Phase I Study of the Intratumoral Administration of Recombinant Canarypox Viruses Expressing B7.1 and Interleukin 12 in Patients with Metastatic Melanoma

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Abstract The objective of this study was to evaluate the safety and activity of the intratumoral administration of the immune costimulatory molecule, B7.1, encoded by a vector derived from the canarypox virus, ALVAC (ALVAC-B7.1), alone and with the intratumoral injection of ALVAC encoding the immune-stimulatory cytokine, interleukin 12 (ALVAC-IL-12). Fourteen patients with metastatic melanoma who had s.c. nodules received intratumoral injections on days 1, 4, 8, and 11. Nine patients were given escalating doses of up to 25×10^8 plaque-forming units of ALVAC-B7.1. Five patients were given 25×10^8 plaque-forming units of ALVAC-B7.1 combined with ALVAC-IL-12 50% tissue culture infective dose of 2×10^6. Toxicity was mild to moderate and consisted of inflammatory reactions at the injection site and fever, chills, myalgia, and fatigue. Higher levels of B7.1 mRNA were observed in ALVAC-B7.1–injected tumors compared with saline-injected control tumors. Higher levels of intratumoral vascular endothelial growth factor and IL-10, cytokines with immune suppressive activities, were also observed in ALVAC-B7.1– and ALVAC-IL-12–injected tumors compared with saline-injected controls. Serum levels of vascular endothelial growth factor increased at day 18 and returned to baseline at day 43. All patients developed antibody to ALVAC. Intratumoral IL-12 and IFN-γ mRNA decreased. Changes in serum IL-12 and IFN-γ levels were not observed. Tumor regressions were not observed. The intratumoral injections of ALVAC-B7.1 and ALVAC-IL-12 were well tolerated at these dose levels and at this schedule and resulted in measurable biological response. This response included the production of factors that may suppress the antitumor immunologic activity of these vectors.

Several lines of evidence have supported the use of immunotherapy in the management of malignant melanoma, an increasingly prevalent cancer that is refractory to conventional chemotherapy. The intratumoral administration of immune-stimulatory cytokines to modify the cytokine profile *in situ* has been an attractive and aggressively pursued approach. Tumor regressions have been observed in patients with melanoma treated with the intratumoral administration of several cytokines, including IFN-α (1), IFN-β (2), granulocyte macrophage colony-stimulating factor (3), and interleukin (IL)-2 (4). Injecting the desired cytokine cDNA encoded by recombinant viruses directly into the tumor has also emerged as an approach to sustain high levels of cytokines locoregionally (5–7). The ALVAC vector derived from a highly attenuated strain of the canarypox virus is particularly well suited to this application. ALVAC infects mammalian cells with high efficiency and induces high levels of recombinant gene expression. ALVAC constructs are likely to be safe because productive viral replication is restricted to avian species and the risk of genomic integration is diminished because of cytosolic transcription (8). Tumor regressions have been reported in early-phase clinical trials of the intratumoral injection of ALVAC vectors expressing IL-2, granulocyte macrophage colony-stimulating factor, and IL-12 (7, 9).

Activation of T cells to produce cytokines requires at least two signals. The first results after the antigen, presented by major histocompatibility molecules, engages the T-cell receptor. The second or costimulatory signal is mediated by costimulatory ligands on the antigen-presenting cell interacting with costimulatory receptors on the T cell. One such signal is B7.1 (CD80; ref. 10). Binding of B7.1 to the CD28 receptor on T cells results in the production of multiple cytokines, including IL-2, IL-12, and IFN-γ, T-helper 1 cytokines critical to the development of antitumor immunity. In contrast, binding of B7.1 to CTL antigen 4 (CD152) on T cells has an inhibitory effect on T-cell activation (11). The importance of B7.1 in the development of an effective antitumor immune response has been shown by transfecting B7.1 into many tumor models. B7.1-transfected melanoma cells are rejected and also stimulate lasting immunity against wild-type tumor cells in murine models. Analysis of this response has revealed production of cytokines consistent with a T-helper 1 and a CTL response (12, 13).

