Identification and Characterization of a Human Agonist Cytotoxic T-Lymphocyte Epitope of Human Prostate-specific Antigen

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Abstract

One potential target of vaccine therapy for human prostate cancer is the prostate-specific antigen (PSA). One strategy to enhance the immunogenicity of a self-antigen such as PSA is to develop agonist epitopes that are potentially more immunogenic. The studies described here report the design and analysis of an agonist epitope designated PSA-3A ("A" for agonist) of the PSA-3 CTL epitope. Studies demonstrate that when compared with the native PSA-3 epitope, the PSA-3A agonist demonstrates enhanced binding to the MHC class I A2 allele as well as enhanced stability of the peptide-MHC complex. T-cell lines generated with either the PSA-3 or the PSA-3A peptide showed higher levels of lysis of targets pulsed with the PSA-3A peptide than those targets pulsed with the PSA-3 peptide; this was observed when both the concentration of peptide and the ratio of effector to target cells were titrated. T cells stimulated with dendritic cells (DCs) pulsed with PSA-3A peptide produced higher levels of IFN-γ than did DCs pulsed with PSA-3 peptide; however, no increase in apoptosis was seen in T cells stimulated with the PSA-3A agonist compared with those stimulated with PSA-3. Human T-cell lines generated with the PSA-3A agonist had the ability to lyse human prostate carcinoma cells expressing native PSA in an MHC-restricted manner. Reombinant vaccinia viruses were also constructed that contained the entire PSA transgene with and without the single amino acid change that constitutes the PSA-3A epitope; DCs infected with the recombinant vector containing the agonist amino acid change within the entire PSA gene (designated rV-PSA-3A) were more effective than DCs infected with the rV-PSA vector in enhancing IFN-γ production by T cells. Finally, the PSA-3A agonist was shown to induce higher levels of T-cell activation, compared with the PSA-3 peptide, in an in vivo model using HLA-A2.1/Kb transgenic mice. These studies thus demonstrate the potential use of the PSA-3A agonist epitope in both peptide- and vector-mediated immunotherapy protocols for prostate cancer.

Introduction

At present, the treatment for prostate cancer involves surgery, radiation, chemotherapy, and/or hormonal therapy. At this time, however, 31,900 men die of prostate cancer each year in the United States alone (1). One new potential modality for the treatment of prostate cancer is vaccine therapy. One of the antigens expressed on prostate carcinoma, which can serve as a potential target for vaccine therapy, is PSA (2). Although PSA is expressed on normal prostatic epithelium, it is not expressed to any appreciable level on other normal adult tissues. Because PSA is clearly a “self-antigen,” questions of tolerance to PSA arise.

Two vaccine clinical trials have now been conducted using a recombinant vaccinia virus expressing the PSA gene (designated rV-PSA) as an immunogen. These studies demonstrated that both anti-PSA antibody responses (3) and PSA-specific T-cell responses (4) could be induced in vaccinated patients. In one of these trials, T-cell responses to a 10-mer PSA peptide could be induced in five of seven prostate cancer patients following vaccination with rV-PSA. These results were achieved using an ELISPOT assay in which PBMCs of patients were incubated overnight with peptide-pulsed APCs; this short incubation period was used to rule out the possibility that the T-cell responses observed were attributable to prolonged cycles of in vitro peptide stimulation of T cells (4). No such PSA-specific T-cell responses were observed when PBMCs were used from the same patient prior to vaccination with rV-PSA.

The peptide used to monitor immune responses in patients receiving rV-PSA was an HLA-A2 binding 10-mer peptide, which has been designated PSA-3 (VISNDVCAQV; Refs. 5, 6). The HLA-A2 binding peptide was used to monitor patients who were positive for the HLA-A2 allele. Patients possessing the HLA-A2 allele were chosen for monitoring because it is the most common HLA allele in individuals in North America and is expressed by ~50% of the Caucasian population (7). Previous studies have also demonstrated that the PSA-3 epitope is naturally processed by tumor cells and bound to MHC class I A2 molecules on the surface of prostate cancer cells, which renders...
them susceptible to lysis by specific T cells in an MHC-restricted manner (5, 6).

Previous studies have analyzed the amino acid sequence of the PSA molecule by both computer algorithms and peptide-binding studies to the HLA-A2-positive T2A2 cell line (8). Studies also analyzed the ability of various PSA peptides to generate CTL lines in vitro; these studies demonstrated that the PSA-3 peptide is optimal for the above properties. Thus, the PSA-3 peptide was subsequently used in the ELISPOT assay described above to monitor immune responses in vaccinated patients (4, 9).

The increases in precursor frequencies observed in the ELISPOT assay as a result of vaccination with rV-PSA were relatively modest (i.e., 2–4-fold; Ref. 4). Studies thus have now focused on enhancing the immunogenicity of PSA with emphasis on the PSA-3 epitope. One way to modify the immunogenicity of a peptide is to modify the amino acid residues that interact with the HLA molecule. Some amino acid modification in the anchor residues of peptides may result in enhanced binding to MHC class I and enhanced T-cell activation, whereas most other amino acid modifications will either have no effect or act to antagonize T-cell activation (10–13). Enhancement of T-cell activation by modifying HLA anchor residues has been demonstrated for some human melanoma-associated antigens (14, 15), but has not been demonstrated for antigens associated with most solid tumors, leukemias, or lymphomas.

