

A Phase I Study of Active Immunotherapy with Carcinoembryonic Antigen Peptide (CAP-1)-pulsed, Autologous Human Cultured Dendritic Cells in Patients with Metastatic Malignancies Expressing Carcinoembryonic Antigen¹

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ABSTRACT

Dendritic cells (DCs), antigen-presenting cells capable of priming naive T cells to specific antigens in an HLA-restricted fashion, have been demonstrated to induce protective T cell-mediated immunity in tumor-bearing animals. We performed this study to test the safety, feasibility, and clinical response of immunizations with *in vitro*-generated DCs, loaded with an HLA-A2-restricted peptide fragment of the tumor antigen carcinoembryonic antigen (CEA), for the treatment of patients with advanced CEA-expressing malignancies. Cell preparations enriched for autologous DCs were generated from the patients' plastic adherent peripheral blood mononuclear cells in serum-free media supplemented with granulocyte macrophage colony-stimulating factor and interleukin-4. Within the cell preparation, 66% of the cells expressed the phenotype typical for DCs (CD86^{high}, HLA-DR^{high}, and CD14^{low}). The DCs were loaded with the CEA peptide CAP-1 and cryopreserved. Groups of three to six patients received four weekly or biweekly *i.v.* infusions of the CAP-1-loaded DC in escalating dose levels of 1×10^7 , 3×10^7 , and 1×10^8 cells/dose. A subset of the patients in the last group also received intradermal injections of 1×10^6 DCs. There were no toxicities directly referable to the treatments. One patient had a minor response, and one had stable disease. Skin punch biopsy at DC injection sites dem-

onstrated pleomorphic infiltrates in the three patients evaluated. We conclude that it is feasible and safe to generate and administer large numbers of previously cryopreserved DCs loaded with CAP-1 peptide to patients with advanced malignancies.

INTRODUCTION

Interest in the application of immunotherapy to the treatment of malignancies has increased with a better understanding of the two major requirements for a tumor-specific immune response: (a) the expression by tumors of unique antigens [such as CEA³ (1), MAGE-1 (2), HER-2/neu (3), MART-1 (4), and MUC1 (5)] that can be recognized by T lymphocytes; and (b) the presentation of these antigens to naive T cells in a manner that stimulates their proliferation and tumor-specific activity. DCs seem to be the most potent antigen-presenting cells (6) for inducing antitumor immunity *in vitro* and *in vivo* (7–13). The development of methods for generating large numbers of DCs from PBMCs *in vitro* (14–16) has now made it possible to study the application of DCs in active immunotherapy strategies for human tumors (17), and several studies (18–20) have been completed, demonstrating the possible clinical efficacy of this approach. The number of DCs used in these studies has been low (range, 1×10^6 to 1×10^7), and DCs have been administered as fresh preparations either intradermally or *i.v.* Because the broader application of DCs may be facilitated by the ability to generate and administer large numbers, it is important to determine whether large numbers of DCs may be generated and cryopreserved while still retaining their phenotype and function and whether administration of these larger numbers will be safe and induce a potent immune response.

We have chosen CEA as the index antigen for evaluating immune responses because of its expression by many gastrointestinal, breast, and lung adenocarcinomas (1), and immunogenicity in murine (21), primate (22), and human (23) recipients of vaccinia virus vectors carrying the CEA gene. A HLA-A2-restricted peptide fragment of CEA called CAP-1 has been shown to be a target for T cell lines derived from patients previously vaccinated with the vaccinia-CEA vector (24). On the basis of our previous demonstration that DCs loaded with CAP-1 could induce CAP-1-specific CTL activity *in vitro* (25), we performed a Phase I clinical trial to assess the safety and

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³ The abbreviations used are: CEA, carcinoembryonic antigen; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorting; ANA, antinuclear antibody; ESR, erythrocyte sedimentation rate; IL, interleukin; MLR, mixed lymphocyte reaction; DTH, delayed-type hypersensitivity.

feasibility of administering escalating doses of autologous, *in vitro*-generated, cryopreserved DCs loaded with CAP-1 to HLA-A2-positive patients with metastatic, CEA-expressing malignancies. Patients were sequentially entered into three dose levels of CEA-loaded DCs and were monitored for toxicity. In addition, the induction of *in vivo* CEA-specific immune responses was monitored. The results support the hypothesis that large numbers of DCs, generated *in vitro* in serum-free media and cryopreserved, may be safely administered and may induce *in vivo* antigen-specific immune responses.

