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Inability to Immunize Patients with Metastatic Melanoma Using Plasmid DNA Encoding the gp100 Melanoma-Melanocyte Antigen

Steven A. Rosenberg, James C. Yang, Richard M. Sherry, Patrick Hwu, Suzanne L. Topalian, Douglas J. Schwartzentruber, Nicholas P. Restifo, Leah R. Haworth, Claudia A. Seipp, Linda J. Freezer, Kathleen E. Morton, Sharon A. Mavroukakis, and Donald E. White
Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

Abstract

Immunization with plasmid DNA represents a theoretically attractive method for increasing T cell responses against cancer antigens. We administered plasmid DNA encoding the gp100 melanoma-melanocyte differentiation antigen to 22 patients with metastatic melanoma and evaluated immunologic and clinical responses. Patients were randomized to receive plasmid DNA either intradermally ($n = 10$) or intramuscularly ($n = 12$). One patient (4.5%) exhibited a partial response of several subcentimeter cutaneous nodules. All other patients had progressive disease. Of 13 patients with cells available before and after immunization, no patient exhibited evidence of the development of anti-gp100 cell responses using *in vitro* boost assays. The same assays were capable of demonstrating immunologic precursors after immunization with fowl poxvirus encoding gp100 or with gp100 peptides. We were thus unable to demonstrate significant clinical or immunologic responses to plasmid DNA encoding the “self” nonmutated gp100 tumor antigen.

OVERVIEW SUMMARY

Multiple approaches to immunizing patients against cancer antigens are being explored. Immunization with DNA encoding cancer antigens has the advantage of immunizing only against the transgene and not against other vector components. In this work we have explored the ability to immunize patients with metastatic melanoma utilizing DNA encoding the melanoma-melanocyte differentiation antigen gp100. We were unable to demonstrate any consistent immunization against gp100 by this approach, using sensitive assays for the detection of immune T cell precursors.

INTRODUCTION

Active immunization strategies for the immunotherapy of patients with cancer are based on the ability to raise T lymphocytes capable of reacting against tumor antigens. Many of the genes encoding cancer antigens have been cloned and extensive studies are being performed to determine the most effective means for immunizing patients against these autologous tumor antigens (Rosenberg, 2001). Immunization with recombinant viruses such as adenovirus, fowl poxvirus, and vaccinia virus engineered to encode full-length tumor antigens can generate modest levels of immune precursors in some patients; however, the ability to perform repeated immunizations with these viruses is severely impeded by the neutralizing immune reactions that occur against the viral envelope proteins (Rosenberg *et al.*, 1998b). Thus alternative approaches have been sought that enable repeated boosting immunizations against the same antigen. Attempts to immunize with full-length recombinant proteins have been limited by the

difficulties and expense of producing sufficient quantities under GMP (Good Manufacturing Practice) conditions suitable for human use. The relative ease of producing large amounts of plasmid DNA has thus led to exploration of the use of DNA encoding full-length antigens as an immunizing vector in animals and in humans.

DNA-based immunization strategies have been shown to elicit cellular and humoral immunity to a broad range of agents including bacteria, viruses, and cancer in many nonhuman species (reviewed in White and Conry, 2000). Preliminary studies in humans, using bacterial or viral antigens, have suggested that cellular immune responses can be generated in humans as well. These studies have prompted us to explore the ability of DNA immunization to generate cellular immune responses against the gp100 human cancer antigen and to explore the therapeutic efficacy of this approach in patients with metastatic melanoma.

gp100 is a nonmutated, differentiation antigen expressed on melanocytes and overexpressed on melanomas (Kawakami *et al.*, 1994, 1995). The immunogenicity of gp100 can be significantly increased by introducing amino acid modifications in the protein at positions 210 (methionine for threonine) and 288 (valine for alanine) that produce peptides with increased binding affinity for HLA-A*0201 MHC class I molecules (Parkhurst *et al.*, 1996; Rosenberg *et al.*, 1998a). In previous studies we have shown that immunization with these modified gp100 peptides or with fowl poxvirus encoding full-length gp100 containing these modifications can generate specific anti-gp100 T cells in patients with metastatic melanoma (Rosenberg *et al.*, 1998a, 2003).

