A Phase I Trial of SD-9427 (Progenipoietin) with a Multipeptide Vaccine for Resected Metastatic Melanoma


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ABSTRACT

Purpose: The melanoma tumor antigen epitope peptides MART-126–35 (27L) gp100209–217 (210M) and tyrosinase368–376 (370D) were emulsified with incomplete Freund’s adjuvant and administered with SD-9427 (progenipoietin), an agonist of granulocyte colony-stimulating factor and the FLT-3 receptor, to evaluate the toxins of adjuvant responses to this regimen as primary end points and time to relapse and survival as secondary end points.

Experimental Design: Fifteen patients with high-risk resected stage III and IV melanoma were enrolled. Each patient received peptides + incomplete Freund’s adjuvant with SD-9427 at doses of either 10, 20, or 40 μg/kg s.c. for 3 days before and 7 days after each vaccination. Immunizations were administered every month for 6 months and then administered once 6 months later. A leukapheresis to obtain peripheral blood mononuclear cells for immune analyses as well as skin testing with peptides and recall antigens was performed before and after vaccination. IFN-γ release assay, ELISPOT, and MHC-peptide tetramer analysis were performed using peripheral blood mononuclear cells collected before and after vaccination to evaluate peptide-specific cytotoxic T-cell responses.

Results: Local pain and granuloma formation and fatigue of grade I or II were the most common side effects. One patient developed antibody-mediated leukopenia and transient grade III neutropenia that resolved after stopping SD-9427. Six of 12 patients tested developed a positive skin test response to one or more of the peptides. Seven of 10 patients tested demonstrated an immune response to at least one peptide when evaluated by IFN-γ release assay and ELISPOT assay after vaccination, as did 11 of 12 patients analyzed by MHC-peptide tetramer assay. Four of 15 patients have relapsed with a median follow-up of 20 months, and 1 patient in this high-risk group has died of disease.

Conclusions: SD-9427 with a multipeptide vaccine was generally well tolerated, although one patient developed reversible antibody-mediated neutropenia. These data suggest that the majority of patients with resected melanoma mount an antigen-specific immune response against a multipeptide vaccine administered with SD-9427.

INTRODUCTION

The generation and detection of tumor-specific immune responses in humans have been enormously simplified by the discovery that T cells recognize tumor antigen peptide-MHC complexes and that many of these antigens are shared between tumors of different types (1–3). Clinical trials of peptide vaccines with or without adjuvants in patients with metastatic and resected cancer have been facilitated by the identification of T-cell epitopes from several classes of tumor-associated and tumor-specific antigens on melanomas as well as breast, ovarian, gastrointestinal, prostate, and lung cancers that are recognized by CD8+ lymphocytes in association with the HLA class I alleles that are frequently expressed in the population (4).

Several groups have conducted trials of peptides derived from melanoma differentiation antigens that comprise melanosome-related “neo-antigens” derived from gene products produced in normal cells. The antigens Pmel 17/gp100, a transmembrane glycoprotein of 100 kDa, tyrosinase, an intracellular enzyme in the melanin pathway, and MART-1/MelanA, another component of the melanosome, have all been shown to encode 9–10-amino acid epitope peptides recognized by CD8+ T cells and presented by the HLA-A2 antigen to CTLs reactive with human melanoma cells (5–9). The gp100209–217 peptide substituted with methionine at the 210 position (210M) sequence IMDQVPFSV and the MART-126–35 peptide substituted with leucine at the 27 position (27L) sequence ELAGILTV strongly bind to HLA A*0201 and are recognized by antigen-specific CTLs (5–8). The tyrosinase368–376 peptide, YMGTV-

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\(^3\) The abbreviations used are: HLA, human leukocyte antigen; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; DTH, delayed type hypersensitivity; IFA, incomplete Freund’s adjuvant; PBMC, peripheral blood mononuclear cell; IL, interleukin; NCI, National Cancer Institute; PE, phycoerythrin; AGC, granulocyte count.
MSQV, was shown to be posttranslationally modified by deamidation of asparagine to aspartic acid resulting in a HLA A2-restricted sequence recognized by human CTLs, YMDGT-MSQV, known as tyrosinase,\textsuperscript{368–376 (370D)} (9).

A variety of cytokine adjuvants have been tested in small trials of melanoma peptide vaccination in an attempt to augment tumor antigen presentation and overcome suppressive influences in tumor-bearing patients. Jager et al. (10) showed enhanced immune responses to peptides encoded by melanoma differentiation antigens after the use of systemic GM-CSF in three patients, all of whom had objective clinical responses. Lee et al. (11) showed that IL-12 was an effective vaccine adjuvant. Rosenberg et al. (12) found decreased levels of circulating peptide-reactive T-cell precursors after IL-2, GM-CSF, or IL-12 administration in conjunction with the gp100\textsuperscript{209–217 (210M)} peptide emulsified with IFA. However, clinical responses were observed in some of these patients, suggesting that detecting immune responses in blood may not completely reflect vaccine immunogenicity. Several trials of GM-CSF added to vaccines in animal models and cancer patients have demonstrated that its ability to function as an adjuvant is associated with augmented numbers of antigen-presenting DCs peripherally and in draining lymph nodes (10, 13–15).

SD-9427 (progenipoietin) is a chimeric protein comprising G-CSF and FLT-3 receptor agonists. Both FLT-3 ligand and G-CSF have been shown to augment the number of DC precursors in peripheral blood (16). Whereas FLT-3 ligand expands both mDC (CD11c+), and pDC (CD11c–) subsets, G-CSF administration increases only the plasmacytoid subset (16). FLT-3 ligand in particular has been demonstrated to increase mDC numbers by 48-fold in normal volunteers (16) and circulating total DCs by 20-fold in tumor-bearing patients (17). In murine experiments, SD-9427 administration was found to markedly increase DC numbers in the peripheral blood and spleen of treated mice in a dose-dependent fashion (18, 19). Murine SD-9427-generated DCs were highly efficient in presenting CTLs and T helper peptides and generated potent peptide-specific immune responses \textit{in vivo} (20).

