Dendritic Cell-Based Xenoantigen Vaccination for Prostate Cancer Immunotherapy[1]

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Many tumor-associated Ags represent tissue differentiation Ags that are poorly immunogenic. Their weak immunogenicity may be due to immune tolerance to self-Ags. Prostatic acid phosphatase (PAP) is just such an Ag that is expressed by both normal and malignant prostate tissue. We have previously demonstrated that PAP can be immunogenic in a rodent model. However, generation of prostate-specific autoimmunity was seen only when a xenogeneic homolog of PAP was used as the immunogen. To explore the potential role of xenoantigen immunization in cancer patients, we performed a phase I clinical trial using dendritic cells pulsed with recombinant mouse PAP as a tumor vaccine. Twenty-one patients with metastatic prostate cancer received two monthly vaccinations of xenoantigen-loaded dendritic cells with minimal treatment-associated side effects. All patients developed T cell immunity to mouse PAP following immunization. Eleven of the 21 patients also developed T cell proliferative responses to the homologous self-Ag. These responses were associated with Ag-specific IFN-γ and/or TNF-α secretion, but not IL-4, consistent with induction of Th1 immunity. Finally, 6 of 21 patients had clinical stabilization of their previously progressing prostate cancer. All six of these patients developed T cell immunity to human PAP following vaccination. These results demonstrate that xenoantigen immunization can break tolerance to a self-Ag in humans, resulting in a clinically significant antitumor effect.   *The Journal of Immunology,* 2001, 167: 7150–7156.

Prostate cancer represents the most common malignancy of men and the second leading cause of cancer-related mortality in the United States, with nearly 31,500 men projected to die from this disease in 2001 (1). While the treatment of metastatic prostate cancer is noncurative and has not significantly changed in over 20 years, a number of new approaches are under investigation, including vaccines designed to induce systemic immunity to prostate cancer cells (2–6). However, the development of such vaccines relies on the identification of relevant target Ags as well as approaches to deliver Ags to generate immunity. Prostate tissue and prostate cancer express several potential target Ags, including prostate-specific Ag (PSA),[4] prostate-specific membrane Ag, and prostatic acid phosphatase (PAP). Although PSA and prostate-specific membrane Ag have been detected in other tissues (7–9), PAP appears to be restricted in its tissue distribution to the prostate and prostate carcinoma (10). mAbs with specificity for PAP stain only normal prostate and prostate cancer cells (11), and the use of PAP cDNA to probe Northern blots from a variety of tissues revealed PAP RNA only in benign and malignant prostate cells (12). The gene for human PAP (hPAP) contains a 1065-nt coding region that gives rise to a 354-aa polypeptide with an estimated molecular mass of 41 kDa (13). Homologs of hPAP have been identified in rat PAP (14) and mouse PAP (mPAP) (our unpublished observations). These proteins share 75 and 81% homology with the human protein on an amino acid level.

Because PAP represents a self-Ag, it is not inherently immunogenic. T cells that recognize self-Ags with high avidity are believed to undergo negative selection through clonal deletion in the thymus or anergy in the periphery (15). We have demonstrated that PAP can be immunogenic in a rodent model (16). However, only immunization with a xenogeneic PAP (hPAP into rat) generated cytotoxic T lymphocytes and prostate-specific autoimmunity, suggesting that xenoantigens may be capable of overcoming tolerance against the homologous self-Ag. Other groups have made similar observations with melanoma Ags (17, 18). Therefore, we wished to examine the use of xenogeneic PAP immunization in humans as a potential cancer vaccine.

One way to potentially immunize patients against PAP is to isolate their dendritic cells (DC), load them with Ag, and then reinfuse these cells as the vaccine. DC represent the most potent APC of the immune system, uniquely capable of sensitizing naive T cells to new Ags. Moreover, when Ag-loaded DC are administered systemically, they home to lymphoid organs where they activate Ag-specific T cells, thereby inducing an immune response. This approach has been used successfully to vaccinate cancer patients, resulting in antitumor immunity and clinical response (19). In the described clinical trial, we examined the ability of DC loaded with the xenogeneic homolog of hPAP derived from mice, mPAP, to generate immune responses in patients with advanced prostate cancer.

Materials and Methods

Patient selection

Men ($n = 21$) enrolled in this clinical study were required to have histologically documented prostate adenocarcinoma with rising serum PSA levels as well as measurable serum PAP. Patients could be hormone refractory or hormone sensitive so long as no hormonal manipulations were performed within the 4 wk before enrolling in the trial to control for antiandrogen withdrawal effects (6 wk if the patient had received bicalutamide). Other eligibility requirements included Karnofsky performance status >70; negative serological tests for HIV, human T cell leukemia virus-1, hepatitis B, and hepatitis C; white blood cell count ≥2,000/mm^{3}; absolute neutrophil count ≥1,000/mm^{3}; platelets ≥100,000/mm^{3}; creatinine ≤2.0 mg/dl; total bilirubin equal to or less than twice the upper limit of normal; and liver transaminases equal to or less than five times the upper limit of normal. The protocol was approved by the Stanford University School of Medicine (Palo Alto, CA) institutional review board, and trial subjects provided signed informed consent before completing the screening process.