In the present study we have administered plasmid DNA encoding the modified gp100 molecule either intradermally or intramuscularly to patients with metastatic melanoma and evaluated the impact on tumor growth and the generation of anti-gp100 T cells.

MATERIALS AND METHODS

Patient protocol

All patients were treated in the Surgery Branch, National Cancer Institute (NCI, Bethesda, MD) and signed an informed consent approved by the NCI Institutional Review Board before entering this clinical trial. All patients were HLA-A*0201 positive, had measurable metastatic melanoma, and were expected to survive more than 3 months. All patients had progressive disease and had not undergone any other form of therapy in the 3 weeks before entering this protocol. Patients requiring steroid therapy or who had any form of primary or secondary immunodeficiency disease were not included. All patients were evaluated by complete physical and radiologic examination before and at 2-month intervals during the protocol.

Patients were prospectively randomized to receive 1 mg of DNA either intradermally or intramuscularly every 4 weeks for up to four doses. Patient clinical status was evaluated after two doses and if disease progressed, patients were removed from the protocol or switched to other treatments. Patients randomized to receive DNA by the intramuscular route received 0.5 mg intramuscularly into each of two proximal extremities. Patients randomized to receive DNA by intradermal injection received 0.1 mg of DNA at five sites within 10 cm² of skin on each of two proximal extremities.

A partial response was defined as a 50% reduction in the sum of the products of perpendicular diameters of all lesions, lasting at least 1 month with no growth of any lesions or appearance of any new lesions. Any patient not achieving at least a partial response was considered a nonresponder.

DNA encoding the gp100 melanoma-melanocyte antigen

Plasmid DNA encoding the full-length gp100 molecule containing methionine and valine amino acid substitutions at positions 210 and 288, respectively, was provided by Vical (San Diego, CA) under a Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute. The DNA was produced in *Escherichia coli*, using the VR4951 plasmid designed to optimize expression of the transgene. The plasmid contained the gp100 gene promoted by the human cytomegalovirus immediate-early (IE) promoter, intron A derived from human cytomegalovirus, the rabbit β -globin/Proudfoot terminator sequence, and the kanamycin resistance gene. The purified DNA was resuspended in phosphate-buffered saline, filtered through a 0.1- μ m pore size filter, and stored in vials containing 1.2 ml at 1 mg/ml. The DNA was stored at -20°C and injected immediately after thawing at room temperature.

The plasmid underwent extensive testing for functionality in mice. A single DNA immunization with the plasmid was found to induce vigorous gp100-specific antibody responses. A single immunization with the DNA plasmid used also induced gp100-specific T cell responses in HLA-A*0201-transgenic mice. Finally, when coated onto gold beads and administered by Helios gene gun (Bio-Rad, Hercules, CA), the plasmid was shown to be effective at inducing protection from challenge with the gp100-expressing B16 mouse melanoma.

In vitro assessment of gp100-specific immune precursors

Immunologic reactivity against the immunizing gp100 antigen was tested with a 12-day *in vitro* boost assay as previously described (Rosenberg *et al.*, 1998a). In brief, cryopreserved peripheral blood mononuclear cells (PBMCs) obtained before and 4 weeks after immunization were thawed and washed in complete culture medium containing 10% human AB serum. Peptides from the gp100 molecule (gp100:209–217 and gp100:280–288) were added at 1 μM concentration and the cells were cultured in interleukin 2 (IL-2) at 300 IU/ml (Chiron, Emeryville, CA) for approximately 12 days. Cells were harvested and tested for specific cytokine release after coculture for 18 hr with T2 cells pulsed with peptides or with HLA-A*0201-positive and -negative tumor cells. As in previous studies a positive response was defined as secretion of interferon γ at greater than 100 pg/ml in response to the specific peptide and at least two times that released in response to a control peptide (Rosenberg *et al.*, 1998a).