The studies described here report the design and analysis of an agonist of the PSA-3 CTL epitope, designated PSA-3A (“A” for agonist). These studies demonstrate that when compared with the native PSA-3 epitope, the PSA-3A agonist demonstrates enhanced binding to the MHC class I A2 allele and enhanced stability of the peptide–MHC complex. T-cell lines generated with either the PSA-3 or the PSA-3A peptide showed higher levels of target lysis when pulsed with the PSA-3A peptide than with the PSA-3 peptide; this was observed when both the concentration of peptide and the ratio of effector to target cells were titrated. T cells stimulated with DCs pulsed with PSA-3A peptide produced higher levels of IFN-γ than did DCs pulsed with PSA-3 peptide. However, no increase in apoptosis was seen in T cells stimulated with the PSA-3A agonist compared with those stimulated with PSA-3. More importantly, human T-cell lines generated with the PSA-3 agonist had the ability to lyse human prostate carcinoma cells expressing native PSA in an MHC-A2-restricted manner. Recombinant vaccinia viruses were also constructed that contained the entire PSA transgene with and without the single amino acid change that constitutes the PSA-3A epitope. DCs infected with the recombinant vector containing the agonist amino acid change within the entire PSA gene (designated rV-PSA-3A) were more effective than the rV-PSA vector in enhancing IFN-γ production by T cells. Finally, the PSA-3A agonist was shown to induce higher levels of T-cell activation, compared with the PSA-3 peptide, in an in vivo model using HLA-A2.1/Kb transgenic mice (16, 17). These studies thus demonstrate the potential use of the PSA-3A agonist epitope in both peptide- and vector-mediated immunotherapy protocols for prostate cancer.

Materials and Methods

Cell Cultures

The human prostate carcinoma cell line LNCaP (Ref. 18; HLA-A2 positive and PSA positive) and SK-MEL-24 (19, a human melanoma cell line (HLA-A2 positive and PSA negative), were purchased from American Type Culture Collection (Manassas, VA). The cultures were free of Mycoplasma and were maintained in complete medium (RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.)). The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or -B antigens (8). C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1 (20). These cells were obtained from Dr. William E. Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). The 174CEM-T2 cell line (T2) transport deletion mutant (21) was provided by Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT). C1R-A2 cells and T2 cells were Mycoplasma free and were maintained in complete RPMI 1640 and in Iscove’s modified Dulbecco’s complete medium (Life Technologies, Inc.), respectively. The V8T cell line, a CTL line directed against the CEA CAP-1 epitope, was established from a patient with metastatic colon carcinoma who was enrolled in a Phase I trial using rV-CEA as immunogen (22). V8T cells were cultured in complete RPMI 1640 containing 10% human AB serum and IL-2 (20 units/ml; provided by the National Cancer Institute, Surgery Branch). V8T cells were restimulated with CAP-1 peptide (25 μg/ml) on day 16 after prior restimulation at an effector-to-APC ratio of 1:3. Irradiated (23,000 rads) autologous EBV-transformed B cells were used as APCs.

Peptides

A panel of analogues with single or double amino acid substitution to positions P1, P2, and P10 of PSA peptide PSA-3 (Ref. 5; Fig. 1), and a CEA peptide, CAP-1-6D (13), were >96% pure; they were made by Multiple Peptide System (San Diego, CA).

Flow Cytometric Analysis

Single-Color Flow Cytometric Analysis. The method for single-color flow cytometric analysis has been described previously (23). Briefly, cells were washed three times with cold Ca2+- and Mg2+-free Dulbecco’s phosphate-buffered saline and then stained for 1 h with monoclonal antibody against HLA-A2 (A2,69; One Lambda, Inc., Canoga Park, CA), at a ratio of 10 μg of the 1× working dilution/106 cells. Mineral oil plasmacytoma-104E (Cappel/Organon Teknika Corp., West Chester, PA) was used as an isotype control. The cells were then washed three times and incubated with a 1:100 dilution of FITC-labeled goat antimouse immunoglobulin (IgG; Kirkegaard and Perry Laboratories, Gaithersburg, MD). The cells were immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm. Data were gathered from 10,000 live cells, stored, and used to generate results.

Dual-Color Flow Cytometric Analysis. The procedure for dual-color flow cytometric analysis was similar to that
for single-color analysis with the following exceptions. The antibodies used were anti-CD4-FITC/anti-CD8-PE, anti-CD45R0-FITC/anti-CD49d-PE, anti-CD28-FITC/anti-CD56-PE, anti-CD86-FITC/anti-CD80-PE, anti-CD58-FITC/anti-CD54-PE, anti-MHC class I-FITC/anti-MHC class II-PE, and anti-IgG1-FITC/anti-IgG2a-PE (isotype controls). Antibodies to CD4, CD8, CD14, CD28, CD45RO, CD56, CD49d, CD54, CD80, CD86, and CD58 were purchased from Becton Dickinson (San Diego, CA). Antibodies to MHC class I and class II were purchased from Serotec (Oxford, United Kingdom). Staining was done simultaneously for 1 h, after which cells were washed three times, resuspended as above, and immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm with the use of the CELLQuest program.

Peptide Binding to HLA-A2

Binding of PSA-3 analogues to HLA-A2 molecules was evaluated by binding to T2A2 cells as demonstrated by flow cytometry (24). Briefly, $1 \times 10^6$ cells in serum-free Iscove’s modified Dulbecco’s complete medium were incubated with peptides at a concentration of 50 $\mu$g/ml in 24-well culture plates at 37°C in 5% CO$_2$. Flow cytometry for peptide binding was performed using T2 cells and single-color analysis. After cells were washed three times in DPBS, as described above, they were incubated for 1 h with HLA-A2-specific monoclonal antibody (One Lambda, Inc.) at a ratio of 10 $\mu$g of a 1× working dilution/$10^6$ cells. UPC-10 (Cappel/Organon Teknika) was used as isotype control. The cells were then washed three times and incubated with a 1:100 dilution of FITC-labeled antimouse IgG (Becton Dickinson). Analysis was conducted with the FACS-cant, as described above. Cells were maintained on ice during all cell preparation and staining.

