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Biochemical Nature and Mapping of PSMA Epitopes Recognized by Human Antibodies Induced after Immunization with Gene-based Vaccines

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Abstract. *Background: A possible new target for immunotherapy is the prostate-specific membrane antigen (PSMA). The aim of the present study was to define potential PSMA epitopes for antibody binding using sera from patients immunized with gene-based anti-PSMA vaccines. Materials and Methods: Sera from prostate cancer patients, immunized repeatedly with plasmid and adenoviral vectors, each encoding for the extracellular portion of human PSMA, were tested for anti-PSMA antibodies by Western blot. PSMA-producing LNCaP cells were used as a control. Recombinant PSMA protein cleaved with different proteinases was used for epitope mapping. Different enzymes were used to cleave the PSMA molecule. Results: Specific anti-PSMA antibodies were detected in the studied patients' sera, mainly against the PSMA protein core. An alignment of the predicted enzyme-cleavage fragments was compared with Western blot results and several antibody epitopes were determined. Conclusion: These data demonstrate that multiple gene-based vaccinations induce an anti-PSMA humoral immune response. The antibodies are predominantly specific for the PSMA protein core.*

A potential marker for the diagnosis, monitoring and treatment of prostate cancer is the prostate specific membrane antigen (PSMA), which is a prostate epithelial cell membrane glycoprotein of approximately 100 kDa (1-5). PSMA has been characterized by a monoclonal antibody (mAb) 7E11-C5.3, a murine IgG1, derived after immunization with purified human prostatic adenocarcinoma cell (LNCaP) membrane fractions. The mAb 7E11 binds specifically to the intracellular domain of PSMA (4, 5).

PSMA has been detected in normal prostate, benign prostate hyperplasia (BPH) and prostate carcinoma (3, 6, 7), and significant expression has been detected in tumor-associated neovasculature (8, 9). Additionally, the high PSMA expression level is maintained in poorly-differentiated tumors and prostate carcinoma metastasis, while levels are low in normal prostatic epithelium (2). Therefore, PSMA is an attractive target for immunotherapy of prostate carcinoma.

Gene-based vaccination is a promising strategy for immunotherapy of prostate cancer. Recently, we have shown that vaccinating of advanced prostate cancer patients with "naked" plasmid DNA and a recombinant adenoviral vector carrying an expression cassette for the human PSMA induces antibodies against PSMA (10).

The aim of the present study was to define the PSMA epitopes recognized by anti-PSMA antibodies, induced after PSMA gene-based vaccinations. PSMA is heavily glycosylated and the antibodies, present in patients' sera, could recognize the carbohydrate moieties as well as portions of the protein core.

Materials and Methods

Vectors used. The PSMA plasmid vectors used in the immunization studies contain an expression cassette for the extracellular portion (AA 44-750) of the human PSMA (XC-PSMA) and have already been described elsewhere (11). For the construction of the recombinant virus (Ad5PSMA), a replication deficient (E1, E3 deletions) adenovirus (Ad5), with expression cassette including the gene for XC-PSMA (11), was constructed.

Patients. The prostate cancer study in Bulgaria was phase I/II and was approved by the Bulgarian Drug Agency (12). Beginning in May 1998, the study was initiated with a series of two immunizations with a PSMA plasmid/GM-CSF cocktail (11). Three weeks following the second plasmid DNA immunization, the

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patients received intradermal inoculation with $5x10^8$ PFUs of adenovirus Ad5-PSMA. After priming, the patients received a booster immunizations, alternating between the PSMA plasmid and the Ad5-PSMA vectors. Blood sera samples were collected from all patients at 3-month intervals throughout the study.

Forty-two patients were enrolled in the study. None of these had received any chemotherapy during the course of the study. The average age of patients was 65 years old (from 49 to 80 years old). Sera from the same patients before the first vaccination and sera from normal men and women were used as negative controls.

Antibodies and reagents. Anti-PSMA polyclonal rabbit serum and the recombinant extracellular portion of the human PSMA (rPSMA, AA 44-750), isolated using an insect expression system (*Drosophila Schneider* cells), were kindly provided by Dr. Jan Konvalinka (13).