PATIENTS AND METHODS

Patients. Patients were recruited from the medical and surgical oncology clinics of Duke University Medical Center from December 1996 through January 1998. For inclusion, patients were required: (a) to be HLA-A2 positive and have a metastatic cancer-expressing CEA, as defined by immunohistochemical analysis; (b) to have adequate hematological function with an absolute neutrophil count >1000 mm³ (3.), an absolute lymphocyte count >1000 /mm³, hemoglobin >9 mg/dl, and platelets $>100,000$ /mm³; (c) to have adequate renal and hepatic function with serum creatinine <2.5 mg/dl and bilirubin <2.0 mg/dl; and (d) to have at least one recall antigen response of >3 mm on skin testing with either tetanus, mumps, *Candida*, *Trichophyton*, or *Histoplasma*. Exclusion criteria were: (a) chemotherapy, radiation therapy, or immunotherapy within the prior 4 weeks; (b) history of autoimmune disease, including inflammatory bowel disease; (c) presence of an active acute or chronic infection, HIV, or viral hepatitis; (d) and the use of immunosuppressives, such as azathioprine, prednisone, or cyclosporine A in the prior 4 weeks.

Generation of DCs. Patients provided signed informed consent, which fulfilled Duke University Medical Center Institutional Review Board guidelines, before enrollment. Eligible patients ($n = 21$) underwent an unmobilized peripheral blood leukapheresis consisting of up to a 4.5-h processing of 7–12 liters of blood, with each collection containing a minimum of 1.0×10^8 nucleated cells/kg recipient weight. Autologous plasma (250 ml) was expressed from the leukapheresis product, which was then separated by density gradient centrifugation over Ficoll in a Cell Separator (Cobe) to obtain PBMCs. Three-quarters of the PBMCs were used to generate DCs, whereas the remainder were cryopreserved for baseline immunological studies. The PBMCs were resuspended in AIM V media (Life Technologies, Inc., Grand Island, NY) at 6×10^6 cells/ml and plated onto T225 tissue culture flasks (60 ml/flask). The flasks were incubated in 5% CO₂ at 37°C, and after 2 h the nonadherent cells were gently resuspended by rocking the flasks and removed. The adherent cells were then cultured in AIM V media containing rh-granulocyte macrophage colony-stimulating factor (800 units/ml) and rh-IL-4 (500 units/ml; both were gifts from M-E. R.) for 7 days to generate DCs. After 7 days, the DCs were harvested by vigorous washing from the flasks, and the remaining cells were removed with cell dissociation buffer (Life Technologies, Inc.). The cells were resuspended at 5×10^6 /ml AIM V and mixed with the CEA peptide CAP-1 [YLSGAN-LNL; 10 μg/ml; a gift from Dr. Jeffrey Schlom and Cancer Therapy Evaluation Program (CTEP), National Cancer Institute,

Bethesda, MD] for 4 h and washed. For the first 12 patients, the cell preparation was cryopreserved in vials containing 10×10^6 cells/ml/vial in 90% autologous serum/10% DMSO (Cryoserve; Research Industries, Midvale, UT), whereas in the last 9 patients, the first dose of the cell preparation was mixed in autologous plasma to be administered fresh, and the remainder of the cells were cryopreserved for later doses. All assays were performed on previously cryopreserved, thawed, and washed cells.

Immunofluorescence Staining and FACS Analysis.

Before immunofluorescence staining, the viability of the cells was assessed by trypan blue exclusion. Staining and FACS analysis were performed at the Duke University Flow Cytometry Laboratory with HLA-DR-PerCp (Becton Dickinson, San Jose, CA), CD86-PE (PharMingen, San Diego, CA), and CD14-FITC (Becton Dickinson). Immunofluorescence staining was performed by washing cells with medium supplemented with 2% FCS, followed by incubation on ice for 20 min with the appropriate antibodies. Labeled cells were washed with PBS containing 2% FCS and fixed with 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO). More than 10,000 events were collected on a FACScan using a 488 argon laser for fluorescence excitation (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson) on a Macintosh computer. In all experiments, isotypically stained cells were used to set cursors so that $<1\%$ of the cells were considered positive.

