Delayed Disease Progression after Allogeneic Cell Vaccination in Hormone-Resistant Prostate Cancer and Correlation with Immunologic Variables

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Abstract

Purpose: There are a significant number of patients with asymptomatic hormone-resistant prostate cancer who have increasing prostate-specific antigen (PSA) levels but little or no evaluable disease. The immunogenicity and minimal toxicity associated with cell-based vaccine therapy makes this approach attractive for these patients.

Experimental Design: We have evaluated a vaccine comprising monthly intradermal injection of three irradiated allogeneic prostate cell lines (8 × 10^6 cells each) over 1 year. The first two doses were supplemented with bacille Calmette-Guérin as vaccine adjuvant. Twenty-eight hormone-resistant prostate cancer patients were enrolled. Patients were assessed clinically and PSA levels were measured monthly. Radiologic scans (X-ray, computed tomography, and bone scan) were taken at baseline and at intervals throughout the treatment period. Comprehensive monthly immunologic monitoring was undertaken including proliferation studies, activation markers, cytokine protein expression, and gene copy number. This longitudinal data was analyzed through predictive modeling using artificial neural network feed-forward/back-propagation algorithms with multilayer perceptron architecture.

Results: Eleven of the 28 patients showed statistically significant, prolonged decreases in their PSA velocity (PSAV). None experienced any significant toxicity. Median time to disease progression was 58 weeks, compared with recent studies of other agents and historical control values of around 28 weeks. PSAV-responding patients showed a titratable T helper 1 cytokine release profile in response to restimulation with a vaccine lysate, while nonresponders showed a mixed T helper 1 and T helper 2 response. Furthermore, immunologic profile correlated with PSAV response by artificial neural network analysis. We found predictive power not only in expression of cytokines after maximal stimulation with phorbol 12-myristate 13-acetate, but also the method of analysis (qPCR measurement of IFN-γ > qPCR measurement tumor necrosis factor-α > protein expression of IFN-γ > protein expression of interleukin 2).

Conclusions: Whole cell allogeneic vaccination in hormone-resistant prostate cancer is nontoxic and improves the natural history of the disease. Longitudinal changes in immunologic function in vaccinated patients may be better interpreted through predictive modeling using tools such as the artificial neural network rather than periodic "snapshot" readouts.

Prostate cancer is the second most common malignancy in the United Kingdom. Despite local therapy, a significant number of men will develop recurrent and subsequently hormone-resistant disease (hormone-resistant prostate cancer; refs. 1–3). Asymptomatic patients with little or no evaluable disease, but with increasing prostate-specific antigen (PSA) levels represent a particularly problematic group. For these individuals, conventional therapies are largely palliative and often toxic. Immunomodulation as a therapeutic measure to control disease progression is particularly attractive at this stage, in view of documented antitumor activity, low toxicity, and the potential to alter the natural history of the disease (4). As most patients will have little or no clinically or radiologically evaluable disease, in vitro monitoring of the effects of new immunotherapies has been problematic with poor reproducibility and correlation with eventual outcomes.

Allogeneic whole cell vaccination may be more effective than peptide, protein, single or polyepitope DNA vaccine approaches. The potential advantages include presentation and processing of multiple antigens and applicability across a variety of patient MHC haplotypes. The use of whole tumor cells should provide both MHC class I and class II epitopes...
leading to a diverse immune response including both polyclonal CTL and CD4 activation.

Several studies suggest that tumor antigens are conserved between individual tumors (5, 6). Murine studies have shown cross-protective immunity to allogeneic whole tumor cell vaccination (7–9). There is recent evidence that cross-priming may occur through the transfer of intact proteins or protein fragments, rather than exogenous or endogenous peptides, from donor cells to antigen-presenting cells. Studies indicated that cross-priming favored antigenic peptides located within mature proteins and revealed an unexpected bias against epitopes located within functional signal peptides (10–12).

Allogeneic whole cell vaccination is currently being evaluated in stage III and stage IV malignant melanoma patients following reports of antitumor activity and antigen-specific responses, most likely through antigen processing occurring in dendritic cells and cross-priming (13–16). Our group has previously shown evidence of T cell activation in response to allogeneic whole cell vaccination even in men with very advanced symptomatic hormone-resistant prostate cancer (17).

In this study, we have evaluated a whole cell vaccine comprising three irradiated allogeneic cell lines in men with early, asymptomatic relapse of their hormone-resistant prostate cancer. In addition to clinical, biochemical, and radiologic variables, detailed longitudinal immunologic monitoring has been undertaken over a 12-month treatment period. Patients at this stage have little or no evaluable disease and, hence, it is possible that immunologic responses may provide surrogate markers of vaccine efficacy. However, the nature of these responses is likely to be complex and therefore multiparametric.