Mouse monoclonal anti-rabbit antibody conjugated with alkaline phosphatase and goat anti-human IgG alkaline phosphatase (AP) conjugated antibodies (Sigma Chemical Co., St. Louis, MO, USA) were used in dilution 1:10,000 in phosphate-buffered saline (PBS) containing 3% dry milk.

Cell lines and reagents. LNCaP and human adrenal carcinoma (NCI-H295R) cells (American Type Culture Collection) were routinely grown in RPMI 1640, supplemented with 10% FCS and L-glutamine at 37˚C, 5% CO_2. When confluent, the cells were trypsinized, harvested by gentle scraping, centrifuged at 440 x g, rinsed twice with 10 ml of PBS and re-plated.

Enzymes. Glycopeptidase F (N-glycosidase F; peptide N-glycosidase; PNGase F; EC 3.5.1.52) was obtained from *Flavobacterium meningosepticum*; β-glucuronidase (EC 3.2.1.31); β-galactosidase (EC 3.2.1.22) from *E. coli*, lyophilized; β-glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, lyophilized; β-fructosidase (EC 3.2.1.26) from yeast, lyophilized; pepsin (EC 3.4.23.1) from pig gastric mucosa (stomach membrane), lyophilized, salt-free; pronase E (EC not defined), from *Streptomyces griseusnonspecific*, lyophilized; proteinase K (EC 3.4.21.14) from *Tritirachum album limber* (fungi); subtilizin (EC 3.4.21.14) from *Bacillus subtilis*; and trypsin (EC 3.4.21.4), lyophilized, salt-free was purchased from (Sigma Chemical Co.).

Ten mM periodic acid (PA) in PBS was used to oxidize the peripheral carbohydrate groups of the PSMA molecule.

Enzyme treatment of samples and immunoblotting. Twenty-five μg LNCaP lysate was treated for 4 h at 37˚C with 5 units glycopeptidase F enzyme, dissolved in 50 mM phosphate buffer (PB) (pH 7.5), containing 0.1% (w/v) SDS /lauryl sulfate/, 50 mM β-mercaptoethanol, 0.75% (v/v) TritonX-100. In separate experiments, LNCaP lysate from $1x10^3$ cells or 12 μg rPSMA were treated with: pepsin - 0.5% solution in 10 mM HCl, pH 2.2; pronase E - 100 μg/ml in 50 mM Tris buffer, pH 7.5 with 2 mM $CaCl_2$; proteinase K -100 μg/ml in 50 mM Tris-HCl buffer, pH 8 with 1mM $CaCl_2$; subtilizin - 100 μg/ml in 50 mM Tris buffer with 0.1M $CaCl_2$; trypsin - 0.5% in PBS buffer, pH 7.5 with 20 mM $CaCl_2$. All incubations were performed at 37˚C for 10 min and then stopped with the protease inhibitor phenylmethyl-sulphonylfluoride (PMSF). Lysates from LNCaP cells and rPSMA, with and without enzyme pretreatment, were then run under reduced conditions in a 12% SDS-PAGE gels for 50 min at 80-90 V. The separated proteins were transferred onto polyvinylidene diflenoride (PVDF) membranes (Sigma Chemical Co.).