Clinical monitoring

Patients were monitored by interval histories, physical examinations, blood counts, serum chemistries, and measurements of PSA and PAP monthly during vaccination and then monthly until evidence of disease progression. Patients were evaluated for toxicity by the National Cancer Institute common toxicity criteria. Patients were assessed for antinuclear Abs and rheumatoid factor before and following vaccination. HLA typing was performed at the Stanford Blood Center. Tumor burden was evaluated by computed tomography and bone scans prevaccination, 4 mo following vaccination, and then every 6 mo until clinical progression. Unless accompanied by clinical progression, increasing levels of PSA or PAP before completion of the vaccination were not taken as reasons for discontinuation. Following vaccination, clinical progression was defined by rising serum PSA levels >50% above baseline and/or new or enlarged lesions on computerized tomography or bone scans. Stable disease was defined as a clinical course that met criteria for neither progressive disease nor partial response (≥50% reduction of measurable or evaluable disease).

PAP Ags

cDNA encoding mPAP was cloned into the pBacPAK8 baculovirus recombination vector (Clontech Laboratories, Palo Alto, CA) to generate recombinant baculovirus. rmPAP was expressed as a His[6] fusion protein. Recombinant baculovirus was then cloned by plaque purification and propagated in Sf21 cells adapted to growth in protein-free suspension. mPAP was purified from culture supernatants with Ni-NTA chromatography (Qiagen, Hilden, Germany) to >95% purity by SDS-PAGE. hPAP used for in vitro assays was purified from human seminal fluid (Biodesign International, Kennebunk, ME).

DC vaccine preparation

The patients underwent unmobilized peripheral blood leukapheresis, with two total body blood volumes (8–14 L of blood) processed with a Cobe cell separator (Gambro, Lakewood, CO). PBMC were obtained by centrifugation over Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden), and then monocytes were depleted by density centrifugation through Percoll (Amersham Biosciences) density 1.065 as previously described (20–22). Monocyte-depleted PBMC were incubated with mPAP (2 μg/ml) in RPMI 1640 (BioWhittaker, Walkersville, MD) with 10% pooled human AB serum without the addition of exogenous cytokines. After a 24-h culture in a humidified incubator at 37°C with 10% CO_2, DC were further enriched from lymphocytes by centrifugation through a 15% (w/v) metrizamide gradient (Sigma-Aldrich, St. Louis, MO). The enriched DC were cultured overnight in medium containing 50 μg/ml mPAP, washed free of Ag, resuspended in normal saline with 5% autologous serum, and infused. The DC dose was calculated by determining the percentage of DC in the vaccine by flow cytometric analysis assessing lineage-negative (CD3, -14, -19, -56), HLA-DR-positive, and CD11c-positive cells performed using a FACSCalibur (BD Biosciences, Mountain View, CA). The mean DC purity in the administered vaccination was 30%. While the enriched DC product contained some contaminating T cells, the number of T cells transferred ($<5 \times 10^7$) is less than the sizeable dose required to produce any systemically measurable immune response (23, 24). The DC product also contained some B cells and monocytes, although these cell types have not been demonstrated to prime immunity in vivo in humans. Nevertheless, their potential contribution to the immune response cannot be excluded.

DC vaccination

Twenty-one prostate cancer patients were immunized twice with mPAP-loaded DC 4 wk apart. Patients were sequentially assigned to three cohorts to receive both DC immunizations via i.v., intradermal (i.d.), or intralymphatic (i.l.) injections. For i.v. administration, DC were suspended in 100 ml of normal saline with 5% autologous serum and infused by a peripheral i.v. catheter following premedication with acetaminophen and diphenhydramine. For i.d. administration, DC were suspended in 4 ml of normal saline with 5% autologous serum and were administered 12–20 i.d. injections into the medial thighs following application of topical anesthetic. For i.l. administration, DC were also suspended in a volume of 4 ml, but were infused via a catheter cannulating a lymphatic channel in the dorsum of the foot that was identified through a small incision. This volume is a typical volume for i.l. injection of radiographic contrast for lymphangiography. All adverse events were evaluated for their possible relationship to treatment with DC injections, and the severity was scored according to the National Cancer Institute common toxicity criteria.