We hypothesized that SD-9427 would augment DC numbers and function, both systemically and at vaccine injection sites, as an adjuvant, resulting in potent peptide-specific CTL responses. A Phase I trial of melanoma peptides + IFA with escalating doses of SD-9427 was conducted in which the primary end points were a determination of the toxicities and the biological and immunological effects of SD-9427, and relapse-free and overall survival were secondary end points.

\section*{PATIENTS AND METHODS}

\subsection*{Trial Eligibility}

All patients had stage III or IV melanoma by the 1988 modified American Joint Commission on Cancer staging system and were rendered free of disease surgically. They were required to have a magnetic resonance imaging or computed tomography scan of the head and computed tomography scans of the chest, abdomen, and pelvis showing no evidence of disease within 30 days of therapy to verify that they were clinically free of disease. Eligibility criteria included age of \(\geq 18\) years, creatinine of \(< 2.0\) mg/dl, bilirubin of \(< 2.0\) mg/dl, platelets of \(\geq 100,000/mm^3\), hemoglobin of \(\geq 9\) g/dl, and total WBC of \(\geq 3,000/mm^3\). Patients were required to be negative for HIV, hepatitis C antibody, and hepatitis B surface antigen, and all patients were HLA-A2 antigen positive by a microcytotoxicity assay. In view of the high frequency (>95%) of HLA-A*0201 in the United States population, allele subtyping was not performed. Tumor specimens were required to express at least one of the three vaccine antigens as detected by immunohistochemistry. All patients were required to comprehend and sign an informed consent form approved by the NCI and the University of Southern California Institutional Review Board. The trial was conducted under United States Food and Drug Administration Investigational New Drug Application BB 8840.

\subsection*{Study Design}

The primary end points were a determination of the toxicities and the biological and immunological effects of SD-9427, and relapse-free and overall survival were secondary end points. Patients were successively assigned to six to eight patient cohorts to receive peptides + IFA with SD-9427 at escalating doses of 10, 20, or 40 \(\mu g/kg\) s.c. for 3 days before and 7 days after each vaccination. Seven patients were accrued at the 10 and 40 \(\mu g/kg\) doses, but only one patient was treated at the 20 \(\mu g/kg\) dose due to a protocol amendment allowing a more rapid dose escalation. Escalation to the next cohort occurred if no autoimmune toxicity of grade II or more or other grade III/IV toxicity was observed within the first 2 months on trial. Immunizations were administered every month for 6 months and then administered once 6 months later. Toxicity was graded according to the NCI Common Toxicity Scale. DTH skin testing with peptides and recall antigens, IFN-\(\gamma\) release assay, ELISPOT, and MHC-peptide tetramer analysis were performed using PBMCs collected before and after vaccination to evaluate the peptide-specific cytotoxic T-cell response to the vaccine. Patients had a leukapheresis with an exchange of approximately 5 liters of blood volume performed within 2 weeks before beginning vaccinations and within 3 weeks after the sixth vaccination to collect PBMCs that were frozen for future analysis. Patients who could not undergo postvaccine pheresis due to poor venous access had 80 ml of heparinized peripheral blood collected for MHC-peptide tetramer assay.

\subsection*{Clinical Grade Peptides}

Peptides gp100\textsuperscript{209–217 (210M)}, MART-1\textsuperscript{26–35 (27L)}, and tyrosinase\textsuperscript{368–376 (370D)} were produced by solid-phase synthesis using 9-fluorenlymethoxy carbonyl chemistry and reverse-phase high-performance liquid chromatography purification and supplied by Ben Venue Laboratories, Inc. (Bedford, OH). The chemical identity was verified by mass spectrometry, and the finished injectable dosage form was manufactured by the Monoclonal Antibody/Recombinant Protein Production Facility, NCI (NCI/Science Applications International Corporation-Frederick, Frederick, MD). Peptide was provided by the Cancer Therapy Evaluation Program of the NCI (Bethesda, MD) under an Investigational New Drug application BB 6123 held by the NCI. The vials of peptide contained no preservative.

The tyrosinase\textsuperscript{368–376 (370D)} peptide (NSC 699048), MART-1\textsuperscript{26–35 (27L)} peptide (NSC 709401), and gp-
100_{209–217}^{210M} \text{ peptide (NSC 683472) are HLA-A2-restricted 9- or 10-amino acid epitope peptides and have the abbreviated amino acid sequences YMDGTMSEQV, ELAGIGILT, and IMDQVPFSV, respectively. The gp100 and tyrosinase peptides were supplied in vials containing 1 ml of a sterile 1 mg/ml solution for injection with 0.1 N HCl added to adjust the pH. The MART-1 peptide was supplied in vials containing 1 mg of lyophilized peptide, sodium phosphate, dibasic. United States Pharmacopea 14.2 mg, sucrose 35 mg, phosphoric acid 5% to adjust pH, and sodium hydroxide, to adjust pH.

Adjuvant

Montanide ISA-51 (also known as IFA) was manufactured by Seppic, Inc. (Franklin Lakes, NJ) and supplied in glass ampoules containing 3 ml of sterile adjuvant solution without preservative.