Allogeneic MLR. Allogeneic responder PBMCs (1.5×10^5) obtained from healthy donors were cultured in RPMI 1640 supplemented with 10% fetal bovine serum or 10% human AB serum in 96-well U-bottomed tissue culture plates. Irradiated (3500 rads) DC preparations (or the patient's PBMCs used as controls) were added in graded doses of 150–15,000 cells/well in a total volume of 200 μl. Cell proliferation after 96 h was quantified by adding 1 μCi of [*methyl*³H] thymidine (NEN-DuPont, Boston, MA) to each well. After 16 h, the cells were harvested onto filters, and radioactivity was measured in a scintillation counter, with results presented as the mean cpm for triplicate cultures.

Patient Treatment. A three-tiered dose escalation schema was used with the first three evaluable patients treated at low dose (1×10^7 total cells/infusion for four infusions). The next six patients were treated at medium dose (3×10^7 total cells/infusion for four infusions), and the remainder of the patients at were treated at high dose (1×10^8 total cells/infusion for four infusions). The first 12 patients were to receive the DCs i.v. over 2–3 min every week for four immunizations. The last 9 patients were to receive the DCs i.v. along with 1×10^6 CEA peptide-loaded DCs in a volume of 0.1 ml of autologous plasma intradermally into the volar aspect of the forearm or thigh every 2 weeks for four immunizations. The last patient received IL-2 (Aldesleukine; Chiron Corporation, Emoryville, CA), 1.2×10^6 units s.c. each day for 4 days after each DC injection.

Toxicity was graded using the standard National Cancer Institute toxicity grading scale. At the completion of all four immunizations, patients underwent a repeat leukapheresis to obtain enough cells for immunological analysis. In addition, a repeat recall antigen panel plus CAP-1 peptide at three concentrations (0.1, 1, or 10 μg/ml in 0.1 ml) was injected intradermally, and 48 h later the diameters of the erythema and indu-

Table 1 Patient characteristics

Patients enrolled	21
Gender	Female, 12; male, 9
Age	Median, 55; range, 34–82
Tumor type	
Colorectal	11
Breast	5
Ovarian	3
Pancreatic	1
Ampulla of Vater	1
Number of metastatic sites ^a (median)	2 (range, 1–3)
Number of prior treatments (median)	2 (range, 0–6)
Elapsed time since last chemotherapy or radiotherapy	4 months (range, 1–39)
Karnofsky performance scale (median)	80% (range, 50–100%)

^a Each organ or tissue type counts as one site (*e.g.*, liver, bone = 2 sites of metastases); an elevated tumor marker alone, although acceptable for enrollment, was not counted as a site of disease.

ration were measured. Tumor markers (CEA, CA-125, and CA15–3) and imaging studies (computed tomography scans, bone scans, and chest radiographs) were reviewed as available before and after all four immunizations to determine the clinical response.

Histological Analysis of Skin Biopsy Specimens. In eight patients, the diameters of the erythema and induration at the DC intradermal injection site were measured after 48 h. In three of the patients, a 3-mm punch biopsy was performed after induction of local anesthesia with 1% lidocaine, and the skin specimen was rapidly frozen in liquid nitrogen for histological analysis. Frozen tissue sections were cut at 4 μ m in a cryostat, placed on a slide, heat-fixed momentarily, and stained with H&E.

RESULTS

Patient Characteristics. The characteristics of the 21 patients initially enrolled in the study are listed in Table 1. There were 9 men and 12 women with the median age of 55 years (range, 34–82). All had CEA-expressing metastatic malignancies originating from the colon or rectum (11), breast (5), ovary (3), pancreas (1), and ampulla of Vater (1). CEA expression ranged from 50–100% for all but the ovarian cancer patients (who had 25% and 40%). The extent of disease ranged from as few as two moderately enlarged abdominal lymph nodes in a patient with ovarian cancer to multiple sites of involvement including the bones, lungs, liver, skin, and peritoneum. The median number of tissues involved was two (range, 1–3). The median Karnofsky performance scale was 80% (range, 50–100%). Although two patients had not received chemotherapy or radiotherapy, the median number of prior regimens was three (range, 0–6), including high-dose chemotherapy with autologous stem cell transplant in two women with breast cancer. Most of the patients were experiencing progression of their tumor despite chemotherapy or hormonal therapy. In those previously treated with chemotherapy or radiotherapy, a median time of 4 months (range, 1–39) had elapsed from their last treatment to the time of their leukapheresis. Two patients who had been receiving hormones (tamoxifen and megestrol acetate) for prolonged periods were allowed to remain on them at the request of their attending physicians.