T cell functional assays

Blood was obtained from patients before immunization, 1 mo following the DC immunizations, monthly for the initial 6 mo following vaccination, and then every 3 mo thereafter until clinical progression. PBMC were obtained by centrifugation over Ficoll-Hypaque (Amersham Biosciences) and were cultured at 100,000 cells/well in triplicate in 96-well U-bottom plates (Costar, Cambridge, MA) in medium containing 10–50 μg/ml mPAP or hPAP. Other T cell stimulators used for in vitro assays included influenza protein (Aventis Pasteur Connaught, Swiftwater, PA) and PMA with ionomycin (Sigma-Aldrich). T cell proliferation was assessed on the basis of 18-h [^{3}H]thymidine incorporation after 6 days of culture as measured in a Microbeta counter (Wallac, Turku, Finland). The results are expressed as stimulation indexes representing counts per minute relative to baseline counts without Ag. To establish the background for the proliferation assays, a control population of 20 volunteer blood donors was assessed with the same methodology and had average stimulation indexes of 1.15 ± 0.34 and 0.86 ± 0.46 to mPAP and hPAP, respectively. A stimulation index >2 was therefore defined as a positive response. Supernatants were also collected from cell cultures, frozen, and assessed for cytokine secretion by ELISA as described below.

Cytokine ELISA

Ninety-six-well Immulon-4 plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 50 μl of the primary Ab to IL-4, IL-10, IFN-γ, and TNF-α (BD PharMingen, San Diego, CA) in 0.1 M carbonate-bicarbonate buffer, pH 9.5. Wells were blocked with Blotto (5% nonfat dry milk in 0.05% Tween 20) for 2 h at room temperature. Frozen cell supernatants were added to the wells and incubated at room temperature for 3 h, after which the appropriate biotinylated secondary Ab resuspended in Blotto was added and incubated for 1 h at room temperature. Following washing with 0.05% Tween 20, HRP-conjugated rabbit anti-mouse Ab was added and incubated for 30 min at room temperature. The plates were washed and developed with the substrate tetramethyl benzidine (Zymed Laboratories, South San Francisco, CA). The reaction was stopped with 1 N HCl, and the OD was read at 450 nm on a microplate reader (Bio-Rad, Hercules, CA). The limit of detection for the three cytokines assayed was 25 pg/ml. To establish the background levels of cytokine production, a control population of 20 healthy volunteer adult blood donors was assessed with the same methodology to mPAP and hPAP. The average cytokine levels measured in these cultures were 1.0 ± 3.0, 13.6 ± 37.9, and 16.7 ± 24.7 pg/ml for IL-4, IFN-γ, and TNF-α, respectively. Cytokine production >100 pg/ml was defined as a positive response.

Cytokine ELISPOT

Ninety-six-well polyvinylidene difluoride-backed plates (MAHA S 45; Millipore, Bedford, MA) were coated with primary Ab to IFN-γ or IL-4 (Mabtech, Stockholm, Sweden) overnight at 4°C. Plates were then washed six times with PBS and blocked with RPMI 1640 supplemented with L-glutamine, penicillin, and 10% heat-inactivated pooled human AB serum (complete medium (CM)) for 1 h. PBMC (10^5) were added in 100 μl/well CM to the precoated plates. Ag was resuspended in CM, and 100 μl was added to the cells in duplicate or triplicate wells. The cells were incubated for 72 h at 37°C in 5% CO_2 and washed six times with PBS, and the appropriate biotinylated detection Ab (Mabtech) was then added. After 2 h of incubation plates were washed with PBS, and a streptavidin-alkaline phosphatase conjugate (Mabtech) was added to the wells. The plates were then incubated for 2 h at room temperature. Wells were washed and chromogenic alkaline phosphatase substrate (Bio-Rad) was added. After 30–60

min, the colorimetric reaction was terminated by washing with water, and plates were air-dried. Spots were counted at ×20 with a stereomicroscope (Leitz GZ6; Leitz, Wetzlar, Germany).

Anti-PAP ELISA

Sera collected simultaneously with the PBMC were frozen and analyzed in batches. Ninety-six-well Immulon-4 plates were coated overnight at 4°C with either mPAP or hPAP, blocked with 5% dehydrated nonfat milk in 50 mM TBS and 0.05% Tween 20, and washed with 0.05% Tween 20. Patient sera were diluted in PBS, added to wells, and incubated for 1 h at room temperature. Plates were washed and incubated with goat anti-human total Ig Ab labeled with HRP (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for an additional hour at room temperature. The plates were washed and developed with the substrate tetramethyl benzidine (Zymed Laboratories). The reaction was stopped with 1 N HCl, and the OD was read at 450 nm on a microplate reader (Bio-Rad).

Statistical analyses

T cell proliferative responses before and after DC vaccination were analyzed with the paired sign test (StatView; SAS Institute, Cary, NC). Correlations among baseline absolute lymphocyte count, PSA, PAP, DC route of administration, and DC doses with T cell proliferation and clinical response were analyzed with two-tailed ANOVA. PSA slope and confidence intervals were calculated with a simple linear regression. The Kaplan-Meier plot for progression-free survival was assessed for significance with the Breslow-Gehan-Wilcoxon test.