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Among T-helper 1 cytokines, IL-12 has shown significant antitumor effects by a variety of activities, including the production of other T-helper 1 cytokines, such as IFN-γ, activation of CTL and natural killer cells, as well as inhibition of tumor angiogenesis (14). Several studies have shown the benefits of combining costimulation with B7.1 with IL-12. B7.1 tumor transfection and systemic IL-12 have been shown to induce effective antitumor immunity in mouse melanoma and mammary cancer models (15). IL-12 was shown to increase *in vitro* lymphoproliferative responses versus autologous melanoma tumors transfected with B7.1 (16). In addition, it has been shown that IL-12 required initial B7.1-mediated T-cell activation to support immune responses toward human breast and ovarian carcinoma *in vitro* (17). The intratumoral injection of adenovirus-mediated coexpression of B7.1 and IL-12 effectively elicited antitumor immunity in a nonimmunogenic, therapy-resistant, mouse pancreatic cancer model (18).

We examined the safety and activity of the intratumoral injection of ALVAC expressing B7.1, alone and combined with the intratumoral injection of ALVAC-IL-12, in patients with metastatic melanoma. As the intratumoral injection of ALVAC-B7.1 had not been previously examined clinically, we first examined escalating doses of ALVAC-B7.1 alone. We then examine the combination of ALVAC-B7.1 and ALVAC-IL-12, using a dose of ALVAC-IL-12 that had been previously shown to be safe and biologically active intratumorally (7, 9). The intratumoral injections of ALVAC-B7.1 and ALVAC-IL-12 were well tolerated and resulted in measurable biological responses. These responses included the production of factors that may negatively regulate the immunologic activity of these vectors.

Materials and Methods

Patient eligibility. Patients with surgically incurable melanoma and at least one s.c. or superficial lymph node metastasis accessible for injection were eligible for this trial. The study protocol was approved by the institutional Scientific Review, Biosafety, and Human Protection Committees of the University of Alabama at Birmingham (Birmingham, AL). A signed written informed consent satisfying all federal and institutional requirements was obtained as a condition of patient registration. Minimum eligibility requirements of the protocol included the following: an Eastern Cooperative Oncology Group performance status of ≤2; WBC count ≥ 3,000/μL; absolute neutrophil count ≥ 1,500/μL; platelet count ≥ 100,000/μL; prothrombin time within normal limits; serum creatinine ≤1.5 mg/dL or creatinine clearance ≥60 mL/min; total bilirubin <1.5 mg/dl; and aspartate aminotransferase and alanine aminotransferase ≤2 times the upper limit of normal. Therapy was initiated at least 4 weeks after prior chemotherapy, immunotherapy, or radiation therapy.

Treatment. ALVAC-B7.1 was manufactured by Virogenetics Corporation (Albany, NY) with Pasteur Mérieux Connaught USA (Swiftwater, PA), and consisted of the ALVAC virus engineered to encode the human B7.1 molecule under transcriptional control of the vaccinia H6 promoter. ALVAC-B7.1 was formulated in vials containing 5×10^9 plaque-forming units (pfu)/mL in 5% lactose solution. ALVAC-IL-12 was manufactured by Pasteur Mérieux Connaught, Inc. (Lyon, France) and consisted of ALVAC and the genes encoding the human IL-12 p35 and p40 subunits inserted at the *C6* nonessential locus under transcriptional regulatory control of the vaccinia E3L and entomopox 42K promoters, respectively. ALVAC-IL-12 was formulated at 2×10^6 50% tissue culture infectious dose ($TCID_{50}$) per vial lyophilized from an initial volume of 1 mL normal saline. ALVAC-B7.1 and ALVAC-IL-12 were distributed by the Cancer Therapy Evaluation Program of the National Cancer Institute and stored in single-use vials at −20°C.

Patients were enrolled into one of four dosing cohorts in which they received ALVAC-B7.1 alone or into a cohort in which they received ALVAC-B7.1 combined with ALVAC-IL-12. All patients received ALVAC-B7.1 by intratumoral injection on days 1, 4, 8, and 11 (i.e., every Monday and Thursday for 2 weeks). Patients in cohort I received 2.5×10^8 pfu ALVAC-B7.1 in an injection volume of 50 μL of undiluted 5% lactose stock solution; cohort II, 10×10^8 pfu in injection volume of 200 μL; cohort III, 25×10^8 pfu in an injection volume of 500 μL. Patients in cohort IV received the maximally tolerated dose of ALVAC-B7.1 followed by 2×10^6 $TCID_{50}$ ALVAC-IL-12 in 500 μL of normal saline. Injections were administered by a 1.0 mL tuberculin syringe with a 23-gauge needle with distribution of the injection volume along 5 to 10 distinct needle tracts. If a second lesion was available, it was injected with 500 μL of normal saline to provide control tissue. Patients in cohort IV with three or more tumors received ALVAC injections into two tumors and 500 μL of normal saline in a third tumor to provide control tissue. Escalation of the dose to the next higher level proceeded after all three patients had completed the injections and each was observed for at least 21 days without evidence of a dose-limiting toxicity. The occurrence of a dose-limiting toxicity in two patients from any cohort of three to six patients established the preceding dose level as the maximally tolerated dose.