Artificial neural networks have been used to predict specific biological outcomes for individual patients by learning patterns of responses derived from a cohort of similarly treated patients. Recently, artificial neural networks have been used in oncology to predict symptomatic lung injury after lung irradiation and outcomes for patients after radical radiotherapy (18–22). Cell surface markers, peripheral blood mononuclear cell proliferation to the vaccine and cytokine responses were measured and correlated with changes in PSA kinetics using advanced artificial neural network analysis techniques due to the nonlinear nature of the data over this time period.

Materials and Methods

Eligibility criteria. Patients with histologically confirmed prostate cancer, who completed optimal first line treatment with luteinizing hormone–releasing hormone blocking drugs, 150 mg bicalutamide or orchidectomy were included in the trial. Patients were required to have progressive disease as indicated by two consecutive increases in PSA, separated by at least 4 weeks, from a minimum level of 2 ng/mL; no evidence of bone or visceral metastases, WHO performance status of 0 to 2 and a life expectancy of at least 6 months. Biochemical trial entry variables included adequate bone marrow function, as defined by white blood cell count of ≥3,500/mm^3, hemoglobin of more than 9 g/dL, platelet count of at least 100,000/mm^3; adequate renal function with serum creatinine of <2.0 mg/dL, and adequate hepatic function defined as less than twice the upper limit of normal values for aspartate transaminase and alanine transaminase. Serum cortisol was tested at the entry to the study and to have been between 60 and 240 nmol/24 hours. As the vaccine was applicable to all haplotypes, patients were not HLA-typed. Blood was collected at weeks −4 and −2 to be used as reference samples once the patient had entered the treatment phase of the trial.

Vaccine. The vaccine used for treatment was made from three allogeneic prostate cell lines; OnyCap23, LnCaP, and P4E6. The cell lines were selected to represent tumor found at the major sites of the disease. P4E6 is derived from a primary prostate cancer biopsy, (23), LnCaP from a lymph node metastasis, and OnyCap23 is a subclone of the established prostate cell line PNT2-C2 (24) that was originally derived from a biopsy of a normal prostate gland. Elements of its gene expression profile are similar to bone metastases. A single dose contained $8 \times 10^6$ irradiated (150 Gy) cells from each of the three lines, mixed together, and administered as eight intradermal injections. The total volume of the vaccine was evenly distributed between the four lymph drainage sites: twoinguinal areas and the right and left axillae.

Schedule of administration. The total treatment period for each individual patient was 12 months. The first two injections were given with adjuvant bacille Calmette-Guérin (Oncotice, Organon, Netherlands) at a dose of $0.6 \times 10^6$ to $2.4 \times 10^6$ cfu mixed with the cell lines just prior to administration. The first three doses of vaccine were injected at two weekly intervals and once a month thereafter.

Delayed-type hypersensitivity test. To determine immune competency at the time of trial entry, all patients had a delayed-type hypersensitivity test to common skin antigens and cell lines. Seven common antigens were administered as intracutaneous tests (Multili, Pasteur Merieux, Lyons, France) and the positive reaction was determined 48 hours later as induration. To qualify for entry, at least one site had to generate an induration response ≥2 mm.

Clinical monitoring. Adverse events were graded according to WHO toxicity criteria. All patients underwent assessment of tumor status at baseline, 6 months, and 1 year or at the time of withdrawal, using computed tomography or magnetic resonance imaging. Technicium bone scan, and chest X-ray. PSA monitoring and International Prostate Symptom Score was done in all patients at the beginning of the study and at each vaccination visit. All patients were asked to fill the European Organization for Research and Treatment of Cancer quality of life (QLQ-30) questionnaire at the entry to the study, at week 20, and at week 52.

Assessment of safety. The patients were observed for 2 hours for the first two vaccinations and monitored for vital signs every 15 minutes for the first 30 minutes, and thereafter every 30 minutes until they were allowed to leave the hospital. For all subsequent vaccinations, patients remained under observation for 15 minutes only.

Criteria for removal from protocol. Patients were withdrawn from the study whenever any of the following occurred: disease progression (defined by appearance of new lesions bone scan and/or ≥25% increase in tumor volume on magnetic resonance imaging or computed tomography, appearance of prostate cancer–related symptoms or accelerated increase in PSA); patient withdrawal for any reason; development of intercurrent illness requiring treatment with corticosteroids; development of any severe (≥grade 3) toxicity or lack of compliance with the protocol.

Statistical considerations. The study was designed to be exploratory in nature but it was calculated that with 34 patients and an expected immunologic response rate of 35%, a two-sided 95% confidence interval around the response rate will extend from 19% to 51% using the large sample normal approximation.

Prostate-specific antigen velocity. PSA velocity (PSAV) was calculated for each patient pretreatment and posttreatment, by fitting a regression model to the log of the PSA values. The model used was ln(PSA)/t, where t is the time between enrollment on the trial and the date of the PSA sample. PSA doubling time was calculated for each patient as ln(2)/PSAV.