RESULTS

Eleven patients were randomized to receive DNA by the intramuscular route and 12 patients were randomized to receive DNA by the intradermal route. One patient randomized to receive intramuscular DNA was subsequently found not to have measurable melanoma and was excluded from the analysis. The details of the 22 evaluable patients are shown in Table 1. Patients ranged from 26 to 70 years of age and all had received systemic treatment for metastatic melanoma before entering the protocol. Sixteen patients had received prior chemotherapy, 20 had received some prior form of immunotherapy, and 8 had received prior radiation therapy. Most patients had multiple sites of metastatic melanoma. Most patients received at least two cycles of DNA treatment although four patients were taken off protocol at 1 month after one cycle of treatment because of substantial disease progression. Five patients received more than two cycles of immunization.

Mild but transient pain at the injection sites in some patients was the only toxicity seen. One of the 22 patients (4.5%) experienced a partial response of three subcentimeter metastatic deposits on the ankle that were the only sites of disease. All other patients had progressive disease.

Of the 22 evaluable patients, 6 had previously received immunization to different forms of gp100, either as peptides or recombinant viruses, and thus were not evaluable for immunologic assessment of precursor generation. Three patients had no postimmunization cells available for assay. Thus 13 patients (5 had received intramuscular injections and 8 had received intradermal injections) were tested for the ability of the DNA immunization to raise immunologic precursors directed against the two gp100 immunodominant peptides: gp100:209–217 and gp100:280–288. An example of an assay performed is shown in Table 2. *In vitro* boosting was performed with the native gp100:280–288 peptide or with the native gp100:209–217 peptide, and the coculture was tested against the native peptides as well. To assess the immunologic competence of the cells, an HLA-A*0201-restricted flu peptide was often included in the assays because most patients had previous exposure to influenza virus. As a positive control in the assay, patients who had been successfully immunized with the immunodominant gp100:209–217 peptide in other protocols were included as well (patient 4 in Table 2) (Rosenberg *et al.*, 1998a). As shown in Table 2, there was no generation of reactivity against the immunodominant gp100 peptides tested, although lymphocytes were immunologically competent, as evidenced by reactivity to flu peptide. PBMCs from the patient who had received prior peptide immunization (included as a control in this assay) were strongly positive against the gp100:209–217 peptide.

None of the 13 patients tested showed any evidence of immunization against the gp100:209–217 or gp100:280–288 peptide when tested 4 weeks after two cycles of immunizations. In addition, three patients were assessed after four cycles of immunization (one intramuscular and two intradermal) and neither had any *in vitro* evidence of immunization, nor did the patient who received eight sequential cycles of intradermal injections. No attempt was made to measure antibody responses to gp100.

Because we could identify no evidence of immunogenicity against these peptides after a single *in vitro* boost assay, PBMCs obtained after two administrations of plasmid DNA were tested after three weekly *in vitro* stimulations with the immunizing peptide. We had previously shown that repeated *in vitro* stimulations (rather than the single 12-day *in vitro* sensitization reported above) were more sensitive at eliciting low levels of immunization (Salgaller *et al.*, 1996; Cormier *et al.*, 1997). PBMCs from 10 patients were subjected to three *in vitro* stimulations (4 intramuscular and 6 intradermal patients) and again, no reactivity could be elicited against the two immunodominant peptides in postimmunization samples.

Results obtained from PBMCs of patients in this study were compared with similar patients with metastatic melanoma treated during approximately the same time period with fowl poxvirus encoding the same modified DNA and utilizing the same 12-day *in vitro* sensitization assay (Table 3). Four of 14 patients and 7 of 14 patients receiving this recombinant fowl poxvirus were successfully immunized against the gp100:209–217 peptide and the gp100:280–288 peptide, respectively. In another clinical trial, 9 of 10 patients were successfully immunized after receiving the gp100:209–217(210M) peptide administered in Freund's incomplete adjuvant, also assessed using this same 12-day *in vitro* sensitization assay (our unpublished data).