Table 2 Characteristics of DCs

Length of leukapheresis (median)	3 hr, 45 min
Total number of cells collected	$2.04 \pm 0.81 \times 10^8$ nucleated cells/kg
PBMCs after Ficoll	$1.03 \pm 0.43 \times 10^8$ cells/kg
PBMCs plated	$8.35 \pm 3.30 \times 10^7$ cells/kg
Cells harvested (washing)	$1.67 \pm 0.71 \times 10^7$ cells/kg
Cells harvested (cell diss buffer)	$0.07 \pm 0.05 \times 10^7$ cells/kg
Viability (at harvest)	92 \pm 5%
Cells remaining (after peptide loading)	$1.43 \pm 0.70 \times 10^7$ cells/kg
Viability (after peptide loading)	90 \pm 8%
Cells/vial before cryopreservation	$2.50 \pm 0.77 \times 10^5$ cells/kg
Cells/vial thawed	$1.37 \pm 0.46 \times 10^5$ cells/kg
Viability of thawed cells	80 \pm 12%
FACS analysis of cultured cells	
CD14+	15 \pm 6%
CD86	63 \pm 17%
HLA-DR	69 \pm 17%

Because of concern that DCs might lodge in the pulmonary vasculature, causing respiratory decompensation, pulmonary function testing was required of all patients before enrollment in the study. The mean results were: forced expiratory volume₁ 77 \pm 19% predicted, forced vital capacity 80 \pm 20% predicted, and D_LCO 88 \pm 20% predicted. The lowest function for a patient who received the infusions was forced expiratory volume₁ 39%, forced vital capacity 53%, and D_LCO 83%. Preexisting organ dysfunction was not uncommon. Two patients, both with ovarian cancer, had an elevated creatinine (1.9 mg/dl and 2.3 mg/dl) because of chronic hydronephrosis and prior platinum-containing regimens. Three patients had liver function test abnormalities greater than two times the upper limit of normal due to hepatic involvement with tumor by the time of their first DC infusion.

DC Characteristics. All enrolled patients underwent the first leukapheresis, and DCs were successfully generated in each case. The characteristics of these DCs are listed in Table 2. The leukaphereses collected $2.04 \pm 0.81 \times 10^8$ cells/kg and, after cell separation to remove granulocytes and erythrocytes, approximately half as many PBMCs were obtained. An average of 2×10^8 cells/kg were archived, and the remainder were used to generate DCs. After 7 days of culture, $1.67 \pm 0.71 \times 10^7$ cells/kg, which represents \sim 15% of the starting number of PBMCs, were recovered with a viability of 92%. By morphology, the harvested population of cells was 60% large dendritic-like cells and 40% small lymphoid-like cells. FACS analysis of the large cell population showed that 69 \pm 17% expressed high levels of HLA-DR, and 63 \pm 17% expressed high levels of CD86, whereas 15 \pm 6% had dim expression of CD14, consistent with DCs (Fig. 1). Sterility testing was negative for contaminants in every preparation. After cryopreservation, 54% of the DCs placed into each vial were recovered with a viability of 80% and a phenotype that was unaltered. When the function of these cryopreserved DCs was tested in the MLR, the DCs demonstrated potent alloreactivity compared with autologous PBMCs (Fig. 2). Thus, the *in vitro*-generated DC preparations used for this study were consistent with previous descriptions of DCs (14).

Patient Treatment Characteristics. Of the 21 individuals enrolled, 19 were evaluable for clinical response and 15