Preparation of blood samples. Patient blood samples were collected in 2× sodium heparin (BD Biosciences, Oxford, United Kingdom) tubes (–10 mL per tube) and separated by density gradient centrifugation. The blood was diluted 1:1 in PBS, and layered onto Histopaque 1077 (Sigma, Dorset, United Kingdom; ratio of blood/ Histopaque = 2:1), and centrifuged (688 × g, 25 minutes). Five milliliters of plasma were removed and stored in 5 × 1 mL aliquots stored at −80°C. The peripheral blood lymphocyte (PBL) layer was
removed and washed with HBSS (Sigma; 688 g, 10 minutes). The PBLs were resuspended in 1 mL of complete medium [RPMI 1640 (Sigma) + 10% FCS (PAA Laboratories, Somerset, United Kingdom), 1% l-glutamine (Sigma)], and counted using the Coulter Particle Counter (Beckman Coulter, Buckinghamshire, United Kingdom) using a size threshold limit of 4.413 μm/L or 45 μm/L. The PBLs were resuspended at 5 × 10^6/mL in complete medium, adding DMSO to a final concentration of 10%. PBL suspension (1 mL volume) were added to 1 mL cryovials and frozen overnight at −80°C before subsequent liquid nitrogen storage.

**Cell surface marker analysis.** Patient whole blood samples were collected in sodium heparin tubes. The whole blood was diluted 1:6 with PBS (Sigma). Diluted blood (100 μL volume) was added to six wells of a 96-well plate. The RBC were lysed with 1 × PermLyse (BD Pharmingen, Oxfordshire, United Kingdom) and washed twice with FACSflow (BD Biosciences). The following antibody mixes (5 μL of each antibody diluted in an equal volume of FACSflow) were added to each of the six wells, respectively—well 1, CD4 FITC/CD3 PE/CD3 PerCP; well 2, CD8 FITC/CD3 PE/CD3 PerCP; well 3, CD62L FITC/CD3 PerCP; well 5, mIgG2a FITC/mIgG2a PE (isotype controls); well 4, CD45RA FITC/CD45RO PE/CD3 PerCP; well 6, IgG1-PE, IgG1-PerCP. The plate was incubated for 30 minutes in the dark at room temperature. The cells were washed once with FACSflow, resuspended in 1% paraformaldehyde and data was acquired using the Cellquest software on FACScan (BD Biosciences) with the following antibody mixes: the multwell automated sampler within 3 days. The data acquired was analyzed using WinMDI software (Joe Trotter, Scripps).

**Proliferation assay.** Patient whole blood samples were collected in BD Vacutainer sodium heparin tubes. The whole blood was diluted 1:5 with proliferation medium [RPMI 1640 (Sigma), supplemented with 50 μg/mL 2-mercaptoethanol (Sigma), and 1% (2 mMol/L) l-glutamine (Sigma)]. Diluted blood (450 μL volumes) were added to six wells of a 48-well plate. The following were added to each of the six wells, respectively; 50 μL of proliferation medium, 50 μL of concanavalin A (25 μg/mL, Sigma), 50 μL of phytohemagglutinin (20 μg/mL, Sigma), 50 μL of the tumor cell line P4E6 lysate at 2 × 10^6 cell equivalents/mL, 50 μL of the tumor cell line OnyCap23 lysate at 2 × 10^6 cell equivalents/mL, 50 μL of the tumor cell line LnCaP lysate at 2 × 10^6 cell equivalents/mL. Lysate was prepared by three successive rounds of freeze-thaw cycles in liquid nitrogen. The proliferation assay was incubated at 37°C, 5% CO_2. After 5 days, 50 μL of bromodeoxyuridine (BrdUrd, 30 μg/mL) (Sigma) was added to each well for 18 hours or overnight. On day 6, 2 × 100 μL volumes from each well were transferred to two individual wells (duplicate) in a 96-well plate [except for the nil control well, from which 3 × 100 μL volumes were transferred to three individual (triplicate) wells in a 96-well plate]. The RBC were lysed with 1 × PermLyse and permeabilized by washing twice with Cytofix and then incubating with 1 × PermWash, with constant shaking, for 10 minutes. The following antibody combinations (diluted in 1 × PermWash) were added to each of the duplicate wells, respectively—(well 1) BrdUrd-FITC, CD4-PE, CD3-PerCP/Cy5.5; (well 2) BrdUrd-FITC, CD8-PE, CD3-PerCP/Cy5.5; (well 3) IgG2a-FITC, IgG2a-PE; (well 4) IgG1-FITC, IgG1-PE, IgG1-PerCP. The plate was incubated for 30 minutes in the dark at room temperature. The cells were washed once with FACSflow, resuspended in 1% paraformaldehyde and data was acquired using the Cellquest software on FACSscan (BD Biosciences) with the following antibody mixes: the multwell automated sampler within 3 days. The data acquired was analyzed using WinMDI software.