Culture of DCs from PBMCs

HLA-A2 normal donor PBMCs were obtained from heparinized blood. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC), as described previously (25). DCs were prepared using a modification of the procedure described by Sallusto et al. (26). PBMCs ($1.5 \times 10^6$) were resuspended in AIM-V medium containing 2 mM glutamine, 50 $\mu$g/ml streptomycin, and 10 $\mu$g/ml gentamicin (Life Technologies, Inc.) and allowed to adhere to a T-150 flask (Corning Costar Corp., Cambridge, MA). After 2 h at 37°C, the nonadherent cells were removed with a gentle rinse. The adherent cells were cultured for 6–7 days in AIM-V medium containing 100 ng/ml recombinant human GM-CSF, 20 ng/ml recombinant human IL-4 (rIL-4), and 20 ng/ml of TNF-$\alpha$. The culture medium was replenished every 3 days.

Recombinant Virus and Infection of DCs with Vaccinia Virus Containing PSA (rV-PSA) or PSA-3A (rV-PSA-3A)

PSA cDNA was inserted under the control of the vaccinia 40K promoter (27) into the HindIII M genomic region of the Wyeth strain of vaccinia virus. The E. coli lacZ gene, under the control of the fowlpox C1 promoter (28), was included as a colorimetric marker for recombinant viruses. Recombinant viruses were identified by a chromogenic assay for the lacZ gene product, as described previously (29). For the construction of rV-PSA-3A, codon 155 in the PSA sequence was changed from ATT (isoleucine) to CTG (leucine) by in vitro mutagenesis. The
resulting PSA sequence, designated PSA-3A, was inserted under the control of the vaccinia 40K promoter into the HindIII M genomic region of a derivative of the Wyeth strain of vaccinia virus, using a host-range selection system, as described previously (30, 31). DCs (1 × 10⁶) were incubated in 1 ml of Opti-MEM medium (Life Technologies, Inc.) at 37°C with rV-PSA, rV-PSA-3A, or control vaccinia virus vector. Titration experiments indicated that 1 × 10⁷ plaque-forming units/ml, equal to an MOI of 10 for 1 h, were able to consistently induce production of PSA. The infected DCs were suspended in 10 ml of fresh, warm complete RPMI 1640 containing 100 ng/ml rhGM-CSF, 20 ng/ml rhIL-4, and 20 ng/ml TNF-α, cultured for 24 h, and then subsequently used as APCs.

Generation of T-Cell Lines

A modification of the protocol described by Tsang et al. (22) was used to generate PSA-specific CTLs. DCs were used as APCs, and nonadherent cells isolated from PBMCs were used as a source of effector cells. PSA-3 or PSA-3A peptide was added to the DCs at a final concentration of 25 μg/ml. Autologous nonadherent cells were then added to APCs at an effector-to-APC ratio of 10:1. Cultures were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO₂. After removal of the peptide-containing medium, the cultures were then supplemented with recombinant human IL-2 at a concentration of 20 units/ml for 7 days; the IL-2-containing medium was replenished every 3 days. The 3-day incubation with peptide and 7-day IL-2 supplement constituted one IVS cycle. Primary cultures were restimulated with PSA peptide (25 μg/ml) on day 11 to begin the next IVS cycle. Irradiated autologous DCs were used as APCs for three IVS cycles. Irradiated (23,000 rads) autologous EBV-transformed B cells were used as APCs after the third IVS cycle.

Cytotoxicity Assay

Target cells (C1R-A2 or tumor cells) were labeled with 50 μCi of ¹¹¹In-labeled oxyquinoline (Medi-Physics Inc., Arlington, IL) for 15 min at room temperature. Target cells (0.3 × 10⁶) in 100 μl of complete RPMI 1640 were added to each of 96 wells in flat-bottomed assay plates (Corning Costar Corp.). Labeled C1R-A2 target cells were incubated with peptides at the concentration indicated for 60 min at 37°C in 5% CO₂ before effector cells were added. No peptide was used when carcinoma cell lines were used as targets. Effector cells were suspended in 100 μl of complete RPMI 1640 supplemented with 10% pooled human AB serum and added to the target cells. The plates were then incubated at 37°C in 5% CO₂ for 4 or 16 h. Supernatant was harvested for gamma counting with the use of harvester frames (Skatron, Inc., Sterling, VA). Determinations were carried out in triplicate, and SDs were calculated. Specific lysis was calculated with the use of the following formula (all values in cpm):

\[
\%\ lysis = \frac{\text{Observed release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100
\]

Spontaneous release was determined from wells to which 100 μl of complete RPMI 1640 was added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

Apoptosis Assay

T cells were incubated for 48 h in the presence of peptide-pulsed autologous B cells, as described above in the IVS procedure, and replated to 96-well plates for 24 h. Apoptosis was analyzed using the TUNEL assay (32).

Transgenic Mice

HLA-A2.1/Kb transgenic mice were kindly provided by Dr. L. Sherman (Scripps Laboratories, San Diego, CA). Transgenic mice express the product of the HLA-A2.1/Kb chimeric gene in which the α₃ domain of the heavy chain is replaced by mouse H-2/Kb domain, but the HLA-A2.1 α₁ and α₂ domains are unaltered (16, 17).