Results

Patient characteristics

Twenty-one patients with recurrent and/or metastatic disease completed the phase I clinical trial (Table I). Seven of these patients had hormone refractory disease. Eleven had measurable metastases, predominantly in bone and lymph nodes, while the remainder had prostate cancer evaluable by serum PSA levels. Four and 15 patients were previously treated with chemotherapy and/or radiation therapy (XRT), respectively. While the patients had essentially normal total white counts on study entry, seven patients had persistent lymphopenia presumably stemming from prior myelosuppressive treatments or bone involvement of their cancer. In fact, all lymphopenic patients had received prior XRT ($p = 0.0284$). As another general measure of immune competence, patients were also tested for delayed-type hypersensitivity to tetanus toxoid, candida, and mumps. Seven patients did not react to any of these recall Ags and therefore would be deemed clinically anergic.

Table I. *Patient characteristics*

| Characteristic	Value
Age (median, range)	67.7 (59–79)
Karnofsky performance status (median, range)	100 (90–100)
Baseline PSA (mean, range)[a]	233.3 (1.23–1935.6)
Baseline PAP (mean, range)[a]	43.6 (1–380)
Gleason score (median, range)	7 (5–9)
Primary therapy (no. of patients)	
Prostatectomy	10/21
Radiation therapy	11/21
Courses of immunosuppressive treatment	
Chemotherapy	4
Radiation therapy	15
Hormone refractory	7/21
Baseline lymphopenia	7/21
Baseline DTH anergy[b]	7/21

[a] Nanograms per milliliter.
[b] Patients were assessed for skin reactivity to candida, mumps, and tetanus. Anergy was defined as <5 mm of induration in response to all of these Ags 2 days following skin testing.

Skin test unresponsiveness did not correlate with lymphopenia or prior XRT.

DC treatment

Patients received two monthly vaccinations with autologous enriched DC obtained from peripheral blood via density gradient centrifugation. Patients underwent leukapheresis preceding each of the vaccinations to obtain PBMC. Committed DC precursors were enriched based upon the change in their buoyant density that occurs as they mature, a process that does not require exogenous cytokines. Cells were cocultured with mPAP during this 2-day ex vivo enrichment. The enriched DC were then administered to the patients as an autologous cellular vaccine with a mean DC dose of 11.2×10^6 per vaccination (range, 0.3–40.4×10^6). All patients tolerated the vaccinations without significant toxicity. Two patients developed grade 2 transfusion reactions at the time of the DC infusions manifesting as self-limited fever and rigors. Three other patients developed grade 1 erythema at the injection sites. Finally, one patient developed a transiently swollen, painful inguinal lymph node following vaccination. Five patients developed elevated antinuclear Abs following vaccination (titers of 1/20–640),
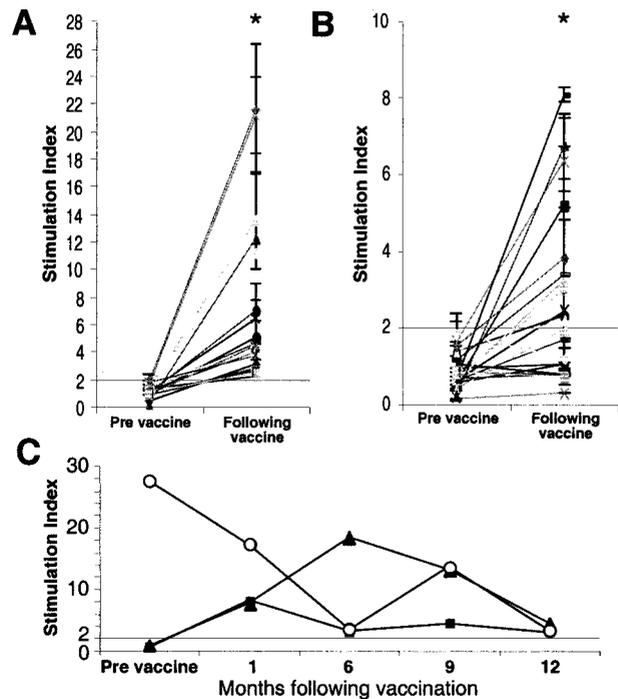
FIGURE 1. Vaccination induces T cell proliferation in response to mPAP and hPAP. PBMC from patients obtained before or following vaccination with mPAP-loaded DC were assessed for their ability to proliferate in response to mPAP and hPAP in vitro. *A*, T cell proliferation in response to 10 μg/ml mPAP was determined before or following vaccination. *, Induced proliferative responses following vaccination were statistically significant compared with preimmunization levels ($p < 0.0001$, by paired sign test) regardless of route. *B*, T cell proliferation in response to 10 μg/ml of the homologous self-Ag hPAP was also determined before or following vaccination. *, Induced proliferative responses following vaccination were again statistically significant compared with preimmunization levels ($p < 0.0072$, by paired sign test) regardless of route. *C*, Proliferative responses to mPAP (▲), hPAP (■), and influenza (○) were measured serially following completion of the vaccination. Results are shown for one patient but are representative of other patients with proliferative responses to mPAP and hPAP. T cell proliferation was assessed by [³H]thymidine incorporation over 18 h following 6 days of culture. Results are expressed as stimulation indexes (counts with Ag/baseline counts without Ag) ± SEM.

and another patient developed an elevated rheumatoid factor following vaccination. None of the patients developed clinically evident autoimmune disease.