Clinical evaluations. Patients were examined on days 4, 8, 11, and 18 to assess the degree of inflammation at the injection site, regional lymphadenopathy, or other signs of toxicity. A complete blood count and serum chemistries were obtained preimmunization and on days 8, 18, and 43. Patients received an additional 4 weeks of clinical monitoring, such that the off-study evaluation was done on day 43. Treatment-related toxicities were evaluated and graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0. Dose-limiting toxicity was defined as any grade 3 or 4 toxicity with the exception of grade 3 fever and grade 3 local toxicity (i.e., ulceration, which was not considered dose limiting). Tumor sizing of the injected and other lesions was done at each injection with a full disease assessment done before the study and at week 43. Response to treatment was determined for both local (injected tumor) and overall disease using standard outcome measures for clinical trials. Tumor measurement consisted of the product of the longest perpendicular diameters as determined by radiographic or physical examination. A complete response was defined as the disappearance of all of the clinical evidence of tumor. Partial responses required a ≥50% decrease in the size of all of the measurable lesions with no new lesions appearing, and stable disease was defined as <25% decrease or <25% increase in the size of tumor lesions with no new lesions appearing. Progressive disease was defined as a ≥25% increase or the appearance of any new lesion. Any response to treatment required a confirmatory staging at least 4 weeks later.

Quantitative real-time PCR. Biopsies of paired ALVAC-injected and saline-injected tumors were obtained from patients with more than one accessible tumor on day 15 to examine intratumoral B7.1 and cytokine mRNA levels. A portion of each tissue sample was examined histopathologically, and the rest was stored in liquid nitrogen until use. Quantitative real-time PCR was conducted on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Commercially available, prestandardized primers and TaqMan probes for B7.1, IFN-γ, IL-10, IL-12(p40), vascular endothelial growth factor (VEGF), and glyceraldehyde-3-phosphate dehydrogenase, to normalize data, were used (Applied Biosystems). Mortar and pestle were used to render the tumor tissue frozen into a fine powder, which was then homogenized in TRIzol (1 mL/100 mg of tissue). RNA was extracted according to the direction of the manufacturer using the RNeasy method (Qiagen, Valencia, CA). RNA was precipitated with isopropanol and 0.8 mol/L sodium citrate and 1.2 mol/L NaCl. The RNA

pellet was collected, washed once with 75% ethanol, air dried, and resuspended in RNase-free water for quantitative real-time PCR. A one-step protocol was used to accomplish the reverse transcription-PCR using standards, controls, and TaqMan Universal Master Mix (Applied Biosciences) according to the recommendations of the manufacturer. The cycle threshold (C_t) values were determined in duplicate, and the number of copies of the cytokine mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase and then compared, following a validation experiment, using the $\Delta\Delta C_t$ method (Relative Quantitation of Gene Expression, User Bulletin #2, ABI Prism 7700 Sequence Detection System, Applied Biosystems). Two separate quantitative real-time PCR analyses were done, and the data represent mean values of the two.

Serum cytokines. Sera obtained preimmunization and on days 15 and 43 were stored at −70°C for analysis of circulating IL-10, IL-12, IFN-γ, and VEGF levels, which were quantified by commercially available ELISA kits (Pierce Endogen, Rockville, IL) according to the recommendations of the manufacturer. The sensitivity of the assays was as follows: IL-10, <3 pg/mL; IL-12, <5 pg/mL; IFN-γ, <2 pg/mL; and VEGF, <8 pg/mL.

ALVAC antibodies. An ELISA was used to detect serum anti-ALVAC antibodies. Microtiter plates were coated overnight with 5×10^6 pfu per well of active parental ALVAC (Virogenetics, Inc., Troy, NY) in PBS with 0.1 g/L calcium and 0.1 g/L magnesium. The plates were blocked with 1% pig skin gelatin and 3% nonfat dry milk in PBS for 90 minutes at 37°C, followed by washing. The plates were then incubated with various dilutions of patient or normal donor sera in blocking buffer for 1 hour at 37°C. Plates were washed and antibody binding was detected with horseradish peroxidase–conjugated goat antihuman immunoglobulin G (heavy and light chain) antiserum (1:5,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). This antiserum binds human immunoglobulin A, immunoglobulin G, and immunoglobulin M by virtue of light chain recognition. Serum titers of anti-ALVAC antibody were calculated as the limiting dilution of serum producing an absorbance of >0.200 and greater than the mean + 3 SD of 10 normal donor sera at the same dilution.