In a separate series of experiments, the proteins were first transferred onto the membranes and then incubated with the following enzymes: β-glucuronidase (2 μg/ml in ddH_2O pH 5, shaking at 37˚C for 1 h and then stopped with PBS); β-galactosidase (0.5 μg/ml in PB, pH 7.8 with 0.1M 2-mercaptoethnol, shaking at 37˚C for 24 h, stopped with citric acid buffer pH 4 for 2 h); β-glucose oxidase (125 μg/ml in 100 mM PB, pH 6.5-7; shaking at 37˚C for 3 h, stopped with $CuSO_4$ buffer); β-fructosidase (250 μg/ml in citric acid buffer, pH 5, shaking at 30˚C for 1.30 h, stopped with PBS, pH7); pepsin (0.5% solution in 10 mM HCl, pH 2.2, shaking for 20 min at 37˚C, and then stopped with PBS, pH 7.2 for 15 min); pronase E (100 μg/ml in 50 mM Tris buffer, pH 7.5, with 2 mM $CaCl_2$ as an activator, shaking for 30 min at 37˚C and stopped with a combination of 5 mM EDTA, PMSF and diisopropyl fluorophosphates (DFP); proteinase K (100 μg/ml in 50 mM Tris-HCl buffer, pH 8 with 1 mM $CaCl_2$, shaking for 30 min at 37˚C, stopped with PMSF); subtilizin (100 μg/ml in 50 mM Tris buffer with 0.1 M $CaCl_2$, shaking for 30 min at 37˚C, stopped with PMSF); trypsin (0.5% in PBS buffer, pH 7.5, with 20 mM $CaCl_2$, shaking for 30 min at 37˚C and then stopped with PMSF). In separate experiments, nitrocellulose membranes were treated with 10 mM PA for 10 min at RT, and then the blots were washed 3 times for 5 min in PBS-0.5% Tween 20 (PBS-T) buffer.

After washing with PBS for 5 min, the membranes were blocked with 3% dry milk in PBS for 1 h at room temperature (RT). The blocked membranes were placed in a multi-screen apparatus (Bio-Rad) and 100 μl of diluted patients' sera (1:50 with 3% milk-PBS) were pipetted into individual lanes. Following 2-h incubation at RT, the blots were removed from the apparatus and washed 3 times for 5 min in PBS-T. The membranes were then incubated for 1 h at RT with anti-human IgG antibodies conjugated with alkaline phosphatase buffer and washed 3 times for 5 min with PBS-T, pH 7.4.

The blots were visualized with nitrotetrazoliumblaurein (NBT) (Sigma Chemical Co.) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.) in AP buffer pH 8, as recommended by the manufacturer. Blots, with and without enzyme treatment, developed either with sera taken from a patient before and after immunization, or with rabbit anti-PSMA polyclonal serum, were compared (10). NCI-H295R lysates served as negative controls.

Verification of the anti-PSMA specificity of the antibodies in the patients' sera was performed as patients' sera, positive for PSMA (dilution 1/50), were mixed with an equal volume of rPSMA (120 μg/ml), or H295R lysate. The sera pre-incubated in that manner were applied as the primary antibody on Western blots of either LNCaP lysates, rPSMA or H295R lysate. Following 2-h incubation at RT, the secondary antibody (anti-human IgG AP conjugate) was added, and the blots were developed as described above (10).

To predict the fragments generated upon digestion with different proteases, we used the Web Tool "Peptide Cutter" on the EXPASY web site:
(www.expasy.org/tools/peptidecutter/peptidecutter_enzymes.html).

Prediction of the proteasome cleavage site for generation of MHC-class I binding peptides was performed using the NetChop 2.0 Prediction Server (http://www.cbs.dtu.dk/services/NetChop/) (14).

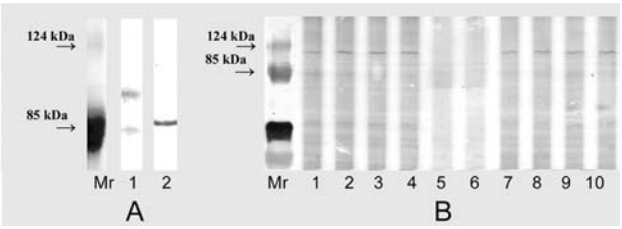

Figure 1. *LNCaP lysates stained with sera from patients immunized against PSMA using gene-based vaccines.*
A – Molecular markers (Mr) and control staining of LNCaP lysates (1) and rPSMA (2) with rabbit polyclonal anti-PSMA serum.
B – Molecular markers (Mr) and LNCaP lysates stained with sera from 10 immunized patients.