SD-9427

SD-9427 was manufactured and provided by Pharmacia Corp. Bioactivity was measured by proliferative responses of BaF3 cells transfected with the human G-CSF receptor. Vials containing 1.0 mg of SD-9427 as a preservative-free, lyophilized powder were reconstituted using 0.5 or 1.0 ml of sterile water for injection, USP. SD-9427 was injected s.c. in a volume of no more than 1 ml for 3 days before and 7 days after each injection and 6 months between the sixth and seventh injection.

Vaccine Preparation and Administration

The peptide vaccine was administered as outpatient therapy. One ml of gp100_{209–217}^{210M}, MART-1_{26–35}^{27L}, or tyrosinase_{368–376}^{370D} peptides with sterile saline was added in a 1:1 volume to IFA and then mixed in a Vortex mixer (Fisher, Inc., Alameda, CA) for 12 min at room temperature. Half of the resulting emulsion for each peptide was injected deeply s.c. in the lateral thigh in a volume of 1 ml using a glass syringe. s.c. as opposed to intradermal administration was chosen because of the volume of injectate (1 ml). Alternating thighs were used for a total of seven injections, which were done over 52 weeks. The intervals between injections were monthly for the first six injections and 6 months between the sixth and seventh injection.

DTH Skin Tests

Skin tests were performed using 50 µg of the gp-100_{209–217}^{210M}, MART-1_{26–35}^{27L}, or tyrosinase_{368–376}^{370D} peptides injected intradermally in a volume of 100 µl using a Tuberculin syringe and a 27-gauge needle. Candida extract, mumps, and trichophyton provided a positive control, and 0.9% saline was a negative control for assessment of DTH. At least 5 mm of induration in one of two perpendicular dimensions, read 48 h after intradermal injection, was required to score a skin test as positive.

Screening for Vitiligo and Eye Changes

All patients had a complete skin examination before therapy and at each visit for vaccination to screen for vitiligo. Slit lamp examinations and iris photography were done by an ophthalmologist before starting therapy in all patients, and hand-held ophthalmoscopic retinal and iris exams were performed at each vaccination visit to assess ocular toxicity.

Processing of Leukapheresis Products

Leukapheresis samples were processed to isolate PBMCs by density gradient centrifugation over Lymphoprep (Nycosmed, Oslo, Norway) followed by extensive washing in HBSS. Cells were frozen in 40% human AB serum (Omega Scientific, Tarzana, CA), 50% RPMI 1640 (Mediatech, Herndon, VA), and 10% DMSO (Sigma, St. Louis, MO) and stored in a liquid nitrogen freezer at −168°C until use.

Analysis of Peripheral Blood DC Number

DC numbers in PBMCs were analyzed by flow cytometry. Total DCs were defined as lineage-negative/HLA-DR+ cells. mDC and pDC subsets were defined as CD11c+/HLA-DR+ and CD123+/HLA-DR+, respectively.

Characterization of Anti-SD-9427 Antibodies

A capture ELISA was used for the detection of antibodies reactive with SD-9427. Anti-SD-9427 antibody in diluted serum samples was captured by the antigen SD-9427, which was preadsorbed to the surface of microtiter wells. The presence of anti-SD-9427 antibody was detected colorimetrically using peroxidase-conjugated goat antihuman IgG (Sigma) and the substrate tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD). The specificity of the assay was determined by evaluating the potential for nonspecific interference in over 50 individual serum samples from normal volunteers and 100 individual serum samples from cancer patients. These data were pooled to determine the negative cutoff, using the average and a multiple of its variance. The titer obtained at the negative cutoff is reported. Positive samples were further analyzed for the presence of neutralizing activities as described below.

Bioassays were used to detect the presence of neutralizing activities to SD-9427, G-CSF, or FLT-3 ligand in patient serum samples. The murine BaF3 cell line was stably transfected with genes encoding a chimeric human FLT-3 receptor (BaF3/FLT3R) or human G-CSF receptor (BaF3/G-CSFR). The ability of these cells to proliferate specifically in response to purified agonists has been described previously (18). To test for neutralizing activities, patient serum samples and reference standards (SD-9427, recombinant G-CSF, and recombinant FLT-3 ligand) were serially diluted in 96-well plates and incubated with fixed numbers of BaF3/FLT3R or BaF3/G-CSFR cells for 4 days. The number of cells present following culture was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyphenyl)-2H-tetrazolium bromide salt (Sigma) and was linear with respect to concentration of each of the reference standards. Samples were considered positive if they had a significant slope:concentration ratio and were linear and parallel to the slope of the reference standard.

Immune Response Assays

Peptides. Peptides used for in vitro studies were synthesized at the University of Southern California/Norris Cancer Center Core Peptide Facility.
Cytokine Release Assay. Assays were performed using peptide-stimulated T cells as effector cells. Peptide-stimulated T cells were produced by incubating $2 \times 10^6$ thawed PBMCs with gp100$_{209-217}\ (210M)$, tyrosinase$_{368-376\ (370D)}$, MART-1$_{26-35\ (27L)}$, MART-1$_{37-55\ (370D)}$, or Flu-M1$_{38-66\ (370D)}$ peptide in each well of a 24-well plate. Cells were plated in AIM-V media with 1% human AB serum. Three days later, recombinant human IL-2 (kindly provided by Chiron (Emeryville, CA) was added at 100 IU/ml. Fresh IL-2 was added every 2–3 days. After 10 days, the T cells were harvested. For the IFN-γ release assay, $1 \times 10^5$ peptide-stimulated T cells were harvested and incubated with $1 \times 10^5$ T2 cells (American Type Culture Collection, Manassas, VA) pulsed with 10 μg/ml of the above-mentioned peptides and the irrelevant HPV-E7$_{86-93}$ peptide as targets in a total volume of 1 ml of RPMI 1640 with 2% human serum for 18 h in a 5% CO$_2$ incubator at 37°C. Neither the effectors nor the targets were irradiated. Supernatants were collected, spun briefly at 14,000 × g to pellet cells and debris, and frozen at −80°C until assays were done. IFN-γ was detected in supernatants by an ELISA using human IFN-γ antibody pairs (PharMingen, San Diego, CA).