In Vivo Immunization and Murine T-Cell Cultures

Three groups of HLA-A2.1/Kb transgenic mice (three mice per group) were immunized s.c. in the base of the tail with PSA-3 peptide-pulsed HLA-A2.1/Kb DCs, PSA-3A peptide-pulsed DCs from HLA-A2.1/Kb transgenic mice, or DCs only. Peptides were used at a concentration of 50 μg/ml, and DCs were used at 1 × 10⁶ cells/mouse/injection. A total of three injections were given to each animal 2 weeks apart. Mice receiving injections of DCs only were used as controls. Mice were sacrificed 7 days after the last injection, and spleen cells were restimulated in vitro with 25 μg/ml PSA-3 or PSA-3A peptide with irradiated syngeneic spleen cells for 6 days. The cytokine production of these bulk cultures was tested. Peptide-pulsed Jurkat A2 (JA2Kb) cells were used as stimulator cells. Jurkat 0201Kb cells are stable transfectants of the human T-cell leukemia line, Jurkat, which expresses the product of HLA-A0201 Kb chimeric gene (33).

Detection of Cytokines

Supernatants of T cells exposed for 24 h to peptide-pulsed uninfected DCs, rV-PSA-infected DCs, or rV-PSA-3A-infected DCs in IL-2-free medium at various responder:stimulator ratios were screened for secretion of IFN-γ, using an ELISA kit (R & D Systems, Minneapolis, MN). The results were expressed in pg/ml.

A CBA system (BD PharMingen, San Diego, CA) was also used to determine the secretion of multiple cytokines by specific T-cell lines. The CBA system uses the fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. The BD human Th1/Th2 cytokine CBA Kit was used to measure IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ protein levels in a single sample. The cytokine capture beads were mixed with PE-conjugated detection antibodies and then incubated with recombinant cytokine standards or test samples to form sandwich complexes. The sample results were generated in graphic and tabular format, using BD PharMingen CBA analysis software. The results were expressed in pg/ml.

Statistical Analysis

Statistical analysis of differences between means was done using a two-tailed paired t test (Stat View statistical software;
Table 1 Peptide analogues

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Initial designation</th>
</tr>
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<tr>
<td>VISNDVCAQV</td>
<td>PSA-3</td>
</tr>
<tr>
<td>VISNDVCAQL</td>
<td>PSA-3 (L-163)</td>
</tr>
<tr>
<td>VLSNDVCAQV</td>
<td>PSA-3 (L-155)</td>
</tr>
<tr>
<td>YISNDVCAQV</td>
<td>PSA-3 (Y-154)</td>
</tr>
<tr>
<td>YLSNDVCAQV</td>
<td>PSA-3 (Y-154/L-155)</td>
</tr>
</tbody>
</table>

Abacus Concepts, Berkeley, CA). Box-Cox power transformation (34) was used to analyze the statistical significance of peptide binding.

Results

Analysis of the primary and secondary HLA-A2 anchor residues at amino acid positions 1, 2, and 10 of the 10-mer PSA-3 peptide revealed that some modifications could possibly enhance binding to HLA-A2. Thus, four different analogues of PSA-3, as shown in Table 1, were synthesized. The four analogues were then tested for binding to the HLA-A2-positive T2A2 cells as described in “Materials and Methods.” The NCA peptide, which has previously been shown to not bind to HLA-A2, was used as a negative control. Peptides were added to T2A2 cells at concentrations of 0–50 μg/ml. As can be seen in Fig. 1A, three of the four analogues bound to HLA-A2; two of these (L155 and Y154) bound to HLA-A2 at higher levels than did PSA-3 at the various peptide concentrations. Peptide analogue L163 exhibited no binding to HLA-A2 (Fig. 1A). An analysis of peptide binding to HLA-A2 at lower peptide concentrations revealed that analogue L155 clearly bound higher levels of HLA-A2 (Fig. 1, B and C). The data indicated that the L155 peptide with a modification in primary anchor position 2 (position 155 of the PSA molecule) was a potential agonist of peptide PSA-3 and was given the designation PSA-3A (‘A’ denoting agonist). For the statistical analysis, both MFIs and the peptide concentrations were logarithmically transformed, and a Box-Cox power transformation (34) was then applied to the log(MFI) values. The Box-Cox parameter 0.58 was chosen because it maximized the $R^2$ statistic of the linear regression fit. The statistic tested was the estimate of the signed area between each pair of fitted lines over the range of the log (concentration) values, and the pairwise $P$s were corrected for multiple comparisons by the method of Hochberg (35). The binding of PSA-3A was significantly different from the parental PSA-3, PSA-3 (Y154), PSA-3 (Y154/L155), and PSA-3 (L163) at $P < 0.0001$. The PSA-3A agonist peptide was thus chosen for further comparative studies with the native PSA-3 peptide. It should be noted from the data in Fig. 1 that a peptide containing the identical change at position 155 (as found in PSA-3A) as well as an additional change at the secondary anchor residue (position 154) actually bound at lower levels to HLA-A2 than did the native PSA-3 peptide.

Studies were then undertaken to examine the stability of the peptide-MHC complex for peptides PSA-3 and PSA-3A. Peptides were incubated with T2A2 cells overnight, washed free of unbound peptide, and incubated with brefeldin-A to block delivery of new class I molecules to the cell surface; at various times, cells were stained for the presence of surface peptide-HLA-A2 complexes. Results are expressed in relative percentage of binding compared with 100% at time 0.