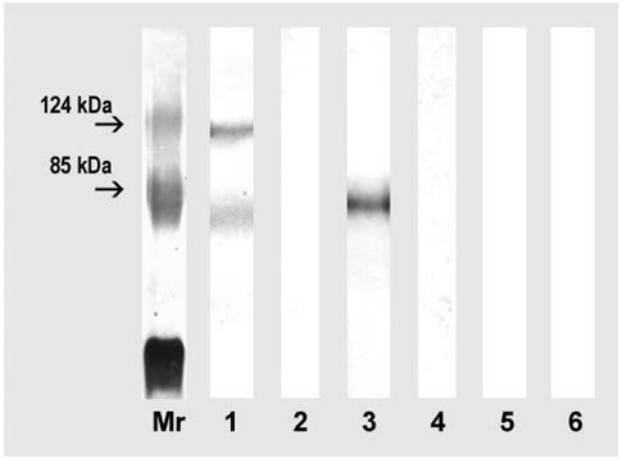

Figure 2. *Anti-PSMA specificity of patients' sera. LNCaP lysates or rPSMA were stained with patients' sera that had or had not been pre-incubated with excess of soluble rPSMA. Mr – molecular markers.*
Lane 1 – LNCaP lysate stained with serum from patient immunized against PSMA.
Lane 2 – LNCaP lysate stained with serum from the same patient after it had been pre-incubated with rPSMA.
Lane 3 – rPSMA stained with serum from patient immunized against PSMA.
Lane 4 – rPSMA stained with serum from the same patient after it had been pre-incubated with rPSMA.
Lane 5 – LNCaP stained with serum from a healthy male volunteer.
Lane 6 – rPSMA stained with serum from a healthy male volunteer.

Results

On Western blot, rabbit polyclonal anti-PSMA serum detected two bands (Mr 100 and 80 kDa) in the LNCaP cell lysates (Figure 1A; line 1) and a single band with Mr of 85 kDa when rPSMA was used as an antigen (Figure 1A, line 2). No PSMA was detected in the H295R cell lysates.

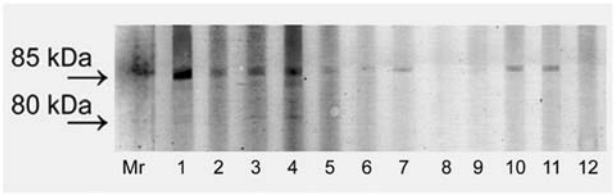

Figure 3. *Anti-PSMA antibodies, present in the sera of immunized patients, recognize the protein core. LNCaP lysates or rPSMA were treated with glycopeptidase F enzyme, then run on SDS-PAGE, transferred to PVDF membrane and stained with patients' sera.*
Mr – molecular markers
1-12 – sera from immunized patients. Note that the numbers identify individual lanes for each Western blot and do not identify patients. For this reason numbers 1 to 10 in Fig. 1B can not be compared with numbers 1 to 12 in Fig. 3. After preliminary deglycosilation, removing the distinct carbohydrate moieties of both rPSMA- and LNCaP-derived PSMA, the resulting bands have the same molecular weight.

Using Western blotting, sera from 42 patients, who had been immunized against PSMA using gene-based vaccines (10-12), were tested. Antibodies against PSMA could be detected in 37 (88%) patients, when the LNCaP lysate was used as an antigen, and in 34 (81%) patients when the antigen was rPSMA. No antibodies had been detected in the sera of those patients before they were immunized, or in the sera of normal male and female volunteers. Sera from the patients and from the volunteers were negative when H295R lysates were used as antigens. The antibodies present in the patients' sera recognized two separate bands (100 kDa and 85 kDa) when the LNCaP lysate was used and one band (85 kDa) when rPSMA was used (Figure 1B).

To determine the anti-PSMA specificity of the antibodies, sera from immunized patients were first incubated with rPSMA or H295R lysates and then used for staining of the blots. Positive sera, that detected bands with Mr of 100kDa and 85 kDa in blots with LNCaP lysates, or 85kDa in blots with rPSMA, became negative after absorption with rPSMA, but remained positive following absorption with H295R lysate (Figure 2). We interpret this as evidence that the antibodies present in immunized patients' sera have anti-PSMA specificity.

