty-two patients were recalled for the following analysis. None had received any chemotherapy during the course of the study. Twenty-four received vaccine therapy combined in various schedules with intermittent hormone therapy on the premise that a reduced tumor load might enhance the effectiveness of the vaccine. Eighteen patients with evidence of disease recurrence following prostatectomy received immunotherapy and no other treatment. Blood sera samples were collected from all 42 patients, and sera from healthy men and women were used as controls. The sera samples were supplied by the National Centre of Haematology and Transfusion Medicine (Sofia, Bulgaria) and by the Laboratory of Clinical Immunology, Medical University Sofia (Sofia, Bulgaria) according to the rules of the Ethical Committee of the Medical University Sofia.

Patients ranged from 49 to 80 years old, and averaged 65 years of age. All sera were collected with the signed consent of the patients after they were informed about the aims of the study (20). Sera from the same patients prior to vaccination and sera from healthy men and women were used as negative controls. All patients were routinely screened for the level of prostate-specific antigen (PSA) at defined intervals during treatment at the National Centre of Haematology and Transfusion Medicine (Sofia, Bulgaria).

#### Antibodies

Anti-PSMA polyclonal rabbit serum, specific for the antigen studied, and a recombinant PSMA protein (250  $\mu\text{g}/\text{mL}$ ) were used throughout these experiments. Dr. Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, the Czech Republic, kindly provided them. Mouse monoclonal antirabbit antibody conjugated with AP (Clone RG-96) and goat antihuman IgG AP conjugated antibodies (Sigma, St. Louis, MO, USA) were diluted 1:10000 in PBS containing 3% dry milk prior to use. FITC-labeled antirabbit IgG (Scottish Antibody Production Unit, Carlisle, Scotland), and FITC antihuman IgG (NCZPB, Sofia, Bulgaria) were diluted 1:50 in PBS 1% BSA prior to use.

#### Cell lines and reagents

LNCaP cells (American Type Culture Collection, Manassas, VA, USA) were routinely grown in RPMI 1640 supplemented with 10% FCS and L-glutamine at 37°C, 5% CO<sub>2</sub>. When confluent, the cells were trypsinized and harvested from the plates by gentle scraping into medium and pelleted by centrifugation at

440 x g. Cells were rinsed twice with 10 ml of PBS (8.0 g NaCl, 0.2 g KCl, 1.44 g NaHPO<sub>4</sub>, 0.24 g KHPO<sub>4</sub>, 1 L dH<sub>2</sub>O, pH 7.2). LNCaP cells for immunofluorescent studies were trypsinized and seeded in Petri dishes containing sterile cover slips. After 24 h incubation, the cells attached to the cover slips were rinsed with sterile PBS and fixed in 4% paraformaldehyde-PBS. The H295R cell line (human adrenocorticocarcinoma, American Type Culture Collection, Manassas, VA, USA) was cultured as recommended by the supplier and used as a control, both for immunofluorescence and immunoblot analysis.

#### Protein G purification of IgG

Sera from 10 patients were purified using a Protein G column (Sigma, St. Louis, MO, USA). Briefly, the serum samples were diluted 1:100 in PBS. The column was equilibrated with phosphate buffer (5.42 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 21.87 g NaH<sub>2</sub>PO<sub>4</sub>·12H<sub>2</sub>O, 1 L dH<sub>2</sub>O, pH 7) several times. Then the sample was loaded into the column, unbound proteins were washed out with phosphate buffer, and bound proteins were eluted with citric acid buffer (0.1 M pH 3). In order to adjust the pH to neutral (pH 7), 100  $\mu\text{l}$  1 M Tris buffer, pH 11, was added to each eluted IgG fraction. IgG fractions were tested by ELISA.