Induction of PAP-specific immunity

To assess the induction of T cell immunity with the vaccination approach, PBMC obtained before and following DC vaccination were assessed for T cell proliferation in response to mPAP (Fig. 1A). Following vaccination, all subjects developed T cell proliferative responses to this Ag that was delivered by the vaccine. More importantly, 11 of the 21 patients developed T cell proliferation in response to the homologous self-Ag hPAP (Fig. 1B). Curiously, there was no correlation among baseline serum PSA, serum PAP, absolute lymphocyte count, or DC dose to hPAP-specific T cell proliferation following vaccination (data not shown). Also, there was no difference in T cell proliferation in response to hPAP between patients who were vaccinated by the different routes (data not shown). T cell proliferation could be measured for months following vaccination, consistent with the induction of T cell memory (Fig. 1C).

Patients' PBMC were also assessed for TNF-α, IFN-γ, IL-4, and IL-10 production in response to mPAP and hPAP before and following vaccination. Seven of 16 evaluated patients produced IFN-γ and one of 16 patients produced TNF-α in response to mPAP (Fig. 2A). Six of 16 evaluated patients produced only IFN-γ, one of 16 patients produced TNF-α, and four of 16 patients produced both cytokines in response to hPAP (Fig. 2B). No patients produced IL-4 or IL-10 (data not shown) in response to either Ag, consistent with the induction of Th1 immunity. To assess the frequency of PAP-reactive T cells induced with the vaccination, IFN-γ and IL-4 secretion was also measured with cytokine ELISPOT assays in a subset of the patients. In the three patients who had IFN-γ responses to both mPAP and hPAP, the frequency of IFN-γ-producing cells was consistently less in response to hPAP compared with mPAP (Fig. 3; mean, 51%; range, 40–64%). Consistent with the cytokine ELISA results, no IL-4 production was detected with the ELISPOT assay (data not shown).

Patients' sera were also assessed for PAP-specific Abs following vaccination (Table II). Four patients developed Abs to both mPAP and hPAP, although only one patient developed Abs at high titer (>1/1000). Four other patients developed low titers of Abs to mPAP, and another two patients developed Abs to hPAP.

Clinical response

While the primary goal in this trial was to assess the feasibility, safety, and immunogenicity of the DC vaccine, patients participating in the trial were also assessed for evidence of clinical response. At study entry candidates were required to have evidence of progressive prostate cancer based primarily on rising serum PSA levels. Study subjects did not receive other therapies during the vaccine study unless they had further clinical progression, at which point they were taken off the protocol. Six of the 21 patients had evidence of disease stabilization following completion of the vaccinations as determined by serum PSA monitoring and confirmed by computed tomography and bone scans. Serial PSA measurements in a representative patient (patient 21) are shown in Fig. 4A. Before vaccination, the patient had a log-linear rising serum PSA (PSA slope, 0.149 ± 0.015). Following vaccination, the patient's PSA stabilized (PSA slope, 0.035 ± 0.019) without any other therapy for well beyond a year. Fig. 4B shows serial PSA measurements in another patient (patient 12), who also had a progressive rise in PSA before vaccination (PSA slope, 0.023 ± 0.001) and then had stabilization of his serum PSA (slope, 0.005 ± 0.001) for over a year. However, this patient eventually went on to develop progressive disease, as shown by the abrupt rise in serum PSA. Interestingly, the patient had lost measurable T cell responses to mPAP and hPAP by this time.

All six patients whose disease stabilized following vaccination had developed T cell proliferation in response to hPAP (Fig. 4C). By contrast, only five of the 15 patients whose disease progressed immediately following vaccination had measurable T cell proliferative responses to hPAP. Moreover, the induction of T cell proliferative responses to hPAP correlated with progression ($p = 0.0087$). There was no correlation between clinical stabilization and the development of anti-PAP Abs. Finally, there was no correlation between clinical stabilization and baseline serum PSA, PAP, lymphocyte count, prior treatment, or route of DC administration (data not shown).