Fig. 2 Comparison of the stability of the complex of the PSA-3 or PSA-3A peptide with HLA-A2. T2A2 cells were incubated overnight with PSA-3 ( □ ) or PSA-3A ( ▪ ) peptide at a concentration of 50 μg/ml and then washed free of unbound peptide and incubated with brefeldin-A to block delivery of new class I molecules to the cell surface. At the indicated times, cells were stained for the presence of surface peptide-HLA-A2 complexes. Results are expressed in relative percentage of binding compared with 100% at time 0.

Studies were then conducted to establish and compare human T-cell lines using APCs pulsed with the PSA-3 and PSA-3A peptides. DCs from an apparently healthy donor were pulsed with either peptide as APC, and autologous PBMCs were used as a source of effector cells. T-cell cultures were established by pulsing with each peptide in the presence of IL-2 as described in “Materials and Methods.” At IVS cycle 3 (IVS-3), T cells were analyzed for the ability to lyse peptide-pulsed targets. The line established with peptide PSA-3A, designated T-PSA-3A, was shown to lyse C1R-A2 cells pulsed with peptide PSA-3A to a greater extent than cells pulsed with the PSA-3 peptide (Fig. 3A). T cells established using the PSA-3 peptide, however, also lysed target cells pulsed with the PSA-3A peptide to a greater extent than those pulsed with the PSA-3A peptide (Fig. 3B). This was seen at two different E:T cell ratios. To further analyze this phenomenon, we pulsed C1R-A2 cells pulsed with different concentrations of the native and agonist peptides and used them as targets for the two T-cell lines. T cells generated with the...
PSA-3A peptide showed a greater percentage of lysis of target cells pulsed with the PSA-3A peptide than cells pulsed with the PSA-3 peptide at each peptide concentration; approximately 8-fold less PSA-3A peptide than PSA-3 peptide was necessary for lysis (Fig. 4A). Moreover, this phenomenon was also observed using T cells established with the native PSA-3 peptide, i.e., these T cells lysed C1R-A2 target cells pulsed with the PSA-3A agonist peptide to greater levels at each peptide concentration than target cells pulsed with the PSA-3 peptide; approximately 8-fold less PSA-3A peptide was required to achieve similar levels of lysis. These studies provided the first indication that a T-cell line derived from the PSA-3A peptide could recognize the native PSA-3 peptide-MHC complex and lyse such cells presenting this complex.

The T-cell lines established with peptide PSA-3 (designated T-PSA-3) and peptide PSA-3A (designated T-PSA-3A) were both shown to be >95% positive for CD49d, >67% positive for CD8\(^+\), <3.0% positive for CD56\(^+\), and >88% positive for CD45RO\(^+\) cells. T cells stimulated with autologous B cells pulsed with either peptide were analyzed at IVS-5 for DNA fragmentation by the TUNEL assay. As seen in Fig. 5, 2.9% apoptotic CD8\(^+\) cells were seen in the absence of peptide (Fig. 5A). 4.9% apoptotic CD8\(^+\) cells were seen in cells stimulated with PSA-3 peptide (Fig. 5B), and 4.5% apoptotic CD8\(^+\) cells were seen when cells were stimulated with PSA-3 peptide (Fig. 5C). Thus, no difference in apoptosis was seen in CD8\(^+\) T cells stimulated with the agonist peptide compared with the native peptide.

The cytokine profile of T cells stimulated with APCs pulsed with either the PSA-3 or the PSA-3A peptide was then analyzed. The T-cell line derived using the PSA-3 peptide was used in these studies, and the APCs used were autologous B cells. A CBA assay was used for analysis (see Materials and Methods) of supernatant fluids obtained 24 h after stimulation. Fig. 6, A, B, and C, illustrate 3 of the 10 internal standards used for the calibration of the results obtained in this assay. Fig. 6, D, E, and F, show the levels of each of six cytokines produced by
T cells stimulated with APCs pulsed with no peptide, PSA-3A peptide, and PSA-3 peptide, respectively. These results demonstrate greater production of the type I cytokines IL-2 and IFN-γ by T cells stimulated with the PSA-3A peptide than with the PSA-3 peptide. Low or undetectable levels of the type 2 cytokines IL-4 and IL-10 were seen with either peptide. No TNF-α could be detected in supernatants at the 24-h time point. The results obtained for IL-5 will be discussed below (“Discussion”). Studies were also conducted to determine the level of IFN-γ production by T cells originally generated with the PSA-3A peptide when stimulated with DCs that were pulsed with the PSA-3 and PSA-3A peptides. As seen in Table 2, T cells stimulated with DCs pulsed with the PSA-3A peptide produced more IFN-γ than did those stimulated with DCs pulsed with the PSA-3 peptide. Cultures devoid of peptide, DCs, or T cells demonstrated the specificity of the IFN-γ production (Table 2) and the lack of nonspecific antiallogeneic reactivity. To ascertain the reproducibility of this observation from T-
Table 2 Production of IFN-γ by a T-cell line stimulated with PSA-3 or agonist PSA-3A peptides

Cells from the PSA-specific T-cell line T-PSA-3A were used as effector cells at IVS-3. T-PSA-3A cells were stimulated with irradiated HLA-A2-positive allogeneic DCs pulsed with PSA-3 or PSA-3A peptide at a concentration of 25 μg/ml and an effector-to-APC ratio of 10:1. Twenty-four-h culture supernatants were collected and screened for the secretion of IFN-γ.