MHC-PEptide Tetramer Assay. The tetramers containing the gp100$_{209-217\ (210M)}$ and tyrosinase$_{368-376\ (370D)}$ peptides were produced following the approach of Altman et al. (21). Briefly, the plasmids encoding the extracellular domain on the HLA-A*0201 heavy chain fused to a biotinylation site and full-length human β-2 microglobulin (kindly provided by Dr. John Altman; Emory University) were expressed in Escherichia coli. Insoluble HLA-A*0201 and β-2 microglobulin were dissolved in 8 M urea, refolded in the presence of 100 μg/ml melanoma peptides, and then purified by gel filtration. The product was biotinylated in the presence of 15 μg of BirA (Avidity, Boulder, CO), 80 μM biotin, 10 mM ATP, 10 mM MgOAc, 20 mM bicine, and 10 mM Tris-HCl (pH 8.3). To remove free biotin, monomeric complexes were then purified by anion exchange (Mono Q), tested for biotinylation efficiency, and tetramerized by the addition of PE-labeled streptavidin (Molecular Probes, Eugene, OR) at a 4:1 ratio. Tetramers were purified by gel filtration and stored at 1–2 mg/ml at 4°C. Tetrameric assessment of CTLs was accomplished by threecolor staining using FITC-labeled anti-CD8, PerCP-labeled anti-CD14/19, and PE-labeled melanoma peptide or irrelevant control tetramer. CD8+ and CD14/19− lymphocytes were analyzed for PE labeling (tetramer binding) using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The percentage of CD8+ cells that stained with tetramer were measured before and after vaccination.

Validation and Titration of Tetramers. Each tetramer is validated by staining against a CTL line or clone specific for HLA-A2 in association with the peptide of interest. Specificity is demonstrated by the lack of staining of irrelevant CTLs. Each tetramer reagent is titrated individually and used at the lowest concentration of tetramers that still gives clearly discernable positive populations (generally, 10–50 μg/ml) to minimize background staining. The limit of detection of each tetramer is determined by titrating known antigen-specific T cells into normal PBMCs. By collecting 106 or more events per sample to increase the number of potentially tetramer-positive events to observe clustering, we established a limit of detection of 0.01% of CD8+ T cells; this is comparable to that of limiting dilution analysis.

Flow Cytometry and Data Analysis. PBMCs from patients were stained with FITC-labeled anti-CD8, PE-conjugated peptide/HLA-A2.1 tetramers, and Cy5-labeled anti-CD4, anti-CD14, and anti-CD19 antibodies at room temperature for 30 min. Cells were washed and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA). One million events were acquired. Data were analyzed using FlowJo (TreeStar, San Carlos, CA). Cells were selected for lymphocytes by forward scatter and side scatter and negatively gated from CD4−, CD14−, and CD19− cells. Selected cells were then plotted for CD8 and tetramer staining. The percentage of CD8 lymphocytes that stain with peptide/HLA-A2 tetramers was given. Samples with tetramer-staining populations were repeated and considered positive only if confirmed.

Table 1: Patient characteristics, treatment, and outcome

<table>
<thead>
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<th>Total no. of patients:</th>
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</thead>
<tbody>
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<td>Male</td>
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<tr>
<td>Female</td>
<td>6</td>
</tr>
<tr>
<td>Mean age (yrs) (range)</td>
<td>55 (33–82)</td>
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<tr>
<td>Stage of disease</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
</tr>
<tr>
<td>Prior therapy:</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
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</tr>
<tr>
<td>Hormonal</td>
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</tr>
<tr>
<td>Immunologic</td>
<td>6</td>
</tr>
<tr>
<td>Radiation</td>
<td>4</td>
</tr>
<tr>
<td>Stem cell transplant</td>
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</tr>
<tr>
<td>Other</td>
<td>0</td>
</tr>
<tr>
<td>Patients at each SD-9427 dose level (μg/kg)</td>
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</tr>
<tr>
<td>10</td>
<td>7</td>
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<tr>
<td>20</td>
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<td>40</td>
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<td>Died of disease</td>
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<td>Relapsed</td>
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<tr>
<td>Median follow-up (months)</td>
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Table 2: Toxicity

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<th>Level 2a (N = 1)</th>
<th>Level 3a (N = 7)</th>
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<tr>
<td>Allergic reaction/hypersensitivity</td>
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<td>1/–</td>
<td>1/–</td>
</tr>
<tr>
<td>Bone pain</td>
<td>1/–</td>
<td>1/–</td>
<td>1/–</td>
</tr>
<tr>
<td>Fatigue (lethargy, malaise, asthenia)</td>
<td>3/1</td>
<td>1/–</td>
<td>3/1</td>
</tr>
<tr>
<td>Fever (in the absence of neutropenia</td>
<td>1/–</td>
<td>1/–</td>
<td>1/–</td>
</tr>
<tr>
<td>Injection site reaction</td>
<td>–/3</td>
<td>2/–</td>
<td>–/3</td>
</tr>
<tr>
<td>Myalgia (muscle ache)</td>
<td>–/1</td>
<td>–/1</td>
<td>–/1</td>
</tr>
<tr>
<td>Nausea</td>
<td>1/–</td>
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<td>1/–</td>
</tr>
<tr>
<td>Ocular-other</td>
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<td>1/–</td>
<td>1/–</td>
</tr>
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<td>Rash/desquamation</td>
<td>1/–</td>
<td>1/–</td>
<td>1/–</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>1/–</td>
<td>1/–</td>
<td>1/–</td>
</tr>
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</table>