FIGURE 2. Vaccination induces a Th1 cytokine profile in response to both mPAP and hPAP. PBMC from patients obtained before or following vaccination were examined for their ability to secrete cytokines in response to mPAP and hPAP following 6 days of culture with the relevant Ags. Supernatants from these cultures were assessed for cytokine production by ELISA. A, Production of TNF-α, IFN-γ, and IL-4 in response to mPAP before and following vaccination was compared. B, Production of TNF-α, IFN-γ, and IL-4 in response to hPAP before and following vaccination was also compared.
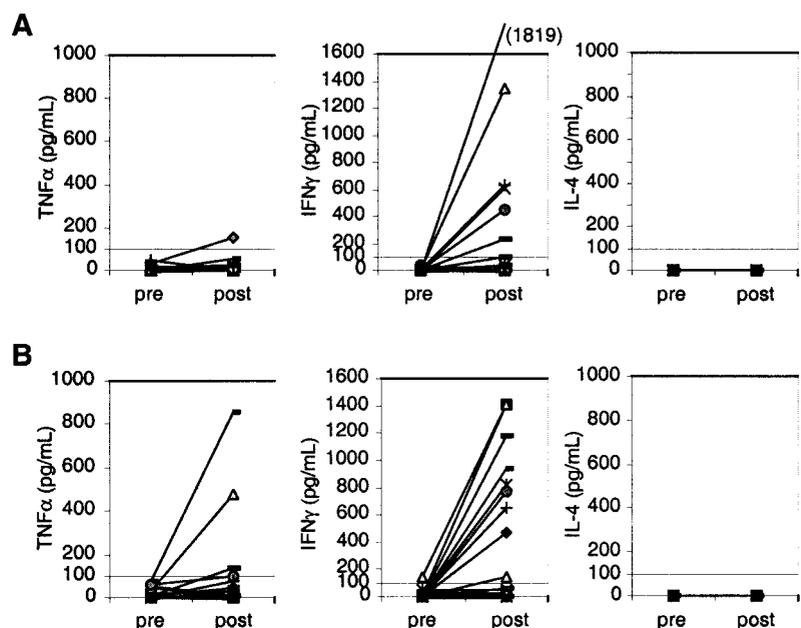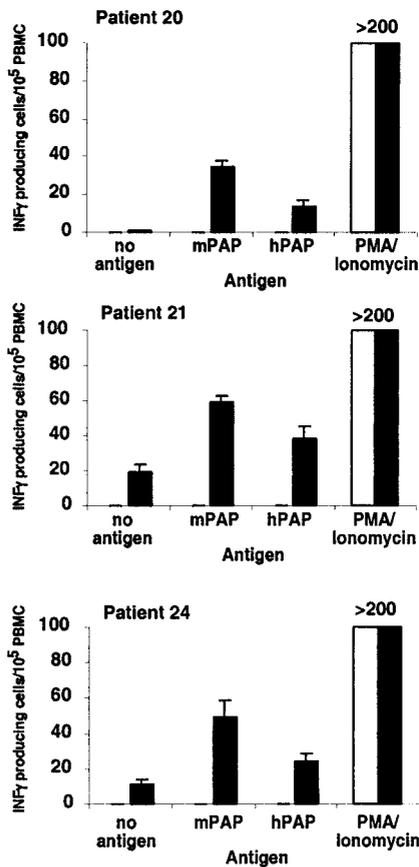
FIGURE 3. Mouse PAP vaccination induces a lower frequency of hPAP-reactive T cells than mPAP-reactive T cells. PBMC obtained from patients before (open bar) or following (filled bar) vaccination were cultured in triplicate at a concentration of 100,000 cells/well without or with 10 μg/ml of the indicated Ags in capture Ab-coated microtiter plates. After 3 days of culture, plates were assessed for cytokine production by ELISPOT. PMA and ionomycin represent nonspecific T cell stimuli. Results are shown for three patients with responses to both mPAP and hPAP.

Discussion

Cancer patients, and prostate cancer patients in particular, represent a challenging population to treat with therapeutic vaccines. As previously discussed, many tumor Ags represent tissue-specific self-Ags to which the host has been rendered immunologically tolerant either through deletion during thymic selection or induction of anergy in the periphery (25). Moreover, tumors may themselves be able to induce tumor Ag-specific T cell anergy (26). Tumor-derived PAP also circulates systemically, conferring a further tolerizing milieu specific to this Ag, because resident T cells could be constantly exposed to the Ag (27, 28). In fact, our patients were required to have measurable serum levels of PAP, which served as an indicator that any residual prostate tissue or cancer was producing this target Ag. Malignant cells may also secrete immunosuppressive factors that inhibit immune recognition, including factors that depress DC maturation and function (29–31). Prostate cancer patients tend to be older patients, and aging has been shown to be associated with narrowing of the naive T cell repertoire (32). As our study demonstrates, these patients also can have residual immunosuppression potentially stemming from their disease or prior myelosuppressive treatments. The latter would be extremely common, since XRT is a first line treatment for prostate cancer. In fact, many vaccine trials exclude patients who are lymphopenic or anergic by skin delayed-type hypersensitivity testing to microbial recall Ags.