<table>
<thead>
<tr>
<th>Antigen-presenting cells</th>
<th>Peptide</th>
<th>T Cell</th>
<th>Production of IFN-γ (pg/ml)</th>
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<tbody>
<tr>
<td>DCs</td>
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<td>+</td>
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</tr>
<tr>
<td>DCs</td>
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<td>+</td>
<td>&lt;15</td>
</tr>
</tbody>
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Table 3 Production of IFN-γ by T-cell lines, generated from two prostate cancer patients, stimulated with PSA-3 and PSA-3A agonist peptide

Cells from four PSA-specific T-cell lines established from two prostate cancer patients (patients 28 and 30) were used as effector cells at IVS-3. These T-cell lines were established by stimulation with PSA-3-pulsed autologous DCs (T-28-PSA-3 and T-30-PSA-3) or PSA-3A-pulsed autologous DCs (T-28-PSA-3A and T-30-PSA-3A). For IFN-γ production, T-cell lines were stimulated with irradiated HLA-A2-positive allogeneic DCs pulsed with either PSA-3 or PSA-3A peptide at a concentration of 25 μg/ml and an effector-to-APC ratio of 10:1. Twenty-four-h culture supernatants were collected and screened for the secretion of IFN-γ.

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<tr>
<th>Peptide</th>
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<td>822.9</td>
<td>384.3</td>
<td>839.9</td>
</tr>
</tbody>
</table>

Fig. 7 Ability of autologous B cells pulsed with native PSA peptide PSA-3 and agonist peptide PSA-3A to induce IFN-γ production by specific T-cell lines. Shown is production of IFN-γ by T-PSA-3A cells stimulated with autologous B cells pulsed with PSA-3 peptide (○) and PSA-3A peptide (●) and by T-PSA-3 cells stimulated with autologous B cells pulsed with PSA-3 peptide (△) and PSA-3A peptide (▲). T-PSA-3A and T-PSA-3 cell lines were T-cell lines generated by stimulation of PBMCs by APCs pulsed with PSA-3A peptide and PSA-3 peptide, respectively. Results are expressed in pg/ml.

PSA-3 and T-PSA-3A cell lines, additional T-cell lines were established from two prostate cancer patients (patients 28 and 30). Four PSA-specific T-cell lines were generated and designated T-28-PSA-3, T-28-PSA-3A, T-30-PSA-3, and T-30-PSA-3A. Cell lines T-28-PSA-3 and T-28-PSA-3A were generated from patient 28 by stimulation of PBMCs by DCs pulsed with PSA-3 and PSA-3A peptide, respectively. Cell lines T-30-PSA-3 and T-30-PSA-3A were generated from patient 30 by stimulation of PBMCs by DCs pulsed with PSA-3 and PSA-3A peptide, respectively. Similar results were obtained with respect to the production of IFN-γ by these T-cell lines stimulated with PSA-3 and PSA-3A peptide (Table 3) compared with the T-PSA-3 and T-PSA-3A cell lines (Table 2). The ability of the T-PSA-3 and T-PSA-3A cell lines to release IFN-γ at various levels of PSA-3 peptide and PSA-3A peptide stimulation were investigated. At each concentration of peptide, PSA-3A peptide led to increases in IFN-γ production by activated T cells, and T-PSA-3A cells released higher levels of IFN-γ at each concentration of peptide compared with T-PSA-3 cells (Fig. 7).

It has previously been shown that the activation of T cells by peptide-pulsed DCs is related to the level of IL-12 production by the DCs. Studies were conducted to determine the levels of IL-12 production by DCs pulsed with PSA-3 and PSA-3A peptides in the presence of T cells. As shown in Table 4, DCs in the absence of peptide, T cells alone, and peptide-pulsed T cells in the absence of DCs all failed to produce detectable levels of IL-12. When DCs pulsed with PSA-3 peptide were incubated with either the T-PSA-3 or the T-PSA-3A T-cell lines, DCs were shown to produce IL-12, with more IL-12 being produced by peptide-pulsed DCs cultured with the T-PSA-3A line (Table 4). When DCs pulsed with the PSA-3A peptide were incubated with both T-cell lines, the DCs incubated with the T-PSA-3A line again produced higher levels of IL-12 than those peptide-pulsed DCs incubated with the T-cell line T-PSA-3.

Studies were then conducted to determine whether T cells generated with the PSA-3A agonist peptide could lyse tumor cells that endogenously express native PSA. As shown in Table 5, the T-PSA-3A line was capable of lysing the LNCaP human prostate carcinoma cell line that expresses native PSA and is HLA-A2 positive. The HLA-A2-positive SK-mel human melanoma line that does not express PSA showed no lysis. The T-PSA-3 line (generated with the native PSA-3 peptide) also lysed the LNCaP line, but at lower levels than those observed with the T-PSA-3A line at each E/T cell ratio (Table 5). To
determine whether the lysis was MHC restricted, antibody-blocking experiments were carried out. As seen in Table 5, lysis of the LNCaP line by the T-PSA-3A line was blocked by the anti-HLA-A2 antibody and not by the isotype-matched control antibody, UPC-10. The PSA-specific T-cell lines T-30-PSA-3 and T-30-PSA-3A, generated from a prostate cancer patient (patient 30), also lysed the LNCaP cells; however, T-30-PSA-3A cells lysed the LNCaP cells at higher levels than those observed with the T-30-PSA-3 cells (Table 6).