**Non specific cytokine release.** PBL from patients were rapidly thawed from prevaccination (week 0) and postvaccination (weeks 12, 36, and 52, where available) time points. After thawing in 9 mL of complete medium (RPMI + 10% FCS + 2 mMol/L l-glutamine), cells were adjusted to make a concentration of 2 × 10^6 cells/mL. Each sample (250 μL) was then aliquoted into five wells, and left for 24 hours in a 37°C, 5% CO_2 incubator. Stimulant (250 μL) was then added to each well. Stimulants used were medium, phytohemagglutinin (final well concentration of 2 μg/mL) and a mixture of lysates made from the three vaccine cell lines at final protein concentrations of 50, 5, and 0.5 μg/mL of each lysate. After 3 days of incubation in a 37°C, 5% CO_2 incubator, wells were harvested and spun at 9,000 × g for 5 minutes. Supernatants were removed and frozen at −80°C and the cell pellets were washed in PBS, then lysed using lysis buffer supplied in the Absolutely RNA RT-PCR Miniprep kit (Stratagene, United Kingdom) prior to being stored at −80°C.

**Lysate-specific cytokine release.** PBL from patients were rapidly thawed from prevaccination (week 0) and postvaccination (weeks 12, 36, and 52, where available) time points. After thawing in 9 mL of complete medium (RPMI + 10% FCS + 2 mMol/L l-glutamine), cells were adjusted to make a concentration of 2 × 10^6 cells/mL. Each sample (250 μL) was then aliquoted into five wells, and left for 24 hours in a 37°C, 5% CO_2 incubator. Stimulant (250 μL) was then added to each well. Stimulants used were medium, phytohemagglutinin (final well concentration of 2 μg/mL) and a mixture of lysates made from the three vaccine cell lines at final protein concentrations of 50, 5, and 0.5 μg/mL of each lysate. After 3 days of incubation in a 37°C, 5% CO_2 incubator, wells were harvested and spun at 9,000× g for 5 minutes. Supernatants were removed and frozen at −80°C and the cell pellets were washed in PBS, then lysed using lysis buffer supplied in the Absolutely RNA RT-PCR Miniprep kit prior to being stored at −80°C.

**Cytometric bead array.** Levels of cytokine protein released into the culture medium were measured using the TH1/TH2 Cytometric Bead Array (CBA) kit (BD Biosciences). Supernatants were thawed rapidly and spun down at 2,000 × g for 3 minutes to remove any cellular debris. Supernatants were used undiluted and diluted 1:50 for the phytohemagglutinin-stimulated samples. A mix of the anti-cytokine beads were added to the neat and dilute supernatant samples and incubated with the included phycoerythrin-detection reagent. Beads and detection reagent were also mixed with a serially diluted standard of all six cytokines. Samples and standards were incubated in the dark at room temperature for 3 hours. The samples were analyzed on a FACScan (BD Biosciences) with the multiwell automated sampler attachment (BD Biosciences) and data was analyzed using the CBA software (BD Biosciences).

**Expression of cytokines by qPCR.** The method used was essentially as described elsewhere (25). Briefly, RNA was extracted from cell pellets using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene) according to the manufacturer’s guidelines. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia, Buckinghamshire, United Kingdom), priming with oligo dT (Invitrogen, Renfrewshire, Scotland). Total cDNA levels were quantified using a modification of the PicoGreen dsDNA quantification kit (Molecular Probes, Cambridge, United Kingdom). cDNA was diluted within triplicate wells of a 96-well optical reaction plate (Applied Biosystems, Warrington, United Kingdom) alongside serially diluted standard λ DNA of known concentration. After the addition of PicoGreen quantitation reagent, the absorbance was measured on an ABI Prism 7700 (Applied Biosystems) under PicoGreen settings. Values for cDNA were read off the linear curve created by the λ DNA standard. Specific controls were constructed for IFN-γ, interleukin (IL)-2, IL-5, IL-10, and tumor necrosis factor-α (TNF-α) by cloning products of real-time PCR reactions into the plBAD-TOPO vector (Invitrogen). Purified plasmid clones were quantified using the PicoGreen quantification method and the copy number subsequently calculated. Real-time PCR runs were done in 96-well optical reaction plates in triplicate, each containing 1 x PCR master mix (Applied Biosystems), 0.3 pmol/μL of forward primer, reverse primer and carboxy fluorescein aminoethyl amide–labeled probe and 2 μL template DNA (either cDNA or plasmid DNA dilutions ranging from 1 in 10^3 to 1 in 5 × 10^6) in a final volume of 25 μL using an ABI 7700 Prism. Default 7700 cycle conditions were as follows: 2 minutes at 50°C then 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A standard curve was drawn by plotting the natural log of the threshold
cycle against the number of molecules. The equation drawn from the graph was used to calculate the precise number of specific cytokine cDNA molecules present per microgram of total cDNA, tested in the same reaction plate as the standard.