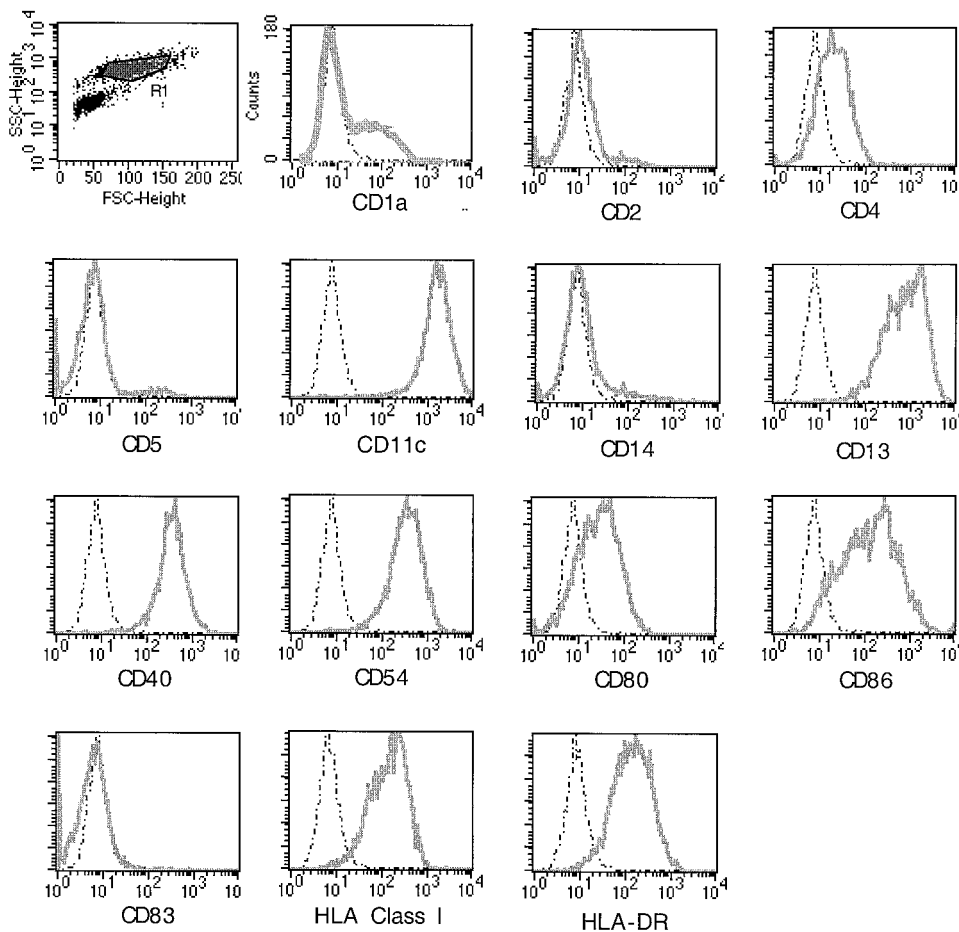


Fig. 1 A representative FACS analysis of the *in vitro*-generated DCs after peptide pulsing and cryopreservation. The analysis was performed on the population of cells with high forward and side scatter. Consistent with prior descriptions of *in vitro*-generated DCs, the cells expressed high levels of CD54, CD86, HLA-DR, and HLA class I. Subpopulations expressed other markers, such as CD1a.

were evaluable for immunological response (Table 3). Two patients had progression of their disease between the leukapheresis and first immunization and received no therapy. Three patients received all four immunizations, but did not return for the follow-up leukapheresis required to determine their immunological response. One patient, treated at low dose, began her therapy with markedly abnormal transaminases and bilirubin due to hepatic involvement with tumor. She had progressively worsening transaminases and bilirubin by the third treatment and was removed from the study after three infusions.

There were no acute toxicities during or immediately after the DC infusions. In particular, no evidence of anaphylactic reactions or other cardiopulmonary compromise was observed. Vital signs monitored included blood pressure, temperature, pulse, cardiac rhythm, respiration, and oxygen saturation. Overall, there were no changes in these parameters, except temperature, which rose a nonsignificant 0.5°C.

There were few adverse events while patients were on the treatment phase of the study or within 1 month of their completion (Table 4). Four patients had increases in liver function test results while receiving the immunizations. Three of these patients had grade 2 abnormalities at the time of initiation of their immunizations and, by the end of the immunizations, their abnormalities had increased to grades 3 and 4. All four patients

had massive involvement of their liver with tumor, and all liver test abnormalities were attributable to tumor progression. One patient with a history of Gaucher's disease, which was manifest as mild thrombocytopenia, developed new right hip and posterior pelvic pain during the immunizations that required narcotics for control. No tumor could be identified in a biopsy of an iliac bone lesion, but Gaucher cells were seen. Thus, it is possible she experienced a flare of her Gaucher's disease in association with the DC infusions. Of note, no patient experienced clinical signs of autoimmune disease. The one patient who received IL-2 had no adverse events other than mild skin erythema and nodule formation at the IL-2 injection sites.