The HLA-A2/Kb transgenic mouse has previously been reported to aid in the determination of the immunogenicity of HLA-A2 peptides for humans. This in vivo system was thus used to further analyze and compare the immunogenicity of the PSA-3A peptide with that of the native PSA-3 peptide. DCs from the HLA-A2/Kb transgenic mice were pulsed with either the PSA-3 or the PSA-3A peptide and used to vaccinate HLA-A2/Kb mice. DCs alone (no peptide) were also used as a control. The PSA recombinant vectors were designated rV-PSA and rV-PSA-3A, and wild-type vaccinia virus did not alter the surface phenotype markers CD80, CD86, CD54, CD58, class I, and class II MHC class I molecule. These mice were developed for the purpose of studying T-cell selection and as a model for predicting the immunogenicity of protein and peptide structures in human class I molecules. It has been demonstrated that the CTL epitopes of human papillomavirus 16 E6 and E7, MUC1, hepatitis B virus, gp-100, and PSA identified by MHC class I binding assays can be immunogenic in HLA-A2/Kbtransgenic mice (6). Replacement of the α3 transmembrane and cytoplasmic domains of H-2Kb by the α3 domain of the HLA-A2.1 molecule expressed a chimeric class I molecule composed of the α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic domains of H-2Kb (33). This has been interpreted as a failure of human MHC molecules to interact efficiently with murine T cells. However, that may be attributable to the fact that the murine processing system does not recognize the PSA-3 epitope in terms of affinity of binding to MHC molecules, avidity of the peptide-MHC complex, and ability to activate CTLs in vitro. The agonist epitope was also more efficient than the native epitope in T-cell activation in an in vivo HLA-A2.1/Kb transgenic mice model (6). HLA-A2.1/Kb transgenic mice express a higher level of IFN-γ production by T cells after vaccination than were DCs infected with rV-PSA or rV-PSA-3A-3A. Recombinant vaccinia viruses were thus constructed with the entire PSA transgene without and with the amino acid change of isoleucine to leucine at position 155; these recombinant vaccinia viruses were designated rV-PSA and rV-PSA-3A, respectively. Initial studies showed that the optimal MOI for human DCs for both recombinant vaccinia viruses was 10 plaque-forming units/cell. Human DCs infected with either of the recombinant vectors showed production of PSA protein at ~63 ng/ml as determined by immunoassay of supernatant fluids. Moreover, infection of human DCs with rV-PSA or rV-PSA-3A-3A and wild-type vaccinia virus did not alter the surface phenotype markers CD80, CD86, CD54, CD58, class I, and class II of DCs. DCs infected with either rV-PSA or rV-PSA-3A were used to stimulate T cells originally derived by pulsing APCs with the native PSA-3 peptide. As seen in Table 8, DCs infected with the rV-PSA-3A recombinant were more effective in activating PSA-specific T cells, as measured by IFN production, than were DCs infected with rV-PSA. For comparison, uninfected DCs pulsed with 25 μg of the PSA-3A peptide were also used to activate T-PSA-3 T cells. As a specificity control, no activation of the CEA-specific T-cell line (V8T) was observed by the PSA recombinant vectors. As an additional control, DCs infected with wild-type vaccinia vector were used. These studies thus demonstrate that the altered PSA epitope can also be processed by human DCs and presented for the activation of specific T cells.

**Discussion**

The data presented here demonstrate that the agonist peptide PSA-3A epitope is superior to the native PSA-3 epitope in terms of affinity of binding to MHC molecules, avidity of the peptide-MHC complex, and ability to activate CTLs in vitro. The agonist epitope was also more efficient than the native epitope in T-cell activation in an in vivo HLA-A2.1/Kb transgenic mice model (6). HLA-A2.1/Kb transgenic mice express a chimeric class I molecule composed of the α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic domains of H-2Kb (33). Replacement of the α3 domain of the heavy chain allows interaction of the murine CD8 molecule on the CD8+ T cells with the syngeneic α3 domain of the hybrid MHC class I molecule. These mice were developed for the purpose of studying T-cell selection and as a model for predicting the immunogenicity of protein and peptide structures in human class I molecules. It has been demonstrated that the CTL epitopes of human papillomavirus 16E6 and E7, MUC1, hepatitis B virus, gp-100, and PSA identified by MHC class I binding assays can be immunogenic in HLA-A2.1/Kb transgenic mice (6, 36–39). The possibility exists that peptide-HLA-A2 complexes that are recognized by murine T cells may be different from those recognized by human T cells. It has been reported that only low levels of viral antigen-specific CTLs were detected in the transgenic mice immunized with the same antigen. This has been interpreted as a failure of human MHC molecules to interact efficiently with murine T cells. However, that may be attributable to the fact that the murine processing system does not recognize the PSA-3 epitope in terms of affinity of binding to MHC molecules, avidity of the peptide-MHC complex, and ability to activate CTLs in vitro.
not produce appropriate peptides for association with human MHC class I molecules. This problem can be overcome by use of peptides for immunization. Results from our study (6) and from other investigators (36–39) suggest that the HLA-A2.1/Kb transgenic mouse may be an important model for the study of immunodominant peptides recognized by T cells. T cells activated by the PSA-3A agonist peptide had the ability to lyse both APCs pulsed with native peptide and human prostate cancer cells expressing native PSA in an MHC-restricted manner. The studies with the vaccinia recombinants demonstrated that the agonist epitope, in the context of a whole PSA molecule, could also be processed and presented by APCs for T-cell activation. Moreover, T cells stimulated by the agonist peptide, compared with the native peptide, were shown to produce higher levels of type I cytokines, but were not subject to increased apoptosis.