Results

Clinical effects. The characteristics of the 14 patients enrolled and their ALVAC-B7.1 and ALVAC-IL-12 injections are displayed in Table 1. Nine patients were enrolled into the ALVAC-B7.1 dose-escalation portion of the trial, and five received ALVAC-B7.1 with ALVAC-IL-12. All patients completed the four planned injections, which were well tolerated. Dose-limiting local or systemic toxic reactions were not observed, and a maximally tolerated dose for ALVAC-B7.1 was not established over the dose range that was tested. Patients in all dosing cohorts developed local inflammatory reactions that peaked following the second and third injections and that resolved by 7 days after the fourth and last injection (Table 2). Injection site reactions were grade 1 to 2 consisting of erythema, induration, warmth, and tenderness. Three of five patients receiving ALVAC-B7.1 with ALVAC-IL-12 (cohort IV) developed superficial vesicles or bullae overlying injected nodules with associated weeping and crusting. No ulceration or necrosis occurred. No inflammation of uninjected or saline-injected control nodules occurred.

Systemic toxicity was also mild to moderate (Table 2). Grade 1 or 2 fever associated with chills, myalgias, and fatigue was observed primarily in patients receiving $\geq 10 \times 10^8$ pfu ALVAC-B7.1. These symptoms were uniformly absent after the first

Table 1. Patient and treatment characteristics

		Dosage*					
Patient number	Age/Sex	ALVAC-B7.1	ALVAC-IL-12	Injected lesions	Prior therapy†	Metastatic disease‡	Response§
Cohort I							
1	48/M	2.5×10^8	—	1	C, I, R	S.C.	PD
2	56/F	2.5×10^8	—	1	I	S.C.	PD
3	80/M	2.5×10^8	—	1	R	S.C.	PD
Cohort II							
4	42/F	10×10^8	—	1	C, I, R	S.C., LN	PD
5	34/M	10×10^8	—	1	C, I	S.C., LN	PD
6	77/F	10×10^8	—	1	C, I	S.C., Liver	SD
Cohort III							
7	56/M	25×10^8	—	1	C, I, R	S.C., LN	PD
8	83/M	25×10^8	—	1	None	S.C., LN	PD
9	83/M	25×10^8	—	1	R	S.C., LN	PD
Cohort IV							
10	63/M	25×10^8	2×10^6	1	C, R, I	S.C., Liver	PD
11	72/F	25×10^8	2×10^6	2	I	S.C., Liver, Lung, LN	PD
12	73/F	25×10^8	2×10^6	2	C, I	S.C.	PD
13	66/M	25×10^8	2×10^6	2	None	S.C.	SD
14	41/F	25×10^8	2×10^6	2	None	S.C., LN	PD

*Dosage per injected lesion; ALVAC- B7.1 in pfu, ALVAC-IL-12 in $TCID_{50}$.
†C, chemotherapy; I, immunotherapy; R, radiation therapy.
‡LN, lymph node; SC, subcutaneous.
§PD, progressive disease; SD, stable disease.

Table 2. Highest grade of adverse events

Patient number	Injection site	Fever	Chills	Rigors	Myalgia	Fatigue	Nausea
Cohort I							
1	1	0	0	0	0	1	0
2	1	0	0	0	0	0	0
3	1	0	2	0	0	0	0
Cohort II							
4	1	0	0	0	0	2	0
5	0	2	0	0	1	2	0
6	0	2	1	0	0	0	0
Cohort III							
7	1	2	0	0	0	2	0
8	1	2	1	0	0	1	0
9	1	0	1	0	0	1	0
Cohort IV							
10	0	2	1	2	1	2	0
11	2	2	1	0	1	2	0
12	1	0	1	0	0	1	1
13	2	2	1	0	0	0	0
14	2	1	2	0	0	0	2

dose, most prominent following the third dose, and resolved usually within 24 hours. No allergic reactions occurred. Grade 1 anemia developed in patients consistent with phlebotomy losses and anemia of chronic disease in this population of patients with metastatic melanoma. There were no changes in the total number of peripheral WBC or in the numbers or percentages of polymorphonuclear leukocytes, lymphocytes, monocytes, or eosinophils. There were no changes in platelet counts. Deterioration in renal or hepatic function was not observed.