The clinical response data are listed in Table 4. There was one minor response, a patient with ovarian cancer who had slight regression of small volume lymphadenopathy after the immunizations. One patient with bone lesions from breast cancer and an elevated CEA had stability of her metastases and a minimal rise in the CEA during therapy and, thus, was considered to have stable disease. The remainder of the patients experienced progressive disease by the time of their restaging studies, 1 month after completing the immunizations. One of these individuals, though, at the 2-month follow-up visit, was found to have stabilization of a liver metastasis from colorectal cancer and a small decrease in his CEA level. Subsequently, his

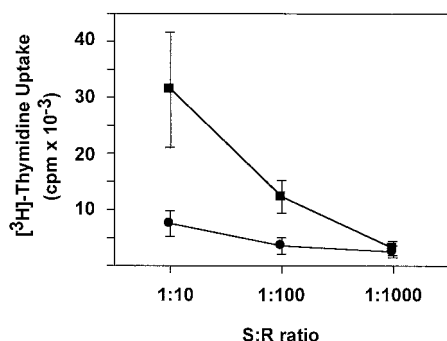


Fig. 2 MLR induced by antigen-loaded, cryopreserved DCs. Aliquots of DCs from the patients were thawed and cocultured with allogeneic responder cells, as described in "Patients and Methods." Alloreactivity was determined by proliferation of responder cells, as determined by thymidine uptake. For comparison with the DCs, the alloreactivity to the patient's PBMCs was also assessed. The figure presents the mean \pm SE of the proliferation of the allogenic T cells to the DCs (■) and PBMCs (●) at various stimulator to responder ratios (S:R) for the patients on the study.

Table 3 Treatment data

No. enrolled	21
No. starting immunizations	19
No. completing immunizations	18
Median no. of doses delivered/patient	4
No. treated/dose level	
Low dose	4; median 10×10^6 cells/dose
Medium dose	8; median 31×10^6 cells/dose
High dose	7; median 100×10^6 cells/dose
No. Evaluable ^a	15
Reason not evaluable	
<4 doses	1
No repeat leukapheresis	3

^a Evaluable, patient has completed all four infusions of DC and the second leukapheresis for immunologic monitoring.

tumor size and CEA levels have begun to slowly increase, but at a rate slower than before enrollment in the study.

The median survival of the treated patients from the time of initiating the DC immunizations is 9 months. Twelve patients, two of whom never received the immunizations, have died since enrolling in this study. The others died between 2 and 13 months after initiating their DC infusions. The cause of death in each case was progression of the malignancy, except one patient who died while having a rod inserted into the humerus for a tumor-associated fracture approximately 1 year after her enrollment in our study.

Clinical Immunological Results. Because of the concern that immunization with DCs might cause autoimmunity, we measured the ANA, rheumatoid factor, anti-thyroglobulin, and ESR as markers of the induction of autoimmunity. Although there were no significant differences in any of these markers among all patients, there were some notable observations. The ANA was positive (at least 1:40) in 14 of the 21 patients examined before the initiation of any therapy, and the median titer in these individuals was 1:160 (range, 1:40 to 1:2560), yet none had clinically manifest autoimmune disease. After all of the DCs infusions, the ANA had increased in three patients,

Table 4 Clinical observations during treatment

Toxicities	
Liver	Grade 2 at baseline; grade 4 at completion
Liver	Grade 3
Liver	Grade 2 at baseline; grade 3 at completion
Liver	Grade 2 at baseline; grade 4 at completion
Miscellaneous	Increased hip pain in patient with Gaucher's
Clinical responses	
CR ^a or PR	0
MR	1
SD	1
PD	17

^a CR, complete response; PR, partial response; MR, minor response; SD, stable disease; PD, progressive disease.

decreased in three patients, and remained the same in the remainder of patients tested. The rheumatoid factor was above the threshold of 30 units in only two patients before the treatment, and there was no change afterward. The anti-thyroglobulin increased in seven individuals, decreased in two patients, and remained the same in two patients after the immunizations. Although some of the increases were small, other increases were more dramatic (18–47 units, 10–153 units, and 38–148 units.) Again, there was no evidence of clinical thyroid disease, although thyroid-stimulating hormone was not measured in these individuals. Finally, the ESR was elevated in 13 of 16 patients tested before the immunizations [the median for all patients was 39 (range, 3–115), but increased in 8 patients after the immunizations (including 2 patients who did not have an elevation previously)], and the median ESR was 56 (range, 9–138.) There were no changes in the immunological parameters in the patient who received IL-2.