Analysis of 10-mer HLA-A2.1 ligands has suggested that residues strongly associated with good HLA-A2.1 binding at primary anchor position 2 are Leu and Met, and Val, Leu, and Ile for position 10 (40). Substitution of Ile at position 2 (amino acid position 155) of the PSA-3 peptide with Leu improved the efficiency of the peptide binding to the HLA-A2.1 molecule. This observation can be explained by the fact that Ile at position 2 can reduce the peptide binding affinity to HLA-A2.1 as well as the stability of the resulting peptide-MHC complexes (40, 41). Replacement of Thr at the NH2-terminal anchoring position 2 of gp-100 peptide 154–162 with Leu or Ile resulted in significantly improved binding to the HLA-A2 molecule. However,
there was no difference in the peptide binding affinity or antigenicity when Ile was substituted for Leu (39). On the other hand, substitution of Ala at position 2 of the nonapeptide Melan-A(27–35) with Leu resulted in increased peptide binding to HLA-A2, but with a >50-fold reduction in antigenic activity (14).

Substitution of Val with Leu at position 10 resulted in a decrease in binding of the PSA-3 peptide to the HLA-A2.1 molecule. This may be explained partially by the fact that Leu at position 9, instead of Val, reduced the peptide-HLA-A2.1 affinity as well as the stability of the complex (39, 42). It has been demonstrated that in the case of Flu A matrix HLA-A2.1 molecule, the actual binding affinity of this peptide was not high.

Table 8  Ability of DCs infected with vaccinia PSA recombinants to process the PSA-3A agonist epitope to activate PSA-specific T cells

<table>
<thead>
<tr>
<th>DCs infected with</th>
<th>Pulsed with peptide</th>
<th>Production of IFN-γ (pg/ml) by</th>
<th>T-PSA-3</th>
<th>V8T</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>rV-PSA</td>
<td>None</td>
<td>195.5</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
<tr>
<td>rV-PSA (PSA-3A)</td>
<td>None</td>
<td>425.5</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
<tr>
<td>V-WT</td>
<td>PSA-3A</td>
<td>946.6</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>PSA-3A</td>
<td>915.9</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
<tr>
<td>V-WT</td>
<td>CAP1-6D</td>
<td>&lt;13.0</td>
<td>787.4</td>
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</tr>
<tr>
<td>V-WT</td>
<td>None</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td>&lt;13.0</td>
<td></td>
</tr>
</tbody>
</table>

It is interesting to note that the analogue peptide made by amino acid substitution with Leu at position 2 (amino acid position 155) and with Tyr at position 1 (amino acid position 154) was not as efficient in binding to the HLA-A2.1 molecule as the native PSA-3 peptide when assayed by binding to T2A cells. This may have resulted from negative effects such as conformational changes, steric hindrances, or repulsive electrostatic interactions. It has been demonstrated that, although a gp-100 peptide 280–288 (YLEPGPVTA) contains the predicted motif residues Tyr at position 1, Leu at position 2, and Ala at position 9, which support good binding to the HLA-A2.1 molecule, the actual binding affinity of this peptide was not high.

It is postulated that the agonist PSA-3A peptide described here can potentially be used either as a peptide vaccine in adjuvant or via peptide-pulsed DCs in vaccine therapy for prostate cancer patients positive for the HLA-A2 allele. Recent studies showing enhanced peptide-pulsed DC activity with molecules such as Flt-3L (48, 49) or TRICOM vectors (vectors containing three costimulatory molecules; Ref. 50) make such DC vaccine protocols even more attractive.

The studies described demonstrate that PSA-specific T cells, generated with the use of either the native PSA-3 epitope or the PSA-3A agonist, produced more IFN-γ in response to the PSA-3A agonist peptide than the PSA-3 peptide. The production of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ was determined by CBA assay as described in “Materials and Methods.” The results indicate an increase in the production of IL-2, IL-5, and IFN-γ, but not IL-4 and IL-10, in T cells stimulated with PSA-3A compared with PSA-3 peptide. Increased production of IL-5 by the activated T cells may in turn activate eosinophils, basophils, B cells, and thymocytes. IL-5 is a chemoattractant and activating factor for eosinophils. IL-5 has also been shown to exhibit a killer-helper factor activity on peanut agglutinin-binding thymocytes in the presence of stimulator cells and IL-2.
resulting in the recruitment of CTLs from among the thymocyte population as a result of the ability of IL-5 to up-regulate IL-2 receptors on target thymocytes (51, 52). These studies thus indicate that the agonist peptide may also potentially be used in more sensitive immunoassays to monitor immune responses of patients involved in PSA vaccine clinical trials.

Several clinical trials involving PSA-based vaccines have now been completed or are in progress with patients with prostate cancer. A recent study in patients with advanced prostate cancer demonstrated that an rV-PSA vaccine induced specific T-cell responses for the PSA-3 peptide in five of seven HLA-A2.1 patients vaccinated (4). The rV-PSA vaccine was shown to be safe, and certain patients remained without evidence of clinical progression for at least 21 months (4). Ongoing clinical trials are using rV-PSA as a primary vaccination, followed by multiple booster vaccinations with a recombinant fowlpox-PSA vaccine. This diversified prime and boost vaccine strategy has shown to be advantageous in both animal models (53) and clinical trials (54) using recombinant CEA vector vaccines. The studies reported here demonstrate that the single amino acid change in the PSA molecule can also be used in recombinant PSA vaccinia or fowlpox vectors, or for that matter in a DNA vector, to activate T cells more efficiently than use of the same vector containing the native PSA gene. Although the agonist epitope may benefit only those patients possessing an HLA-A2 allele, this still constitutes approximately half of the Caucasian population as well as other populations to a lesser extent. Finally, these studies reinforce the concept that a single amino acid change in the composition of a CTL epitope of a relatively weak immunogenic self-antigen, such as PSA, can be used to more efficiently activate specific T-cell populations capable of lysing tumors expressing the native gene product.

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References