a Level 1 = tyrosinase/gp100/MART-1 + SD-9427 (10 μg), Level 2 = tyrosinase/gp100/MART-1 + SD-9427 (20 μg), Level 3 = tyrosinase/gp100/MART-1 + SD-9427 (40 μg).
**ELISPOT Assay.** Briefly, frozen pre- and postvaccine PBMCs were thawed and then cultured overnight in AIM-V medium with 5% human AB serum and 20 IU/ml IL-2. ELISPOT plates (MAHA S45–10; Millipore, Bedford, MA) were prepared by adding 100 μl of primary anti-IFN-γ antibody (MabTech, Nacka, Sweden) diluted to 3 μg/ml to the plates overnight at 4°C in a refrigerator. The next day, plates were washed four times with wash buffer (1× PBS with 0.05% Tween 20) and then incubated for at least 1 h at 37°C with blocking buffer (AIM-V medium with 10% human AB serum). The buffer was discarded, and thawed incubated PBMCs were added at 166,000, 83,000, and 41,500 cells/well in triplicate in a total volume of 100 μl. Phytohemagglutinin (10 μg/ml) was added to six wells as a positive control, and AIM-V medium was added as a negative control. Peptide was then added at 5 μg/ml to all other wells. Plates were then incubated in a 5% CO₂ incubator for 4 h at 37°C. Plates were then washed eight times with wash buffer using an automated washer (Scanwasher 300; Skatron, Lier, Norway). The plate was blotted dry, and 100 μl of secondary antibody (MabTech) at 1 μg/ml was added. The plates were then incubated overnight at 4°C, and plates were again washed eight times with wash buffer. Plates were blotted dry, and streptavidin/alkaline phosphatase (MabTech) diluted 1:1,000 in 1× PBS with 1% BSA (Sigma) was added. Plates were then incubated for 1 h at room temperature, washed eight times with wash buffer using an automated washer, and blotted dry. 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (100 μl; Kirkegaard & Perry) was added at room temperature, and plates were incubated in the dark to develop them. The colorimetric reaction was then halted by washing with running water. Plates were allowed to dry, and the number of spots was read on a Zeiss Axioplan 2 imaging system (Carl Zeiss, Thornwood, NY). Values were normalized to spots/100,000 cells.

**Immunohistochemical Staining for gp100, MART-1, and Tyrosinase.** The T311 antibody for tyrosinase (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), M21 antibody specific for MART-1/MelanA, and HMB-45 antibody for gp100 (Ventana Medical Systems, Tucson, AZ) were used for immunohistochemical staining of paraffin-embedded sections on glass slides by the Vectastain technique (Vector Laboratories, Burlingame, CA) as per the manufacturer’s instructions. Appropriate negative and positive controls were included with each assay.

**Statistics**

Differences in the pre- and postvaccination values for (gp100209–217) (210M) = (gp200–217M) and MART-126–35 (27L) = (MART-1) peptides for the cytokine release, ELISPOT, and tetramer assays were examined. A nonparametric test (Wilcoxon’s rank-sum test) was used to compare these differences between the 10 and 40 μg/kg cohorts. Because only one patient was treated at 20 μg/kg, this cohort was not included in the analyses.

**RESULTS**

**Patient Characteristics**

Fifteen patients with stage III and IV resected melanoma were treated in this trial. The demographic details of this high-risk group are shown in Table 1. The mean age of the nine men and six women was 55 years. Ten patients had resected stage III disease (three with lymph node or s.c. recurrences after adjuvant IFN-α therapy), and five had resected stage IV disease. All had cutaneous melanoma, and none had ocular melanoma. Three of the patients had received a cellular vaccine. Five patients failed to be leukapheresed posttreatment (3 because of disease recurrence before finishing the series of vaccinations, and 2 could not be leukapheresed due to inadequate venous access), leaving 10 patients with leukapheresis samples collected for evaluation both before and after vaccination. Both patients who could not have a postvaccination leukapheresis due to venous access problems had only tetramer analysis performed on a peripheral blood sample.

**Toxicity**

The overall toxicities of the gp100209–217210M/tyrosinase368–376(370D)/MART-126–35(27L) vaccine with SD-9427 for all 15 patients are shown in Table 2. The vaccine + IFA with SD-9427 was generally well tolerated, except for injection site reactions including grade I or II local pain, tenderness, or granuloma formation that occurred in five patients. Four patients developed granulomata at the injection sites, although none needed to be resected due to symptoms. One patient developed antibody-mediated grade III neutropenia necessitating discontinuation of SD-9427. That patient’s clinical course and details of the antibody response are described in greater detail below. Other toxicities observed included bone pain in two patients and fatigue in nine patients. Vitiligo or ocular toxicity was not observed in any patient. No appreciable differences were noted between overall toxicities in the three cohorts of the trial. The details of the toxicities observed are shown in Table 2. In conclusion, the toxicity of the gp100209–217210M/tyrosinase368–376(370D)/MART-126–35(27L) + IFA vaccine administered with SD-9427 was, for the most part, modest and related to the SD-9427, except for the development of autoantibody to G-CSF in one patient, which resulted in neutropenia that resolved after discontinuation of SD-9427.
Case Report