PSMA is heavily glycosylated, and the anti-PSMA antibodies, present in patients' sera (10), could be directed against the carbohydrate moieties or against the protein core. To eliminate carbohydrate residues, the LNCaP lysates or rPSMA were treated with glycopeptidase F as described earlier (15). Most of the patients' sera (33 out of 37 when tested against LNCaP lysate and 33 out of 34 when rPSMA was used as antigen) retained their anti-PSMA reactivity following glycopeptidase treatment (Figure 3). We interpret this as evidence that the antibodies recognize epitopes present in the protein core

Table I. *The percentage of resistant/susceptible epitopes, reacting with Abs in patients' sera against PSMA after enzyme treatment.*

Enzyme	GlycopeptidaseF		Galactosidase		Glucuronidase		Glucoseoxidase		Fructosidase	
Ag	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes
LNCaP	89%	11%	97%	3%	95%	5%	95%	5%	97%	3%
rPSMA	97%	3%	100%	0%	97%	3%	97%	3%	100%	0%

of PSMA. Only in 4 patients (11%) did the antibodies recognize carbohydrate moieties, which were eliminated following glycopeptidase treatment.

Similar results were obtained following treatment with other enzymes that affected carbohydrate PSMA residues (Table I). Thirty-six of the 37 patients retained their reactivity against PSMA following galactosidase, fructosidase, glucuronidase, glucose oxidase or PA treatments. In some cases, epitopes were sensitive to glucose oxidase and glucoronidase treatment but not to treatment with the other enzymes. Bands stained weaker after PA treatment.

In order to check for the presence of anti-PSMA antibodies against the protein PSMA core, the LNCaP lysate or rPSMA was treated with different proteases (subtilysin, pepsin, trypsin, proteniase K, pronase E). In general, the treatment with proteases prior to blotting resulted in numerous bands with Mr between 20 and 90 kDa, which were positively stained by patients' sera (Figure 4). When the LNCaP lysate was treated with protease and then reacted with vaccinated patients' sera, negative reactions were found in 62-65% of 37 patients and a similar percentage of negative reactions were found (68-71% of 34 patients) when rPSMA was used as the target antigen. However, the reactivity to the antigen, treated with any protease, was preserved in 35-38% of the 37 patients for LNCaP lysate and 29-32% of 34 patients for rPSMA. This might be due to the fact that the antibodies recognize epitopes located out of the enzyme cleavage sites in the protein core of PSMA, or to reactions against the carbohydrate PSMA structure (Table II).

The results obtained clearly demonstrate that the patients' sera, containing antibodies against predominantly carbohydrate epitopes, do not have antibodies against peptide epitopes.

According to the amino acid sequence of PSMA, pronase E would cleave the molecule into 3 fragments, composed of 8-10 amino acids, and more than 5 fragments with lengths less then 6 amino acids. Proteinase K would cleave the molecule into 4 fragments with 8-10 amino acids and many smaller fragments. The other enzymes tested in this study – subtilizin, trypsin and pepsin – formed more fragments, which were, however, long enough to be recognized as epitopes by antibodies.