Patients were evaluated for clinical response of the injected and noninjected tumors (Table 1). None of the injected tumors met the criteria for a response. Regressions of noninjected metastatic tumors were also not observed. Two patients manifested stable disease that lasted 3 and 4 months. Patients who manifested progressive disease manifested progression both in injected and noninjected tumors.

B7.1 and cytokine production. The ability of the intratumoral injection of ALVAC-B7.1 and ALVAC-IL-12 to mediate expression of the transgene and modify the cytokine profile *in situ* was analyzed by biopsing ALVAC-injected tumors on day 15 and comparing the levels of B7.1, IL-12, IL-10, IFN-γ, and VEGF mRNA, as determined by quantitative real-time PCR, to levels in saline-injected control tumors also biopsied on day 15 (Fig. 1). Tumors from eight patients were studied. Day 15 control tumor was not available from patients 1, 2, and 7. The glyceraldehyde-3-phosphate dehydrogenase C_t value of either the ALVAC-injected or the control tumors from patients 4, 6, and 14 was $\geq$30, suggesting an inadequate starting amount and quality of total RNA in these samples; these samples were excluded from analysis. There was considerable variability in the level of B7.1 and cytokine transcripts of the control, saline-injected tumors, which did not manifest clinical evidence for an inflammatory response. Log-fold higher levels of B7.1 expression were observed in five of the eight patients tested, all four of the patients tested who received ALVAC-B7.1 alone and one of the four who received ALVAC-B7.1 with ALVAC-IL-12. Higher levels of VEGF in ALVAC-injected tumors were observed in seven of the eight tested; log-fold higher levels in five. Higher levels of IL-10 were also noted in most ALVAC-injected tumors. In contrast, levels of IL-12 and IFN-γ transcripts were usually less than that in saline-injected tumors. Log-fold higher levels in IL-12 and IFN-γ mRNA were observed in one of the four patients tested receiving ALVAC-IL-12.

The serum levels of IL-10, IL-12, IFN-γ, and VEGF were determined in all 14 patients (Fig. 2). Circulating IFN-γ could not be detected in any of the patients at any time point. In contrast, VEGF was detectable in all patients. There was a significant increase in circulating VEGF at day 15 (P = 0.01, paired t test). VEGF levels decreased to pretreatment levels by day 43. Although decreases were observed at day 15, serum levels of IL-12 did not significantly change at day 15 or 43 when compared with preinjection levels. IL-10 levels were also not significantly affected.

ALVAC antibody. A significant increase in anti-ALVAC titers was observed in all of the patients after the first injection (Fig. 3). The highest anti-ALVAC titers were elicited after the fourth injection (at day 15), which was followed by a gradual decrease. There did not appear to be a dose response with regard to the development of anti-ALVAC antibodies nor any differences in the kinetics or peak levels in patients receiving ALVAC-IL-12 versus ALVAC-B7.1.

Discussion

The ligation of CD28 by B7.1 delivers a well-characterized, and perhaps the most potent, antigen-independent costimulatory signal to T cells, resulting in an increase in the production of T-helper 1–associated cytokines important in promoting antitumor CTL responses. B7.1 can also increase expression of the IL-2 receptor on T cells, prevent activation-induced T-cell