A 45-year-old female received treatment with the multi-peptide vaccine + IFA and SD-9427 in the 10 µg/kg cohort. Leukocytosis and neutrophilia occurred predictably during the first and second cycles. The leukocyte response was blunted during the third cycle, and during the fourth cycle, WBC count was 4500/mm³ on day 7 with an AGC of 2320/mm³. SD-9427 was discontinued, and the patient received peptides + IFA alone during further cycles. Despite discontinuation of SD-9427, WBC count and AGC continued to drop to a nadir of 2800/mm³ and 910/mm³, respectively, on day 7 of cycle 6. On day 49 of the sixth vaccination cycle, the WBC count was 2800/mm³ with an AGC of 1100/mm³, and a bone marrow biopsy was performed that showed mild myeloid hypoplasia with a myeloid:erythroid ratio of 1:1. No tumor was seen by histology, and no abnormal cells were observed by flow cytometry. A corticosteroid pulse was administered for 10 days after the bone marrow biopsy. The neutrophil count gradually rose, and 4 months after the nadir counts, WBC count was 4500/mm³, and AGC was 1940/mm³. Serum samples obtained during this period were tested for antibody titer and neutralizing activity against SD-9427 or its components in a G-CSF or FLT-3 receptor-specific cell-based assay. Antibody to SD-9427 was first detected during cycle 3 and peaked during cycles 5 and 6, during which time antibodies to G-CSF and FLT-3 were also detected. Serum samples from cycles 5 and 6 showed neutralizing activity.
against SD-9427 when tested in an assay with cells using G-CSF receptors, but not with cells using FLT-3 receptor. No neutralizing activity was seen in this system against native G-CSF or FLT-3. The peak antibody titers and neutralizing activity were detected during the nadir of neutrophil count. This patient’s leukocyte counts and antibody titers are depicted in Fig. 1. Her WBC count has since returned to normal without evidence of circulating antibodies, and she remains free of disease 2 years after starting therapy.

**WBC and DC Numbers in Peripheral Blood**

Because FLT-3 ligand and G-CSF individually increase the number of predominant mDCs and pDCs, respectively, SD-9427 administration would be expected to increase the number of total peripheral blood circulating WBCs, circulating DCs, and the individual subsets. Total peripheral blood DCs (DR+, lin–) as well as the mDC (CD11c+, DR+) and pDC (DR+, CD123+) subsets were analyzed by flow cytometry at each dose level during the first and fourth cycles of SD-9427 administration at days 1, 4, 7, 10, and 13 of the cycle. Because peak WBC and DC counts were observed on day 7, the increase compared with baseline was calculated for the 10 and 40 μg/kg cohorts, because the 20 μg/kg cohort had only one patient. Total WBC count increased 2.2-fold in the 10 μg/kg cohort and 5.4-fold in the 40 μg/kg cohort, both on day 7 of cycle 1, as shown in Fig. 2A. Total circulating DCs increased 2.5 times in the 10 μg/kg cohort and 5.9 times in the 40 μg/kg cohort, again on day 7 of the first cycle, as seen in Fig. 2B.

mDCs increased 1.8 times in the 10 μg/kg cohort and 6.9 times in the 40 μg/kg cohort on day 7, whereas pDCs increased 1.9 times in the 10 μg/kg cohort and 3.7 times in the 40 μg/kg cohort on day 7. These data are shown in Fig. 2, C and D, respectively. Similar increases in WBC and DC subsets were observed on day 7 of the fourth cycle (data not shown). These data show that SD-9427 treatment resulted in a dose-dependent increase in the number of total WBCs as well as the mDC and pDC subsets in peripheral blood, with the peak response occurring on day 7 in most patients.

**Immune Response**

**DTH Skin Test Results.** DTH skin testing has been shown to be useful in detecting tumor-specific CD8+ T-cell reactivity (22). Skin test reactivity to recall antigens (Candida albicans, trichophyton, or mumps) and gp100209–217 (210M)/MART-126–35 (27L)/tyrosinase368–376 (370D) peptides was assessed before the first and after the sixth vaccine cycles. Three of 15 patients were anergic to all recall antigens before vaccination. None of the 15 patients reacted to the gp100209–217 (210M)/MART-126–35 (27L)/tyrosinase368–376 (370D) peptides before vaccination. After the sixth vaccine cycle, 1 of the 12 patients tested remained anergic to all recall antigens. Twelve patients who completed all six vaccine cycles were tested after the sixth cycle for DTH to the above-mentioned melanoma peptides, and 6 of 12 (50%) were positive for gp100 or MART-1 reactivity, defined as at least 5 mm of induration measured in one of two perpendicular dimensions. Three patients developed reactivity to gp100, three patients developed reactivity to MART-1, and none developed reactivity to tyrosinase (data shown in Table 3). This included the single patient who was anergic to the panel of recall antigens before and after vaccination.