Other immunological parameters measured (Table 5) included: (a) the absolute lymphocyte count, which had a non-significant decrease from 1273 ± 486 to 1069 ± 288 cells/mm³ after the immunizations; and (b) the DTH response to recall antigens. Twenty patients had recall antigen responses on DTH testing before the immunizations, and of the 15 patients retested after the immunizations, 12 patients had a DTH response, including one patient who had had a negative DTH response previously. Interestingly, five patients displayed some skin site reactivity to the CEA peptide before the immunizations (induration size range, 1–5 mm). Two of these patients continued to have reactivity afterward, although none had increased in magnitude. In two patients who had reactivity before the immunizations, there was no detectable reactivity afterward, and one was not repeated, whereas one patient developed new reactivity after the immunizations (2.5 mm). Of the eight patients who received intradermal DCs loaded with CEA peptide, four had signs of skin erythema or induration ranging from 1–3 mm in diameter. We were able to compare the skin response to CEA peptide and DCs loaded with CEA peptide (DC-CEA) in three patients. In one patient with no reactivity to the CEA peptide, there was a 2-mm reactivity to the DC-CEA injection at 24 h. In the other two patients, there was no reactivity to either the peptide or the DC plus peptide injections.

Skin biopsies of DC injection sites were evaluated by H&E staining in the last three patients (one of whom had received IL-2), and all three patients were found to have a perivascular

Table 5 Clinical immunological observations

Absolute lymphocyte count pretreatment	1267 ± 483
Absolute lymphocyte count posttreatment	1051 ± 269
Patients with recall antigen DTH response pretreatment	17
Patients with recall antigen DTH response after treatment	14 (2 not done)
Patients with CEA DTH response pretreatment	5 (range, 1–3 mm)
Patients with CEA DTH response pretreatment who had CEA DTH response posttreatment	2
Patients with no CEA DTH response before treatment who had CEA DTH response posttreatment	2
Number of patients with response to DC-CEA DTH	3 of 5

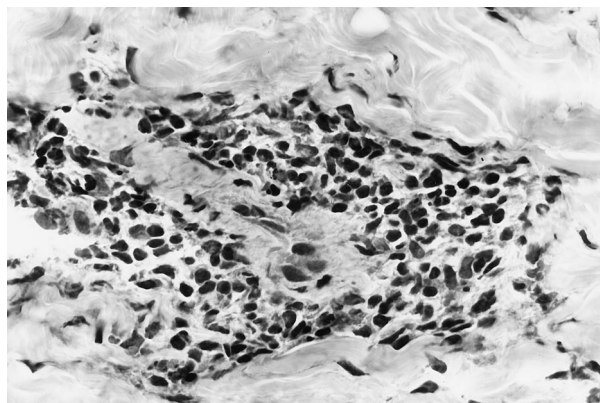


Fig. 3 Photomicrograph of a representative skin biopsy specimen obtained from the intradermal injection site of CAP-1 peptide-loaded DCs. There is a pleomorphic, perivascular infiltrate of cells consistent with a DTH response.

infiltrate, judged by the pathologist as 1–2+ (Fig. 3), despite the fact that only one of the three patients was judged to have any induration or erythema at the injection site. There was no difference in the infiltrate in the patient who received IL-2, although there was a 3-mm area of induration at the DC injection site.

DISCUSSION

The purpose of this study was to evaluate the safety and feasibility of administering a number of DCs that represents the maximum that can be generated from a 4-hour leukapheresis product. We demonstrated that up to four doses of 1×10^8 mononuclear cells (enriched for DCs) could be produced, loaded with the CEA peptide CAP-1 *in vitro*, cryopreserved, and administered to patients with advanced malignancies.

The demonstration of the ability to generate large numbers of functional DCs from patients with advanced malignancies is an important one. Although small-scale generation of DCs *in vitro* has been achieved by a number of groups using either adherent PBMCs (14–16) or CD34+ cells (26–28) as the starting source, the conditions achieved in small preparations do not always mirror those in large clinical preparations. Previous human studies of DC-based immunotherapy have used lower cell numbers and freshly prepared DCs (18–20), and, thus, our study extends these observations on the safety of *i.v.* DC injections.

The characteristics of the cell preparations used for the

immunizations in our study were consistent with previously described properties of DCs. FACS analysis demonstrated that the preparation was enriched for DCs, mixed with bystander lymphocytes. The interpatient variability in the percentage of DCs was broad, despite extensively validated, uniform cell-processing guidelines, and, thus, we believe that this represents a true difference in DC precursors or the cytokine milieu of each individual. The expression of HLA-DR and CD86 were generally high, and when CD14 expression was present it was generally low. In some cases, a separate population of cells that were CD14+ and lower in CD86 or HLA-DR could be discerned. It is not clear whether these cells are monocytes or immature DC or their precursors. The function of the DC as assessed by allostimulatory activity was also quite variable, but they were always more potent than autologous PBMCs as stimulators.