DISCUSSION

DNA-mediated immunization is a theoretically attractive approach to immunization against full-length cancer antigens. DNA is relatively easy to prepare, purify, and sequence in large amounts compared with full-length proteins or recombinant viruses. Plasmid DNA does not result in the expression of non-relevant proteins and, thus, no immune reactions are generated to neutralize the immunizing vector, as is the case for recombinant viruses, thus enabling the repetitive administration of DNA. Unlike many recombinant viruses, DNA vaccines do not

express proteins that can downregulate MHC class I gene expression in transfected cells. In addition, DNA preparations are stable and, because they do not integrate into the genome, represent little risk of insertional mutagenesis or recombinational events that can lead to pathogenic viruses (Nicholas *et al.*, 1995).

Since early reports by Wolff *et al.* demonstrated that skeletal muscle cells could internalize plasmid DNA and result in the episomal expression of the encoded gene (Wolff *et al.*, 1990, 1992), many attempts have been made to utilize DNA-based immunization to raise humoral and cellular immunity to a broad range of agents including bacteria, viruses, and cancer in a wide variety of species (Tang *et al.*, 1992; Cox *et al.*, 1993; Davis *et al.*, 1993; Fynan *et al.*, 1993; Ulmer *et al.*, 1993; Wang *et al.*, 1993; Hoffman *et al.*, 1994; Conry *et al.*, 1995; Xu and Liew, 1995; Irvine *et al.*, 1996). Multiple studies have shown that direct injection of DNA encoding “foreign” model tumor antigens could protect mice from the growth of tumors, including those expressing simian virus 40 (SV40) large T antigen, human carcinoembryonic antigen, mutated p53 antigen, β -galactosidase, as well as a variety of human immunodeficiency virus (HIV)-related proteins (Conry *et al.*, 1995; Wang *et al.*, 1995; Bright *et al.*, 1996; Irvine *et al.*, 1996). The best route to immunize with DNA is unknown, and although the intramuscular route is most commonly used, studies of mouse and nonhuman primates have suggested that intradermal injection or ballistic introduction of DNA-coated gold particles, utilizing a “gene gun,” can be more effective in raising immune responses (Tang *et al.*, 1992; Fynan *et al.*, 1993; Ulmer *et al.*, 1993; Wang *et al.*, 1993).

These preclinical studies led to attempts to use DNA to immunize humans against bacterial or viral proteins. Multiple trials in which HIV-infected individuals were immunized with DNA encoding envelope proteins or the Nef, Rev, and Tat regulatory proteins from HIV have been difficult to interpret because of preexisting immunity in these patients, although several have suggested an increase in cellular and humoral immune reactions (Goepfert *et al.*, 1998; MacGregor *et al.*, 1998; Boyer *et al.*, 1999). DNA vaccine trials against HIV proteins in normal volunteers have resulted in modest immunization in a minority of patients (Goepfert *et al.*, 1998; Gold and Avrett, 1998). DNA vaccination resulted in humoral and cellular responses in humans against malarial, hepatitis virus, and papillomavirus proteins in patients receiving repeated injections of up to 2.5 mg of DNA either by the intramuscular or intradermal route or encapsulated in biodegradable polymer particles (Wang *et al.*, 1998; White and Conry, 2000; Conry *et al.*, 2002; Klencke *et al.*, 2002).