Artificial neural network analysis. All immunologic variables were recorded in a bespoke MySQL database before conversion into an Excel spreadsheet using days on the trial as an index. Prior to the development of back-propagation predictive artificial neural network models, the optimal input variable data set was identified by a stepwise additive approach applied to a simple probabilistic neural network variable selection procedure within the program Statistica (Statsoft, Ltd., Tulsa, OK; www.statsoft.com). This procedure determined a predictive error at each additive step and allowed rapid identification of the optimal input data set prior to training more advanced multilayer perceptron artificial neural network–based models. These have been described by McCulloch and Pitts (26), and achieved widespread application after a suitable learning process was described by Rumelhart et al. (27).

The optimal input set was then used to develop artificial neural network models for the classification of PSAV responders and nonresponders within the population. We used a three-layer multilayer perceptron artificial neural network with a back-propagation algorithm and a sigmoid activation function. Prior to training, the data were scaled linearly between 0 and 1 using minimums and maximums. The network consisted of an input layer representing immunologic variables, a hidden layer for feature detection and an output layer encoded with Boolean representation representing nonresponders (0) and PSAV responders (1). Prior to training each artificial neural network model, the database was subdivided into training data (60%), validation data (20%), and test data (20%) by random extraction. The neural network was trained by weight updates using a feed-forward/back-propagation algorithm (28). Training was stopped for each network when the error reached convergence. In order to develop a model with optimum performance, 4,500 models were trained using different combinations of inputs and numbers of hidden nodes (1-40). The performance of each submodel was determined for validation data and this performance variable was used as the selection criteria for the optimum model. The optimum model when identified (termed model a), and was further evaluated on blind test data and specificity, sensitivity determined by the plotting of receiver operator curves (29) outflow obstruction due to tumor around the bladder neck. The remaining adverse events were of grades 1 and 2 WHO toxicity criteria and the majority were due to injection site reactions (54%), injection site bruising (15%), inflammation (15%), and erythema (12%). Eleven patients (42%) reported gastrointestinal complaints, mostly nausea (15%), diarrhea (12%), and vomiting (12%). Eleven patients reported arthralgia (19%) and back pain (15%), and 15% of patients developed a maculopapular itchy rash that self-resolved.

Out of 26 evaluable patients, 19 patients (68%) completed the entire 12-month study treatment period. Seven patients developed disease progression, three of them confirmed by radiologic investigations and four had clinical symptoms and/or PSA progression. Eleven patients (42%) had a statistically significant reduction in PSAV from pretreatment to posttreatment over 1-year period, hereafter termed “PSAV responders” and the remainder “nonresponders.” For the whole cohort, there was a decrease in mean PSAV from pretreatment to posttreatment of 0.000712 (95% confidence interval, −0.000052 to −0.001372), which was statistically significant (P = 0.0357). Example plots of PSAV for individual patients over time are shown in Fig. 1. The median time to disease progression, as defined by clinical and/or

### Results

**Clinical variables.** A total of 26 patients were recruited over a 1-year period (from February 2002 to February 2003). Twenty-six patients received at least one dose of study medication and were included in the safety and intention-to-treat analysis. The remaining two patients were ineligible as one was receiving an alternative cancer vaccine treatment and the other began steroid treatment, thus affecting the PSA value. Mean age of the patients was 66 years (range 54-78); 88% of patients were Caucasian and 12% Afro-Caribbean. The WHO performance status was 0 in 23 (89%) of the patients and 1 in the remaining 3 (12%; Table 1).

The Gleason score ranged from 6 to 9 and is shown in Table 1. Eleven of the 26 patients had combined Gleason scores of ≥7. The mean PSA at the entry to the study entry was 21.38 (range 3.2-103). All patients received conventional treatment for prostate cancer prior to entry to the study with one patient treated by orchidectomy and the remaining patients receiving luteinizing hormone–releasing hormone analogues alone, antiandrogen alone or the combination of both (Table 1).

| Table 1. Demographics of the entire cohort evaluated in the study |
|------------------|-------------------|
| **Patient demographic data (N = 26)** |
| **Age** |
| mean | 66 years |
| SD | 6.3 years |
| range | 54-78 years |
| **Race** |
| Caucasian | 23 (88.5%) |
| Black | 3 (11.5%) |
| **Histology** |
| (Gleason grade) |
| 6 | 3 (11.5%) |
| 7 | 5 (19.2%) |
| 8 | 8 (30.1%) |
| 9 | 4 (15.3%) |
| missing | 6 (23%) |
| **Initial treatment** |
| radical radiotherapy | 12 (46.1%) |
| surgery | 2 (7.6%) |
| luteinizing hormone – releasing hormone analogues | 11 (42.3%) |
| orchidectomy | 1 (3.5%) |
| **WHO performance status** |
| 0 | 23 (88.5%) |
| 1 | 3 (11.5%) |