Despite the advanced disease and immunocompromised state of the patients in our trial, all patients developed xenoantigen-specific CD4 T cell immunity following immunization. DC used in this trial were enriched directly from the blood in contrast to other trials that use monocyte-derived precursors. Patients received DC via i.v., i.d., or i.l. routes of administration. As we have previously reported (33), all three routes of DC administration generated T cell proliferation to mPAP, although there was a difference in the rate of effector cytokine induction. However, no statistically significant difference in the induction of T cell proliferation to hPAP or clinical responses were seen between the different routes. This

Table II. *Immunologic and clinical response following treatment*

Patient	Route of DC Injection	Average DC Dose[a]	mPAP T Cell SI[b]	hPAP T Cell SI[b]	mPAP Cytokine ELISA[c]	hPAP Cytokine ELISA[c]	Anti-mPAP Ab Titer	Anti-hPAP Ab Titer	Clinical Response[d]
1	i.v.	3.2	3.1	1.0	ND	ND	0	0	PD
2	i.v.	6.3	13.6	5.2	ND	ND	1/3560	1/1280	SD, 11 mo
3	i.v.	14.9	4.0	1.9	ND	ND	1/160	1/80	PD
4	i.v.	3.4	21.1	6.3	TNF-α	TNF-α, IFN-γ	1/640	0	SD, 6 mo
5	i.v.	4.8	6.4	2.4	0	IFN-γ	0	0	PD
6	i.v.	17.7	5.1	2.3	0	TNF-α, IFN-γ	0	0	SD, 18 mo+
7	i.v.	7.6	4.1	1.0	0	0	1/80	1/80	PD
8	i.v.	14.9	2.2	0.8	0	0	1/80	1/80	PD
9	i.v.	18.6	4.6	1.7	0	0	0	0	PD
10	i.d.	24.4	11.2	1.2	IFN-γ	0	0	0	PD
11	i.d.	2.3	4.0	3.0	0	TNF-α	0	0	SD, 12 mo
12	i.d.	14.6	7.0	0.8	0	0	1/40	0	PD
13	i.d.	10.6	2.8	3.4	IFN-γ	IFN-γ	0	0	PD
14	i.d.	5.1	2.5	0.7	0	0	0	1/40	PD
15	i.d.	22.7	12.2	6.7	IFN-γ	TNF-α, IFN-γ	0	0	SD, 10 mo+
16	i.l.	9.5	4.1	2.2	IFN-γ	IFN-γ	1/40	0	PD
17	i.l.	14.4	4.4	0.3	0	0	0	0	PD
18	i.l.	26.9	3.7	0.8	IFN-γ	0	0	0	PD
19	i.l.	2.6	2.6	3.2	IFN-γ	0	0	1/40	PD
20	i.l.	9.7	2.6	8.1	0	IFN-γ	0	0	SD, 10 mo
21	i.l.	10.3	21.6	3.8	IFN-γ	TNF-α, IFN-γ	1/40	0	SD, 12 mo+

[a] Average of two DC doses × 10^6.

[b] SI, Stimulation index following vaccination.

[c] Cytokine production in response to Ag following vaccination. Cytokines assessed included TNF-α, IFN-γ, and IL-4. Listed cytokines represent a statistically significant increase of cytokine production over background levels (>100 pg/mL). ND, Not done.

[d] PD, progressive disease; SD, stable disease.
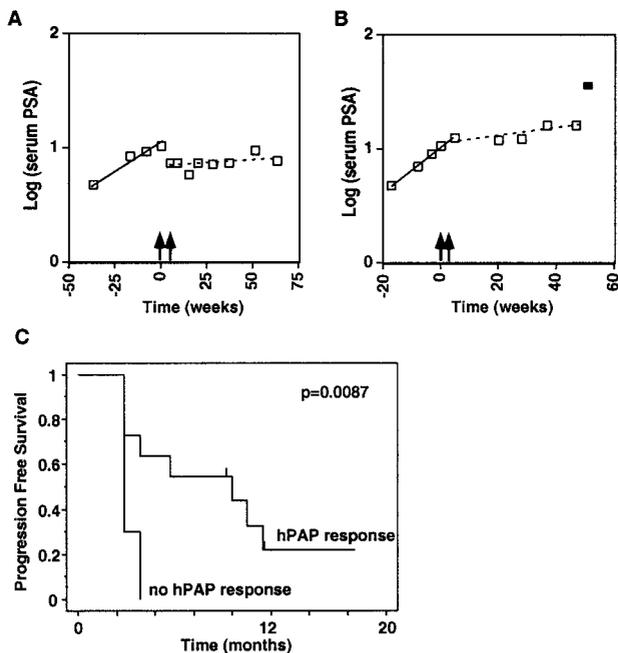
FIGURE 4. Clinical stabilization in a subset of patients. *A*, Serial PSA levels are shown for patient 21 whose disease stabilized following vaccination. Linear regression before vaccination (solid line; slope = 0.149) and after vaccination (dashed line; slope = 0.035) are shown in the context of DC vaccinations (arrows). *B*, Serial serum PSA levels of patient 12 also demonstrate rising levels before vaccination (solid line; slope = 0.023) compared with stable PSA levels following vaccination (dashed line; slope = 0.005). This patient's disease then progressed (■), at which time T cell proliferation to hPAP was no longer detectable (data not shown). *C*, The development of an hPAP-specific T cell response correlated with an increase in progression-free survival. The Kaplan-Meier plot was assessed for significance with the Breslow-Gehan-Wilcoxon test.