#### Indirect immunofluorescence

Immunofluorescent tests were performed using nonpermeabilized LNCaP cells attached to cover slips and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 30 min at RT, washed with 1% BSA-PBS, and then incubated with 5% BSA-PBS for 1 h at RT. Cells were overlaid and incubated overnight at 4°C with 50  $\mu\text{l}$  of sera from healthy donors or from prostate cancer patients (sera diluted 1:50 in 1% BSA-PBS), or with 50  $\mu\text{g}/\text{mL}$  purified IgG from the patients' sera and with sera from positively reacting patients preincubated with recombinant PSMA protein or LNCaP lysate. After washing with PBS (three times, 5 min/wash), the cells were treated for 1 h at RT with FITC-antihuman IgG, diluted 1:50 in 1% BSA-PBS, containing 0.01% Evans blue. The cells were extensively washed in PBS and mounted in Mowiol-118 mounting medium (Hoechst, Frankfurt, Germany).

A parallel set of fixed LNCaP cells were overlaid with polyclonal rabbit anti-PSMA serum, diluted 1:50 in 1% BSA-PBS (as positive control), and incubated overnight at 4°C. After washing with PBS, FITC-goat antirabbit IgG (1:50 in 1% BSA-PBS containing 0.01% Evans blue) was added for 1 h and the cells were then washed extensively with PBS. The cell preparations were then mounted in Mowiol-118 and stored at 4°C overnight. The fluorescence was read on an epifluorescent microscope (Zeiss, Oberkochen, Germany) and documented with film (Konica, ISO 400).

#### Western blot detection of PSMA expression

Lysates from LNCaP cells and recombinant PSMA protein (125  $\mu\text{g}/\text{mL}$ ) were run under reducing conditions in a 12% SDS-PAGE gel. The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Sigma, St. Louis, MO, USA) for 50 min at 80-90 V. The membranes were blocked overnight in 3% milk-PBS (pH 7.2) at RT. The blocked membranes were incubated with anti-PSMA polyclonal rabbit serum (1:400) for 2 h at RT. The membranes were washed three times (5 min/wash) with Tween 20-PBS, pH 7.4 (T-PBS) and incubated 1 h with antirabbit IgG-AP conjugate. The blots were visualized by AP buffer with NBT (nitroblue tetrazolium) and BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (Sigma, St. Louis, MO, USA). These blots served as positive controls. Lysates from H295R cells

were prepared and used as controls in Western blot analysis. Both lysates contained a similar amount of protein.

#### **Antibody detection in patient sera by Western blot assay**

Lysates from LNCaP cells were electrophoresed as described previously, the blocked membranes were placed in a multiscreen apparatus (Bio-Rad Laboratories, Hercules, CA, USA), and approximately 100 µl of diluted serum was pipetted into individual lanes. Serum samples were diluted 1:50 with 3% milk-PBS. Following a 2 h incubation at RT, the blots were removed from the apparatus and washed three times (5 min/wash) in T-PBS. The membranes were then incubated 1 h at RT with antihuman IgG antibodies conjugated with AP and washed three times (5 min/wash) with T-PBS, pH 7.4. The blots were visualized with NBT and BCIP in AP buffer (Sigma, St. Louis, MO, USA), as recommended by the manufacturer.

Verification of the anti-PSMA specificity of the antibodies in the patients' sera was performed in two ways. In the first, LNCaP lysates or recombinant PSMA were run in SDS-PAGE and, following transfer, the blots were treated for 2 h at RT with either a negatively reacting patient's serum, a positively reacting patient's serum, or with serum from a healthy male donor. The blots were incubated for 2 h at RT with rabbit anti-PSMA serum diluted 1:50 in blocking buffer and then for 1 h at RT with antirabbit IgG-AP conjugate. The blots were developed in AP buffer with NBT and BCIP (Sigma, St. Louis, MO, USA), as recommended by the supplier. In the second, sera from positively reacting patients (dilution 1:50) were mixed with an equal volume of recombinant PSMA (120 µg/ml). The preincubated sera were applied as the primary antibody on Western blots of either LNCaP lysates or recombinant PSMA. Following a 2 h incubation at RT, the secondary antibody (antihuman IgG AP conjugate) was added and the blots were developed as described previously. Later, positively reacting sera were preincubated with H295R lysates and then tested against recombinant PSMA or LNCaP lysates in immunoblotting experiments.

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