All 26 patients (100%) reported at least one treatment-emergent adverse event. Three patients reported serious adverse events, none of which were thought to be related to the study treatment. One patient developed a cerebral-vascular accident due to cerebral ischemia, and one patient reported grade 3 nausea and vomiting that was caused by a hiatus hernia. One patient developed renal failure that was caused by urinary outflow obstruction due to tumor around the bladder neck. The remaining adverse events were of grades 1 and 2 WHO toxicity criteria and the majority were due to injection site reactions (54%), injection site bruising (15%), inflammation (15%), and erythema (12%). Eleven patients (42%) reported gastrointestinal complaints, mostly nausea (15%), diarrhea (12%), and vomiting (12%). Eleven patients reported arthralgia (19%) and back pain (15%), and 15% of patients developed a maculopapular itchy rash that self-resolved.
radiologic criteria, was 58 weeks as shown in the Kaplan-Meier plot in Fig. 3. Kaplan-Meier analysis of the 11 PSAV responders against the 15 nonresponders showed a strong trend between reduced PSAV and extended time to disease progression (data not shown). There was no correlation between Gleason score and either PSAV response or time to disease progression.

Cell surface marker analysis. Pre- and posttreatment peripheral blood mononuclear cell samples were routinely screened for a number of cell surface markers by flow cytometry. These markers were divided into three groups: costimulation, activation, and memory. In order to examine the range of pretreatment values, samples taken in the 4-week trial initiation period were used as controls. An average was calculated from all 26 evaluable patients and lines representing average \( \pm \) SD plotted. In no case was a significant deviation outside this broad range observed (data not shown). These studies showed that all patients had a complete, and competent, lymphocyte complement.

Proliferation analysis. Detection of T cells capable of proliferating to the vaccine was carried out using flow cytometry to measure the incorporation of BrdUrd. This assay was carried out in whole blood to allow autologous dendritic cells to cross-prime appropriately. Detection of antigen-specific responses was not possible in this trial given the complex nature of the antigen. Furthermore, simply measuring proliferation to the vaccine cell lines would have been uninformative given the allogeneic nature of the immunization. To address this, a lysate of each cell line was produced by multiple rounds of freeze-thawing. Consequently, this removed the ability of the vaccine to initiate a mixed lymphocyte reaction but still retaining the complex mixture of tumor antigens needed to trigger T cell proliferation.

The disadvantage of this assay is the inherent “noise” due to variable effects of RBC lysis. Each sample was split into two and half-stained with anti-BrdUrd, CD4, and CD3, whereas the remainder was examined with anti-BrdUrd, CD8, and CD3. Comparison of BrdUrd versus CD3 from both samples showed the reproducibility of the technique. T cell proliferation is represented as a stimulation index calculated from the percentage of stimulated cells divided by percentage of unstimulated cells. It is important to note that these stimulation index values are not directly comparable to classical thymidine assays because the BrdUrd assay is significantly less sensitive in our hands. Each vertical bar represents proliferation at a particular time point. Figure 4 represents an example proliferation profile for a particular patient. Proliferation of both CD4 and CD8 cells is seen predominantly to LnCaP after ~200 days on trial. Less pronounced, but still detectable proliferation was seen in both P4E6 and OnyCaP23. Positive controls of phytohemagglutinin showed that the assay was valid at all time points shown. Although the assay clearly shows the presence of vaccine-responsive T cells, no correlation could be made between responders and nonresponders.

Cytokine profile. Measurement of cytokines was attempted as a surrogate for demonstrating that the vaccine had elicited an immune response. This was carried out in two ways: nonspecific maximal stimulation and vaccine-specific response. In the former case, peripheral blood mononuclear cells were stimulated with a mixture of PMA/ionomycin to determine the optimal cytokine release of these cells. A second stimulation using LPS

The quality of life assessment based on a European Organization for Research and Treatment of Cancer questionnaire showed a high quality of life with a median score of 83.3 and no deterioration over the year (data not shown). The median International Prostate Symptom Score showed little change over the first 36 weeks of the study. The median score at baseline was 7.0 and it ranged from 6 to 8 over the first 36 weeks, at week 52, it increased to 11.
was also carried out, because this has been shown to yield the optimal levels of IL-10 release. Consequently, these stimuli effectively provide a measure of the maximal possible cytokine release from vaccinated patients. Figure 5 shows the levels of IL-10 and IL-2 throughout the course of the trial. No obvious trends were discernable and there did not seem to be a distinction between responder and nonresponder groups. All six cytokines tested (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) were detectable to some degree in all samples, suggesting that responses were not obviously polarized towards either TH1 or TH2.

The design of the assay was such that it was possible to make cDNA from the same PBL samples used to produce the cytokine protein measured previously. Using a novel qPCR methodology we quantified the number of cytokine transcripts per μg of cDNA in each sample (25). This method allows direct comparison between samples and permits the longitudinal examination of responses over time. Again, we found a broad spread of responses and no obvious polarization to either TH1 or TH2 phenotype (data not shown).

An alternative protocol for examining cytokine responses is to use a stimulation based on a lysate of all three combined vaccine lines. PBL samples were thawed at four time points (0, 12, 32, and 52 weeks) and stimulated with either medium alone or a titration of 50 to 0.5 μg of cell lysate protein. Positive controls were included using phytohemagglutinin to ensure that the system was functional (data not shown). As may be seen in Fig. 6, no IFN-γ response was demonstrable at time 0, but a clear dose-response was detected at 12 and 36 weeks. Interestingly, cytokine release had diminished by week 52, possibly consistent with progressive immunosuppression from the disease process.