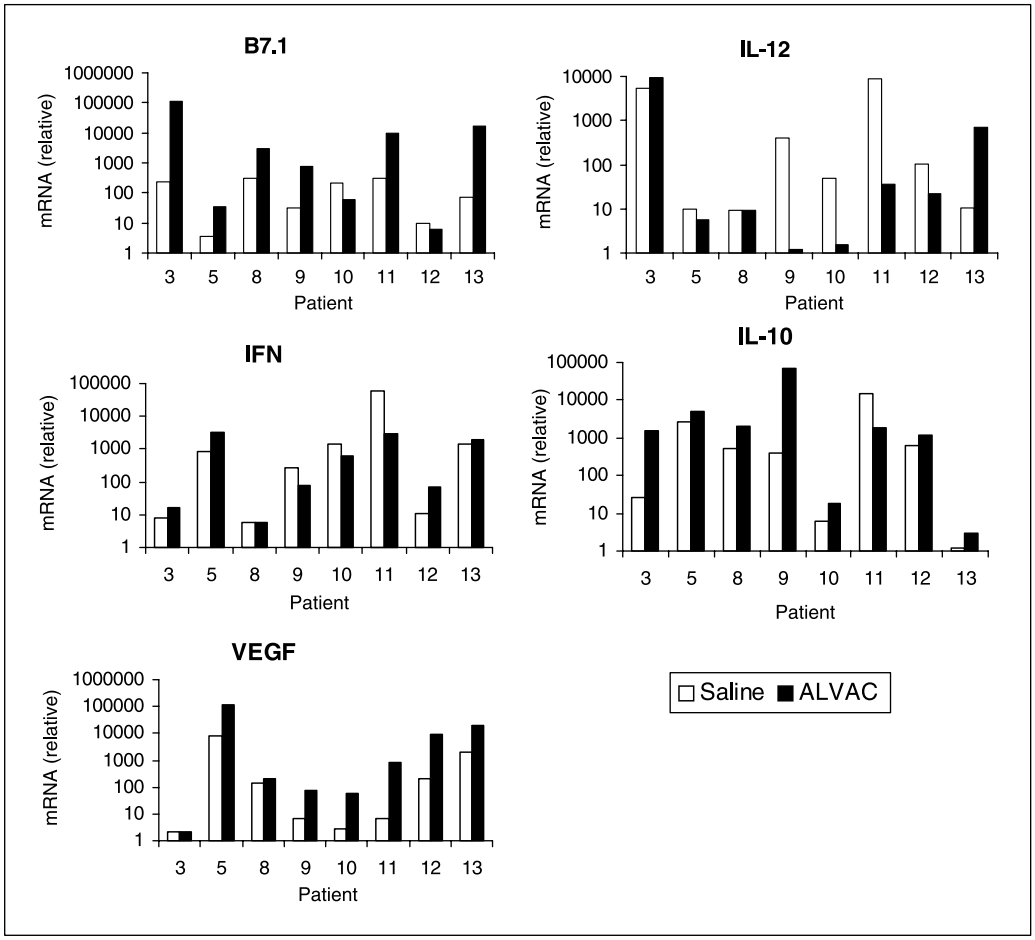

Fig. 1. Intratumoral B7.1 and cytokine mRNA expression. Tumors injected with ALVAC-B7.1 or ALVAC-B7.1 and ALVAC-IL-12 (*closed columns*) and injected with saline (*open columns*) were obtained at day 15. mRNA levels were quantified with quantitative real-time PCR. Patient numbers refer to those in Table 1. Patients 3, 5, 8, and 9 received ALVAC-B7.1 and patients 10, 11, 12, and 13 received ALVAC-B7.1 and ALVAC-IL-12.

death, and trigger natural killer cell cytotoxicity (19, 20). Whereas conferring B7 expression on tumor cells can enhance antitumor responses, it is also apparent that provision of B7 expression is not sufficient to induce immunity against nonimmunogenic tumors (21). Combining B7 expression with immune-stimulatory cytokine expression has been shown to augment tumor immunity. Synergy between B7.1 costimulation and IL-12 has been shown in melanoma models as well as models of breast cancer, lymphoma, hepatocellular cancer, head and neck cancer, and multiple myeloma (15, 22–26). We examined the safety and toxicity of the intratumoral injection of ALVAC canarypox vectors expressing *B7.1* and *IL-12* in patients with melanoma, an immunogenic tumor. Early-phase clinical trials of ALVAC encoding immune-stimulatory cytokines administered by intratumoral injection have shown the feasibility of this approach (7, 9).

We found that the intratumoral injections of up to a total of 50×10^8 pfu of ALVAC-B7.1 and up to a total of 4×10^6 $TCID_{50}$ ALVAC-IL-2 were well tolerated. We were able to administer four intratumoral injections of both vectors without difficulty. Local inflammation was induced but was well tolerated and not limiting. These inflammatory reactions, consisting of local erythema and swelling at the injection site, resolved within 7 days. Although vesicles and bullae were observed, they were not dose limiting, as they have been with the intratumoral administration of vaccinia vectors (27). Systemic toxicity was infrequent and limited to mild flu-like symptoms that usually resolved within 24 hours. The injection of ALVAC-B7.1, with and without ALVAC-IL-12, was biologically active, in addition to the clinical evidence of local inflammatory effects observed. Although the variability of the control tumors, sampling only portions of the tumor in some