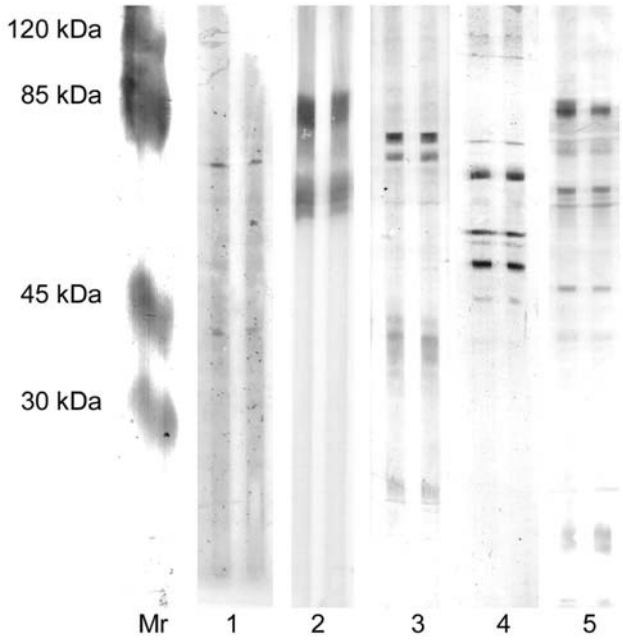

Figure 4. *The anti-PSMA antibodies are directed against different fragments of the PSMA molecule. LNCaP lysates were treated with pronase E (Lane 1); proteinase K (Lane 2); subtilizin (Lane 3); pepsin (Lane 4) or trypsin (Lane 5), and then immunoblotted against sera from immunized patients. Mr – molecular markers.*

Since similar percentages for both sensitive and resistant epitopes were obtained for all patients' sera, it can be assumed that several fragments of the extracellular PSMA domain can be identified, which are most probably the dominant epitopes recognized by antibodies present in the sera. Using the algorithm for the prediction of cleavage sites, NetChop 2.0 (14), we identified the amino acid sequences which are most probably the epitopes recognized by the antibodies. We looked at sequences which were common for fragments obtained after treatment with the 5 enzymes and which corresponded to the fragments predicted by the software. Following this approach, 11 sequences, consisting of 8-11 amino acids, were identified (Figure 5).

Table II. *The percentage of resistant/susceptible epitopes reacting with Abs in patients' sera against PSMA after protease enzyme treatment.*

Enzyme	Pepsin		Pronase E		Proteinase K		Subtilizin		Trypsin	
Ag	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes	resistant epitopes	susceptible epitopes
LNCaP	35%	65%	35%	65%	35%	65%	38%	62%	38%	62%
rPSMA	29%	71%	29%	71%	29%	71%	32%	68%	32%	68%

Figure 5. *Amino acid sequence of PSMA; eleven PSMA fragments, which are the epitopes most probably recognized by antibodies in the patients' sera induced after gene-based vaccination, are bold and underlined.*

Discussion

The possibility of inducing a specific immune response against PSMA after vaccination with DNA has been demonstrated in several studies. Induction of humoral responses directed against PSMA has been shown after vaccination of Balb/c mice immunized with "naked" DNA. Vaccination with NIH3T3 cells, expressing the extracellular portion of human PSMA (PSMc), also induce antibodies to PSMA that strongly and selectively stain PSMA-positive cells. This is accompanied by inhibition of LNCaP tumor development in athymic mice by serum from NIH3T3 PSMc-vaccinated mice (16), a finding in support of the therapeutic potential of anti-PSMA vaccines.

The vaccines, used in the current clinical trial, encode products that are retained in the cytosol and undergo rapid proteasomal degradation (15). This was done deliberately in order to increase the priming of strong cellular immunity to the target antigen (15). Other laboratories have already shown that the nature of the immune response after DNA vaccination is influenced by the type of antigen expressed and its cellular localization (17, 18). Our results clearly fall into this pattern. The fact that antibodies develop following immunization with vectors that encode for proteasomally-degraded proteins may be explained by the release of PSMA fragments by transfected cells that die following vaccination. This will explain why the antibodies in almost all patients were directed against the protein core and against protein fragments, which most probably were generated after proteasomal cleavage. Whether such antibodies may be of any therapeutic value requires further testing and analysis.

Development of antibodies against peptides requires CD4 T cell help. Such help is obviously generated, despite the deliberate design of the vaccines to mainly access the MHC class I presentation pathway, most probably by cross-talk between the two presentation pathways.

In summary, we have shown that:

1. Prostate cancer patients, immunized with gene-based vaccines deliberately designed to access the MHC class I pathway, develop antibodies to the target antigen.
2. The antibodies in almost all patients are directed to the protein core and may recognize fragments that are proteasomally derived.
3. Antibodies develop either against the protein core (majority of patients) or against the carbohydrate residues, but never against both.

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