**Assay validation.** For proliferation assays, we used the same method as previously published (17) and included positive and negative controls as detailed previously. Cell surface markers are self-validating because we show that there is a remarkable consistency of results over time for each patient. CBA analysis is designed such that there are at least 300 of each type of cytokine bead analyzed per sample, thus reducing error. Furthermore, samples were frozen and then analyzed in batches to remove interexperimental variability.

However, the lack of a control arm does make validation of this and the qPCR assay difficult. Therefore, to address this, we conducted two further studies in control individuals to examine background levels and variability of cytokine responses in a population. In the first study, we examined four groups of 10 individuals for both protein and qPCR levels of cytokines. The groups were normal controls, hormone-sensitive prostate cancer, hormone-resistant prostate cancer, and metastatic prostate cancer. There did seem to be an overall general increase in levels as disease severity increased, although this was not significantly different, thus validating our assertion that simple parametric statistics are of limited use in these clinical settings. The second study examined the effect of stimulating cells from eight normal individuals with vaccine lysate, as shown in Fig. 6. In this case, no vaccine-specific cytokine release was detected, thus validating our data.

**Artificial neural network analysis.** The complex nature of these multiparametric immunologic assays requires careful data analysis. It is our contention that conventional statistical approaches are not applicable because it is likely that multiple variables contribute to a clinical outcome. To address this issue, we employed artificial neural network analysis using a data set...
comprising some 20,000 datapoints and 46 categories. The initial screening identified a subset of 14 variables, which originally resulted in a root mean square error of 0.49 for training data. After training 4,500 models with different permutations of the reduced input data set and hidden node numbers, the best model was able to detect a PSAV responder and nonresponder phenotype with 84%, 73%, and 84% accuracy, respectively, for training, validation (blind) and test (blind) data sets, respectively, used in analysis (Table 2). This new model produced an error value of 0.45 for training data. Receiver operator analysis indicated that this model had a high sensitivity and a high specificity with areas under the curve of 0.94, 0.81, and 0.87 for training, test and validation data, respectively. This model used all of the input data from the optimized set (14 inputs), with the exception of protein levels of TNF-α induced by LPS, had 35 nodes in a single hidden layer.

![Fig. 5. Quantitative protein expression of (A) IL-10 after LPS stimulation and (B) IL-2 after PMA/ionomycin stimulation. Results were obtained using CBA and measured by flow cytometry. ●, clinically responding patients; ○, nonresponders. Days on trial indicated on the x-axis.](image)

![Fig. 4. Example proliferation assay for a particular patient over the course of the trial. Results are expressed as stimulation indices relative to an unstimulated control. Black columns, proliferating CD4 cells; open columns, CD8 cells; horizontal dotted line, stimulation index of 1.0, consequently, values above this indicate proliferation. Days on trial indicated on the x-axis. A, proliferation to a lysate of LnCap. B, proliferation to a lysate of OnyCap23. C, proliferation to a lysate of P4E6. D, control proliferation to phytohemagglutinin mitogen.](image)
and had a single output. Furthermore, sensitivity analysis was able to define which immunologic variables had the most predictive power and should therefore be considered to correlate with PSAV response. These data are summarized in Table 3. Somewhat surprisingly, IFN-γ and TNF-α, as detected by qPCR, were the most influential variables, closely followed by IFN-γ and IL-2 as measured by CBA. Therefore, these data show that there is indeed a difference in $T_{H1}/T_{H2}$ ratios after vaccination, although this is not readily apparent simply by examining cytokine release in isolation.

**Discussion**

Hormone-resistant prostate cancer is a significant health problem worldwide. Although patients with symptomatic hormone-resistant prostate cancer may benefit from palliative chemotherapy, there is currently little justification in cytotoxic therapy for patients with minimal symptoms, biochemical PSA relapse or small disease burdens, who are otherwise enjoying an excellent quality of life. For these patients, immunotherapy may offer an alternative less toxic option, but one that may still alter the natural history of their disease. Autologous whole cell vaccines used in the adjuvant setting improve progression-free survival in stage III renal cell carcinoma (30). The use of allogeneic irradiated whole tumor cells has clear advantages in terms of vaccine production, and obviates the need to procure autologous tumor tissue and generate bespoke vaccine. Considerable clinical data exists for the use of autologous whole cell vaccines, particularly in melanomas (14, 15, 31). This approach is further supported by recent observations that cross-priming of CD8+ T cells is based on the transfer of proteasome substrates rather than peptides. Cellular proteins, rather than peptides or heat shock protein/peptide complexes, are the major source of antigens that are transferred from antigen-bearing cells and cross-presented in vivo (10–12). Our group has previously reported induction of proliferative T cell responses in hormone-resistant prostate cancer patients with heavy tumor burdens in a pilot phase I trial of allogeneic whole cell vaccination in advanced prostate cancer (17).