**Cytokine Release Assay.** A peptide-specific CTL response was measured in 10 of the total of 15 patients on the trial who had pre- and postvaccination leukapheresis samples available. Antigen-specific release of IFN-γ was measured by ELISA of culture supernatants from effector cells restimulated once with peptide-pulsed irradiated PBMC stimulators and analyzed on day 10. Effector cells were incubated for 18 h with control HLA-A2 antigen-positive T2 cells and gp100209–217 (210M) or gp100209–217/MART-126–35 (27L) or MART-127–35, or tyrosinase368–376 (370D) peptide-pulsed T2 cells as described in “Materials and Methods.” Seven of 10 patients tested who received peptides + IFA with SD-9427 showed evidence of increased reactivity to gp100209–217 (210M), 10 of 10 patients showed reactivity to MART-126–35 (27L), and only 3 of 10 patients showed response to tyrosinase368–376 (370D) peptide-pulsed T2 targets as well as evidence by at least a 2-fold increase in postvaccine cytokine release over prevaccine values. The pre- and postvaccine mean (of three measurements) IFN-γ release values for gp100209–217 (210M) ranged from 0 to 1,545 and 0 to 13,098 pg/ml, respectively, whereas corresponding values for MART-126–35 (27L) were 0 to 1,627 and 2.9–4,837 pg/ml, respectively. The results of pre- and postvaccine IFN-γ release for gp100209–217 (210M) and MART-126–35 (27L) peptides for individual patients are shown in Fig. 3. The values for wild-type gp100 and MART-1 peptides were similar to those for the modified peptides for each patient (data not shown). The tyrosinase368–376 (370D) peptide gave the weakest response, with mean prevaccine IFN-γ release values ranging from 0 to 1,094 pg/ml, and postvaccine values ranging from 2.4 to 1,375 pg/ml. The increase in reactivity to gp100 and MART-126–35 (27L) peptides after vaccination was not significantly different (P > 0.05) between the 10 and 40 μg/kg cohorts.

The mean (geometric mean, with 95% confidence intervals) IFN-γ release of T2 targets pulsed with the irrelevant HPV-E736–43 peptide and incubated with gp100209–217 (210M) and MART-126–35 (27L) specific effectors were 39 pg/ml.

**Table 3** DTH skin test results (induration) to gp100209–217 (210M) or MART-126–35 (27L) or tyrosinase368–376 (370D) peptides

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<th>gp100 Post</th>
<th>MART-1 Pre</th>
<th>MART-1 Post</th>
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*Values represent average measurement in millimeters of two perpendicular diameters. Pre denotes prevaccination results, and Post denotes postvaccination results.
(16–91 pg/ml) and 6 pg/ml (2–23 pg/ml) for pre- and postvac-
cine samples, respectively. The presence of a HLA-A*0201-
restricted influenza virus matrix protein Flu-M1_58–66 response
was monitored as a positive control for immune integrity in pre-
and postvaccine cytokine release assays. Significant peptide-
specific cytokine release was expected for Flu-M1-stimulated
effector cells both before and after vaccination for all patients,
and equivalent Flu-M1-specific cytokine release was observed
both before and after vaccination in 10 of 10 patients tested
(data not shown).

**ELISPOT Assay.** Sufficient cells were available from
10 patients for ELISPOT assays to be done before and after
vaccination. This assay enumerates IFN-γ-secreting cells that
are detected as spots on a nitrocellulose filter in wells of a
96-well plate and are counted using a digital imaging analysis
system. This technique is capable of enumerating antigen-
specific functional T cells from fresh blood without restimulation.
The results of ELISPOT assays for gp100(209–217) and
MART-1(26–35) peptide-specific response released in Fig. 4
show that 6 of 10 patients tested had an increase in peptide-
specific T cells specific for gp100(209–217) and 7 of 10
patients tested mounted a response to MART-1(26–35), as
shown by more than a 2-fold increase over the prevaccine
sample. One patient (patient 1) showed strong prevaccination
MART-1(26–35) response that remained unchanged after vac-
cination. No statistically significant difference between prevac-
cine and postvaccine values was observed between the 10 and
40 μg/kg cohorts for any of the peptides tested. The mean
(geometric mean, with 95% confidence intervals) number of
spots/3 × 10^5 cells pulsed with the irrelevant HPV-E7(86–93)
peptide was 9.7 (5.1–17.9) and 4 (2.6–5.9) for pre- and postvaccination samples, respectively. No
background values were subtracted, and the actual data are
plotted in Fig. 4.

**MHC-Peptide Tetramer Assay.** Flow cytometry assays
using tetramers constructed with the gp100(209–217), tyro-
sinase(368–376), and MART-1(26–35) peptides were per-
formed to enumerate antigen-specific CD8+ T cells in PBMC
samples. Positive cells were defined as CD8+ cells staining
with the respective PE-labeled HLA-A*0201 peptide tetramer.
The limit of detection was previously determined to be 0.01% or
1:10,000 CD8+ cells. Using this limit of detection, there

![Fig. 3 Prevaccine and postvaccine day 10 cytokine release assay to gp100 and MART-1 peptides. The release of IFN-γ at 18 h by 1 × 10^5 effector cells stimulated with peptide-pulsed stimulators (T2 cells) at a 1:1 ratio is shown on the ordinate in pg/ml. The mean of duplicate samples is plotted. A, gp100 response; B, MART-1 response. The geometric mean and 95% confidence intervals for IFN-γ release of T2 targets pulsed with the irrelevant HPV-E7_86-93 peptide and incubated with gp100- and MART-1-specific effectors are noted in “Results.”]
was no evidence for any preexisting gp100- or tyrosinase-specific immune response before vaccination in any of the 12 patients analyzed. Similar to the ELISPOT assay, patient 1 showed a potent MART-1_26–35 (27L) response similar to the results of the ELISPOT assay. Eleven of 12 patients tested had greater than 0.01% tetramer-positive gp100_209–217 (210M)–specific CD8+ cells after vaccination, whereas 9 of 12 patients mounted a MART-1_26–35 (27L) response (Fig. 5). The range of positive cells was 0.01–0.5% for gp100 and 0.05–0.24% for MART-1_26–35 (27L). Similar to the results of the cytokine release and ELISPOT assays, the vaccine response to gp100_209–217 (210M) and MART-1_26–35 (27L) was similar for the 10 and 40 μg/kg cohorts for the peptides tested. Minimal increases were seen above background after vaccination for staining with the tyrosinase tetramers in six patients (four patients in the 10 μg/kg cohort and two patients in the 40 μg/kg cohort; data not shown), indicating again that it was a weak immunogen.