lack of correlation may stem from the limited number of patients developing responses to hPAP, and additional study patients would be required to clarify this. Nevertheless, hPAP responses were also associated with IFN-γ and TNF-α, but not IL-4, production and are consistent with a Th1 phenotype. More importantly, 11 of the patients developed T cell proliferation in response to the homologous self-Ag, hPAP. Following vaccination, the frequency of T cells reactive to mPAP as measured by ELISPOT was similar in magnitude to the frequency of memory T cells against influenza in this population of patients (data not shown). In contrast, the frequency of hPAP-reactive T cells was about half that for mPAP. This would be expected given the negative selection of self-reactive T cells during thymic development. The induction of hPAP-specific T cells potentially represents the induction of T cells with primary specificity for epitopes in the xenoantigen, but that possess sufficient TCR plasticity to recognize the self-Ag (34). Xenogeneic homologs may also give rise to altered peptide ligands that possess agonistic properties to reactive T cells remaining in the repertoire (35, 36). Alternatively, T cells recognizing cryptic self-determinants may be expanded with such a vaccination (37, 38).

Following DC immunization, six of the 21 patients experienced stabilization of disease as assessed by serum PSA and confirmed with radiographic imaging. This stable disease was maintained without any other therapeutic intervention. These results are intriguing given the patient population studied, the progressive nature of metastatic prostate cancer, and the minimal toxicity associated with the vaccination. A rise in serum PSA associated with

progressive prostate cancer has an exponential growth rate (log-linear) (39), which was seen in all patients at study entry. Patients whose disease stabilized had statistically significant reductions in the rate of PSA rise, consistent with a change in the growth rate of the cancer (40). In fact, all patients who developed clinical stabilization following vaccination had serum PSA doubling times of 1–4 mo before vaccination. Moreover, clinical stabilization correlated quite strikingly with the development of T cell responses to the relevant self-Ag. Thus, all six of the clinical responders developed T cell immunity to hPAP compared with only five of 15 nonresponders ($p = 0.0087$). While the induction of predominantly low-titer PAP-specific Abs was detected, no correlation was seen between the presence of anti-PAP Abs and the induction of T cell immunity or clinical stabilization. Therefore, Abs do not appear to be playing a role in the clinical effect.

Patients participating in this trial were not restricted to particular HLA alleles. As a result there was considerable allelic diversity (data not shown), making detection of CD8 T cell immunity difficult. Nevertheless, induction of CD4 T cell help is required for the generation and maintenance of effective CD8 immunity (41, 42) and would serve as a surrogate for detecting patients with a relevant immune response. There was no clear correlation between clinical or immune responses and HLA alleles, although the study size is too small to exclude such a relationship (data not shown). Ultimately, responses to hPAP and clinical responses may be reliant not only on the ability of immunogenic epitopes to be presented by various HLA haplotypes, but also the presence of T cells with relevant TCR specificities. An expanded trial would be required to clarify any potential correlation between HLA alleles and the capacity to present relevant epitopes.

While our results demonstrate that patients with lymphopenia, delayed-type hypersensitivity anergy, and advanced disease can respond to vaccination, treating patients with earlier stages of disease may prove even more successful. Possibilities would include immunizing patients in an adjuvant setting following primary treatment for prostate cancer or following a course of hormonal therapy in hormone-sensitive patients (4). The latter setting offers an opportunity to induce a minimal disease activity state in patients without myelosuppression. Combining a vaccine approach with other treatments may also be promising. For example, preclinical data demonstrate that the addition of cytokines such as IL-2 can increase the efficacy of DC vaccination (43).

This study represents the first demonstration that immunization with xenogeneic Ags can be used to immunize against poorly immunogenic self-Ags in humans. Ultimately, it will be interesting to determine how broadly applicable xenoantigen vaccination will be. Xenoantigens may potentially represent an "altered self," with sufficient differences from self-Ags to render them immunogenic, but with sufficient similarities to allow reactive T cells to maintain recognition of self, as our study demonstrates. Therefore, trials with other xenogeneic Ags would be warranted.

Acknowledgments

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