Our vaccination approach was safe, nontoxic, and feasible in the outpatient setting. Patients with minimal or no symptoms, no bone involvement, and no pain symptoms were recruited as they were less likely to be immunosuppressed by their cancer; a significant observation in our earlier pilot study (17). PSA kinetics over the study period showed evidence of a significant reduction in PSAV in 42% of patients. As most patients had little evaluable disease and low PSA levels prior to treatment, the purpose of clinical and radiologic restaging was to ascertain the time to disease progression, the best measure of efficacy of treatment in this context (32, 33). Our vaccine approach resulted in an increase in median time to disease progression to 58 weeks versus historical experience which suggests a median time to disease progression of 26 weeks (34). This compares favorably with recent trials involving novel nonhormonal nonsteroidal agents such as an endothelin-A receptor antagonist where median time to disease progression was 26.1 weeks compared with 19.5 weeks in the control group treated conventionally (35), and a trial of autologous dendritic cells stimulated with a prostatic acid phosphatase-granulocyte macrophage colony stimulating factor fusion protein where median time to disease progression was 29 weeks (36).

A secondary end point of the study was a longitudinal evaluation of immunologic responses to vaccination. Measuring immunologic responses in human trials poses significant technical problems. The most difficult issue is that the precise nature of the immunogen in the vaccine is not known. Indeed, it is likely that because the patient cohort was comprised of disparate tissue haplotypes, the dominant antigens may be different between patients. It is even possible that, due to the phenomenon of immunoediting, the patient’s own immune system leads to phenotypic shifts in the tumor itself, with the consequence that the dominant antigens may change over time (37). Therefore, conventional periodic “snapshot” immunologic readouts could not be employed in this study, and led to the development of several novel assays.

The first assessment made was simply to examine cell surface expression of lymphocyte markers over time. It was conceivable that gross changes in circulating lymphocyte subsets may have developed with disease progression or as a response to vaccination. Our data does not support this assertion. All patients had, and maintained, a normal lymphocyte complement throughout the course of the study. These data do, however, show the sustained immunocompetence of the patients throughout the course of the trial. Proliferative responses did develop over time and are suggestive of the initiation of a T cell response. This assay was unable to clearly distinguish patients in the PSAV responding and nonresponding groups.

Conventionally, the induction of a $T_{H1}$-type cytokine response following immunotherapy has been associated with improved outcome compared with a $T_{H2}$ phenotype (38, 39). We were able to monitor the type of cytokine response in real-time before, during, and after vaccination. Two approaches

![Figure 6](image-url)
were taken; PBL were stimulated maximally using calcium ionophore or they were incubated with a lysate of the vaccine. The former quantifies the potential of T cells to produce a particular cytokine, whereas the latter measures antigen-specific cytokine release. Maximal cytokine release produced a very complex pattern, whereas antigen-induced released seemed much more predictable.

In the initial stages of the trial (i.e., first 6 months or so) it was possible to discriminate PSAV responding and non-responding patients based on their maximal cytokine release, because PSAV responders seemed to have increasing levels of both Th1 and Th2 cytokines over time, compared with a predominantly Th2 cytokine phenotype in nonresponders. However, with progressive vaccinations beyond this time, this distinction became less clear.

Consequently, our data analysis became multiparametric and we used advanced pattern recognition rather than conventional statistics. These approaches frequently have improved performance compared with conventional statistical analyses using linear approaches for medical data (28, 40–42). Artificial neural networks provided a powerful approach for patients after radical radiotherapy (19–22). To our knowledge, artificial neural networks have not yet been used to associate complex immunologic variables with clinical responses.

Our artificial neural network analysis has shown that certain cytokine profiles can be used to predict clinical outcome with a high degree of accuracy. If these data are validated in a larger cohort of patients, this methodology may well prove useful in the future management of hormone-resistant prostate cancer patients. Furthermore, vaccinations resulted in titratable proliferative responses to tumor lysate, induction of a proinflammatory immune response and modified the natural history of the disease by extending time to disease progression to 58 weeks. Artificial neural networks provided a powerful approach to the modeling of data, and provided a good indication of the variables driving the predictions of the model.

In conclusion, allogeneic irradiated whole cell vaccination is a safe and nontoxic approach for the treatment of asymptomatic hormone-resistant prostate cancer. Vaccination resulted in a significant, prolonged reduction in PSAV in 42% of patients and an extended time to disease progression which compares favorably with other approaches evaluated at a similar stage of this disease. The safety and efficacy of this form of immunotherapy makes this a realistic treatment option for a significant subgroup of men with hormone-resistant prostate cancer.

References

Delayed Disease Progression after Allogeneic Cell Vaccination in Hormone-Resistant Prostate Cancer and Correlation with Immunologic Variables

Agnieska Michael, Graham Ball, Nadine Quatan, et al.