Clinical Outcome
The patients on this trial have been followed for a median of 20 months since initiation of treatment. Three patients have relapsed, and one of these patients has died. Median survival has not been reached.

DISCUSSION
Cytokines capable of augmenting DC numbers and mobilizing DCs are attractive candidates as adjuvants for cancer vaccines. Promoting both local and systemic DC differentiation and migration would be expected to enhance the immunogenicity of these vaccines. This was shown to be true in the case of
GM-CSF, which promoted migration of DCs into vaccine sites and increased DC numbers in regional lymph nodes and has been shown to augment the immune response to peptide and other vaccines (24, 25). In vivo antigen loading of DCs in local draining lymph nodes has been demonstrated after peptide vaccination (26). Thus SD-9427, a FLT-3 and G-CSF receptor agonist, is a rational choice for a vaccine adjuvant, based on murine data that showed that SD-9427 increased DC numbers in the spleen and peripheral blood and augmented the ability of DCs to efficiently present CTLs and helper peptides (18–20).

Although in this trial SD-9427 was generally well tolerated, the most serious toxicity observed was the development of autoantibody reactive to its G-CSF domain in one patient, resulting in prolonged neutropenia. However, the antibody titers declined over time, and leukocyte counts normalized after SD-9427 was discontinued. Larger trials would be necessary to determine the incidence of this complication. Meanwhile, close attention to leukocyte counts is necessary in any future trials of SD-9427. Because SD-9427 was shown to reproducibly induce initial leukocytosis in all patients, failure of leukocyte counts to rise after SD-9427 administration should arouse suspicion about the development of an antibody response. The precise mechanism of the neutropenia in our patient is unclear. Although antibodies to G-CSF were detected and peaked at the nadir of the neutrophil count, neutralizing activity to native G-CSF was not observed in a cell-based assay. In another trial, where a 7-day course of SD-9427 was administered to patients with advanced malignancies before chemotherapy, SD-9427 was similarly well tolerated. In 33 patients treated, no antibodies to SD-9427 developed, although results of only one cycle of therapy have been reported (27).

SD-9427 is a multifunctional cytokine with a variety of effects on the hematopoetic and immune systems. Its effects include increasing DC numbers in peripheral blood, increasing total and polymorphonuclear leukocyte numbers in peripheral blood, and promoting the generation of colony-forming units-granulocyte macrophage from CD34+ stem cells (18), all of which might be beneficial in the context of cancer immunother-

**Fig. 5** Prevaccine and postvaccine MHC-peptide tetramer assay for gp100 and MART-1 peptide-specific CD8+ T cells. The percent of tetramer-positive CD8+ cells by flow cytometry is shown on the ordinate. A, gp100-specific cells; B, MART-1-specific cells. The limit of detection for the assay was 0.01% or 1:10,000 cells.
apy. In this trial, SD-9427 administration along with a multipeptide vaccine induced potent peptide-specific immune responses in 11 of 12 patients tested before and after six monthly vaccinations. Responses to all three peptides were observed by a variety of immune assays, although they appeared to be at a level similar to those observed in prior trials of peptides + IFN without additional adjuvants. The ELISPOT and MHC-peptide tetramer analyses can be considered more relevant than the cytokine release assays, in that they measure the frequency of peptide-specific CTLs in unmanipulated PBMCs. A dose-titration for T-cell responses to SD-9427 did not appear to exist at the doses tested. Due to the small numbers of patients tested, concordance of the assays could not be carefully evaluated. For both gp100 and MART-1 peptides, however, a vaccine response measured by these two assays was observed in 9 of 10 patients who had both assays performed. As an example, patient 1 showed strong MART-1 reactivity before vaccination in both these assays, and patient 3 had a good response to gp100 by both assays.

Due to the ability of FLT-3 ligand to generate predominantly mDCs and the ability of G-CSF to mobilize mainly pDCs (16), SD-9427 administration would be expected to increase the numbers of both DC subsets in peripheral blood. This in fact was the case in our patients, who showed an increase in both subsets of peripheral blood DCs. Expansion and mobilization of both DC subsets may be more representative of the physiological situation in vivo after a “danger signal” is delivered. Whereas mDCs migrate into sites of inflammation, take up antigens, and subsequently migrate to lymph nodes viaafferent lymphatics, pDCs are capable of migrating across high endothelial venules into T-cell areas of lymph nodes (28). The hypothesis that pDCs predominantly induce Th2 responses has not been proven because studies have shown the ability of pDCs to induce potent Th1 responses (29). Studies on the in vivo function of pDCs would be further facilitated by the recent description and isolation of murine pDCs (30).

In summary, SD-9427 is a cytokine that has multiple effects on the immune system including mobilization of DCs into peripheral blood. Although a dose-response relationship was observed between SD-9427 dose and WBC and DC numbers in peripheral blood, this did not clearly translate into a stronger immune response to the peptide vaccine. Immune responses to both gp100 and MART-1 modified peptides were detected in this small trial at levels similar to that previously seen in trials with either peptide alone. SD-9427 was generally well tolerated at the doses studied, but the incidence of antibody response to the chimeric protein needs to be defined in larger trials. Besides its use as a vaccine adjuvant, other potential applications of SD-9427 would include mobilization of DCs for ex vivo manipulation, enhancing immune reconstitution after high-dose chemotherapy and stem cell transplantation and mobilization of stem cells for transplantation (33).

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*V. Pullarkat, unpublished data.*


A Phase I Trial of SD-9427 (Progenipoiotin) with a Multipeptide Vaccine for Resected Metastatic Melanoma

Vinod Pullarkat, Peter P. Lee, Ronaldo Scotland, et al.


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