Two other important aspects of our procedure for generating DCs were the use of serum-free conditions and cryopreservation to allow for multiple injections without subjecting the patient to repeated leukaphereses. We believe serum-free conditions are necessary in clinical trials to minimize the risk of sensitizing patients to unintended antigens or introducing adventitious organisms. Although some have advocated autologous serum or plasma, we have found that this is unnecessary and, thus, prefer the serum-free condition. Cryopreservation did not significantly alter the phenotype, viability, or function of the DCs, thus, permitting reproducible conditions for each immunization.

It was also important to document the safety of administering large numbers of DCs *i.v.* to patients with advanced malignancies, many of whom had pulmonary involvement and risk factors for coagulopathy. A study using monocyte infusions has reported changes in coagulation parameters (29), but we saw no consistent laboratory or clinical evidence of clotting abnormalities. In fact, one of the patients had previously experienced two deep vein thromboses years earlier and had an inferior vena cava filter in place, but tolerated all infusions well. The addition of IL-2 in one patient did not lead to any discernible toxicity other than erythema and skin nodule formation at the IL-2 injection site.

The first 12 patients enrolled were treated weekly. This schedule was used because of the short life expectancy of most patients in the study. Nonetheless, in this group, DCs were cryopreserved after being generated and not infused until all tests of sterility (bacterial, fungal, *Mycoplasma*, and endotoxin) were reported as negative, a 4-week delay. The last nine patients received the DCs *i.v.* every 2 weeks for four immunizations. Patients chosen for this portion of the study were felt to be able

to tolerate a longer period off standard therapy. At the same time, it was decided to administer the first dose of DCs fresh on the day of harvest (if a Gram's stain was negative for organisms) because our sterility testing on the first 12 patients showed no contamination. This led to a shortening of the overall period that patients were on the treatment phase of the study.

Another area of theoretical concern was the induction of autoimmunity. CEA is found in minute amounts in the colon and has homology with nonspecific cross-reacting antigen found on granulocytes and the biliary tree (1). One safeguard was the fact that the CAP-1 peptide does not have homology with nonspecific cross-reacting antigen, thus reducing the risk of granulocytopenia. No patient experienced diarrhea or other clinical evidence of autoimmunity. It was interesting to note that 7 of 11 patients tested had an increase in the anti-thyroglobulin titer. There has been no evidence of clinical hypothyroidism, but we did not perform extensive laboratory studies of thyroid function. Approximately 2% of the adult population are thought to have antithyroid antibodies, in the absence of clinical hypothyroidism (30). A study of patients with pancreatic adenocarcinoma reported 24% had antithyroid antibodies (31). The increase in ESR in our study may reflect increased inflammatory response either due to tumor progression or in response to the immunizations.

Although not a primary objective of this study, induction of CAP-1-specific immune responses *in vivo* was identified in preliminary studies. The data showing CAP-1-specific T cell activity measured in the peripheral blood will be presented elsewhere. In four of the eight patients receiving intradermal injections with DCs loaded with CAP-1, there were measurable areas of erythema and induration. These areas were biopsied in three patients and showed pleomorphic perivascular infiltrates consistent with a DTH response, despite the fact that in two of the individuals, no erythema or induration was observed. This indicates that lack of an obvious DTH response on visual inspection of the skin does not preclude a measurable response on microscopic exam. Of interest was the fact that CAP-1 peptide alone induced measurable areas of erythema and induration in some patients, even before receiving the DC immunizations. In two patients with no reactivity before the immunizations, there was skin reactivity afterward. This is particularly interesting because the DTH response is generally thought to be class II-dependent, but the CEA peptide is class I-restricted. In murine models, though, class I-restricted peptides have been shown to elicit DTH-like reactions (32), consisting of infiltration with CD8+ cells.

The safety and the feasibility of the immunizations provide the background for further studies of active immunotherapy with DCs. Detailed characterization of the T-cell response to the immunizations is ongoing, as are efforts to improve the function of the DCs, including applying maturation signals such as CD40-ligand (33). Also, because animal models demonstrate the efficacy of active immunotherapy in the setting of minimal tumor burden, we are now studying the application of DC-based immunotherapy in human settings of minimal residual disease. We administered IL-2 to one patient in a "pilot" fashion and observed no increase in toxicity at the doses used. Further evaluation of the role of adjuvant cytokines is also necessary.

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