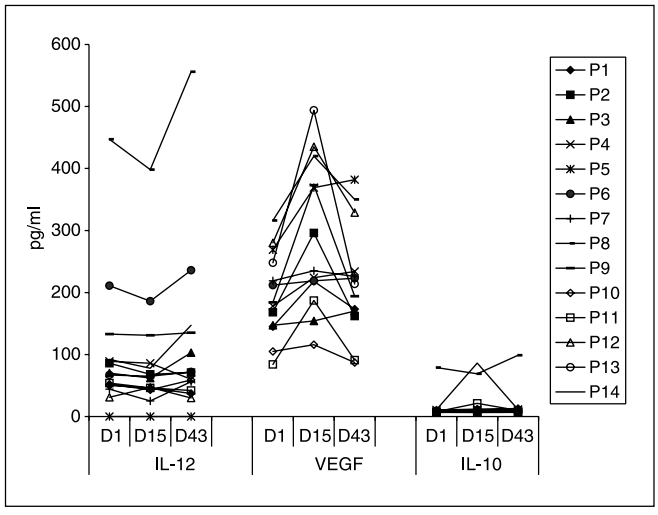

Fig. 2. Serum IL-12, VEGF and IL-10 levels. Serum levels were determined prior to the first injection (*D1*) and on days 15 (*D15*) and 43 (*D43*). Patient (*P*) numbers refer to those in Table 1.

subjects, and the small sample sizes were limiting, we observed higher levels in the intratumoral expression of B7.1 mRNA and in the intratumoral expression of mRNA of several cytokines in ALVAC-B7.1– and ALVAC-IL-12–injected tumors.

The objective of this phase I study was not to determine the tumor response rate. We did not, however, observe any clinical responses, despite the clinical and laboratory evidence for biological activity. It should be noted that costimulatory molecule expression can be frequently detected in melanoma metastases. Although this expression can be attributed to infiltrating immune cells, there is also evidence that some melanoma tumor cells can express costimulatory molecules. In a recent report, expression of B7.1 by tumor cells was found in 9 of 24 metastatic melanoma lesions. No significant correlation between B7.1 and tumor response to biochemotherapy, time to progression, or overall survival was found (28). The antitumor immune response by B7.1 and IL-12 in preclinical studies has been shown to be associated with a strong induction of IFN-γ (18). We did not observe evidence of an increase in intratumoral or systemic IFN-γ production. IFN-γ mRNA actually appeared to decrease in most of the tumors injected with ALVAC-B7.1. This contrasts with observations made in a clinical trial of the intratumoral injection of AVLAC-IL-12 alone in which increases in IFN-γ were observed, albeit in a minority of patients treated (9). There is evidence that expression of costimulatory molecules by tumor cells decreases tumorigenicity but may also reduce systemic antitumor immunity (29). The mechanism of this is not known. Intratumoral injection of an adenovirus encoding murine B7.1 failed to eliminate established murine melanoma despite high-level transgene expression in tumor cells. T-cell inhibitory factor(s) secreted by melanoma cells may have contributed to the failure to achieve protection in this setting (30). Combining costimulatory molecules and cytokines may be a useful therapeutic approach in some, but not all, tumors (31). In a murine transformed liver cell model, coexpression of B7.1 and IL-12 in tumor cells did not result in improved antitumor activity when compared with expression of IL-12 alone. It was suggested that B7.1 expression changed the mechanisms of antitumor immunity and inhibited IFN-γ production induced by IL-12 *in vivo* (32).

We observed higher levels of intratumoral VEGF and IL-10 production and increases in serum VEGF levels with the intratumoral administration of ALVAC-B7.1, alone or combined with ALVAC-IL-12. VEGF is a factor produced by a substantial proportion of solid tumors, including melanoma (33, 34). Increased serum concentration of VEGF in melanoma patients has been reported to confer a worse prognosis (35). VEGF has shown a variety of immune-suppressive effects. VEGF has been shown to impair the functional maturation of dendritic cells, including the ability to down-regulate dendritic cell B7.1 expression (36–38). VEGF also has been reported to decrease the development of T cells (39). IL-12 has been reported to synergize with B7.1-CD28 interaction, not only in inducing IFN-γ production but also in up-regulating IL-10 production by human T cells (40). IL-10, a T-helper 2 type cytokine, also known to be produced by melanoma cells, can counteract the activities of IFN-γ and other T-helper 1 immune-stimulatory cytokines, and blunt antitumor T-cell responses (41–43). IL-10 has also recently

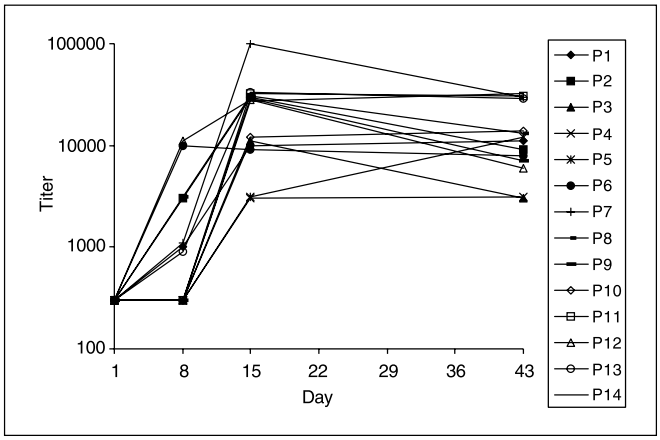

Fig. 3. Anti-ALVAC antibody. Anti-ALVAC antibody titers were determined by ELISA. Patient numbers refer to those in Table 1.

been implicated in the inhibition of the generation of antitumor T cells mediated via CTL antigen 4 (44). In contrast, some investigators have observed that IL-10 can mediate melanoma regression in animal models and advocate administration of IL-10 as an antitumor agent in humans (45, 46). Higher levels of tumoral IL-10 mRNA have been noted in patients with melanoma responding to IL 2–based vaccination programs. Whether this represents an antitumor effect of IL-10 or the ability of IL-2 to reverse IL-10 immune suppression is not known (47).

All patients receiving intratumoral ALVAC in our study developed anti-ALVAC antibody. The clinical significance of a humoral immune response against the vector is not yet known. Anti-ALVAC immunoglobulin G antibody response in patients receiving ALVAC constructs has been previously observed (4, 48, 49). It should be noted that ALVAC vectors do not induce cross-reactive immune responses against vaccinia vectors and their immunogenicity is not altered in vaccinia-experienced individuals (50). The presence of neutralizing antibodies did not seem to prevent expression of the *B7.1* transgene in our study. The high local virus concentrations achieved with injection in tissues would be predicted to overwhelm even a high-titer neutralizing antibody. An anti-vector immune response, however, may alter the kinetics of expression of the encoded transgene, which may modulate its activity.

Because tumor is often easily accessible, intratumoral immunotherapy has been evaluated in several clinical trials in patients with melanoma. The objective of these approaches is not only to generate an immune response *in situ* but also systemically. Intratumoral immunotherapies, including ALVAC-IL-12 when applied alone (7, 9), have shown antitumor activity in patients with melanoma. Responses, however, have been infrequent. Furthermore, none of the intratumoral immunotherapies studied to date has been shown to have consistent effects on uninjected metastases, and none has yet been shown to alter survival of patients with melanoma. Further improvement of the antitumor activity of ALVAC-based intratumoral immunotherapy may be possible by incorporating other costimulatory signals. Combinations of three costimulatory molecules, B7.1, intercellular adhesion molecule 1 (CD54), and lymphocyte function–associated antigen

3 (CD11a/CD18), have recently been shown to activate T cells to a far greater extent than the combination of one or two costimulatory molecules, and a recombinant poxviral vector encoding these molecules has been shown to significantly enhance antitumor immune responses in mouse tumor models (51, 52). A clinical trial has been initiated (53). Although intratumoral B7.1 can mediate direct antigen presentation to T cell by tumor cells, cross-priming of T cell by host professional antigen presenting cells, such as dendritic cells, seems to be central to the development of potent systemic immunity. ALVAC can induce apoptosis of dendritic cells (54). The intratumoral injection of dendritic cells derived *ex vivo* has shown activity in patients with melanoma (55). Preclinical studies have indicated that the intratumoral administration of cytokines and costimulatory molecules with dendritic cells has superior systemic antitumor immunity than either alone, which suggests the potential benefit of applying intratumoral dendritic cell injections with ALVAC-IL-12 and/or ALVAC-B7.1 (56–59).

In summary, although this therapy induced local inflammatory responses and measurably increased levels of intratumoral B7.1, intratumoral levels of T-helper 1 cytokines (IL-12 and IFN) were not increased. Further, levels of potentially negative mediators of antitumor immunity (IL-10 and VEGF) were increased. Whether this is due to *B7.1* transgene expression or anti-ALVAC immune response is unknown. The results of this study underscore the complexity of modifying intratumoral cytokines to promote antitumor immune responses.

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