Few data are available concerning the ability of DNA-mediated immunization to raise immune responses against non-mutated but overexpressed “self” cancer antigens in humans. A dual expression plasmid encoding carcinoembryonic antigen, as well as hepatitis B surface antigen, injected intramuscularly into 17 patients with metastatic colorectal cancer demonstrated effective generation of antibodies against the hepatitis protein but little or no humoral or cellular reactivity against carcinoembryonic antigen (Conry *et al.*, 2002). DNA vaccines encoding a chimeric immunoglobulin molecule containing autologous variable heavy and light chain immunoglobulin sequences derived from each patient’s B cell lymphoma linked to mouse immunoglobulin constant region sequences resulted in cellular immune reactivity against the human idiotype in 1 of 12 patients (Timmerman *et al.*, 2002). No antitumor clinical responses were seen in these trials. DNA encoding the HLA-B7 MHC class I protein was injected into HLA-B7-negative patients, using DNA-liposome complexes designed to enhance the immunogenicity of tumor cells by activating immune responses against these foreign MHC antigens. Some regression of injected nodules was reported in these studies (Nabel *et al.*, 1993).

On the basis of these preclinical and clinical efforts we have conducted a study attempting to immunize HLA-A*0201-positive melanoma patients with DNA encoding the gp100

melanoma antigen containing two amino acid substitutions designed to increase the immunogenicity of the immunodominant epitopes of this antigen (Parkhurst *et al.*, 1996). To determine the most effective method for immunizing patients, they were randomized to receive repeated injections of 1 mg of DNA either intramuscularly or intradermally. The vector we used in these clinical trials (produced and supplied by Vical) had several features designed to optimize expression of the encoded gene, including a eukaryotic cytomegalovirus intron A that has been reported to augment protein production (Chapman *et al.*, 1991). Only one partial response of subcentimeter cutaneous nodules was seen in the 22 patients we treated. All other patients had progressive disease. We were unable to demonstrate any evidence of cellular immune reactions against the two immunodominant epitopes of the gp100 molecule, using assays that were successful in demonstrating immune reactions against these antigens after injection of recombinant fowl poxvirus encoding gp100 or by the direct injection of gp100 peptides in Freund's incomplete adjuvant (Table 2 and Table 3). The *in vitro* sensitization boost assay we used is highly sensitive and can detect immune reactions in patients in whom enzyme-linked immunospot and tetramer assays are negative. Despite the high level of sensitivity of this assay, including efforts at repeated *in vitro* stimulations, we were unable to demonstrate any evidence of cellular immune reactivity in these patients.

We thus conclude that neither intramuscular nor intradermal injection of DNA encoding the gp100 nonmutated melanoma-melanocyte antigen was capable of raising cellular immune reactivity or a significant incidence of antitumor effects in patients with metastatic melanoma. Studies performed in experimental animals have suggested that addition of DNA encoding cytokines (Kim *et al.*, 1997; Xin *et al.*, 1998), additional CpG sequences (Sato *et al.*, 1996), or replicon-based vectors (Leitner *et al.*, 2003) may be more potent approaches to DNA immunization in future. We conclude, however, that although DNA immunization in humans can raise cellular and immune reactivity to "foreign" proteins, little evidence exists today that such reactivity can be generated against nonmutated cancer antigens in humans.

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TABLE 1

PATIENT CHARACTERISTICS

Patient	Age/sex	Prior treatment ^a	Site(s) of disease	DNA, cycles (number)	Response ^b
1	70/F	S, I	Cutaneous	8	PR
2	44/F	S, C, I	Lung	2	NR
3	26/F	S, C, I	Subcutaneous	2	NR
4	39/M	S, C, I, R	Liver, lymph nodes, spleen	4	NR
5	49/M	S, I	Liver, lung, adrenal	2	NR
6	42/F	S, C, I	Lung, lymph nodes	1	NR
7	68/M	C, I, R	Liver, lung	2	NR
8	62/F	S, C, I	Subcutaneous	2	NR
9	69/M	S, C, I	Lung	4	NR
10	60/M	S, C, I, R	Liver, lung, subcutaneous	2	NR
1	51/M	S, C, I	<i>Intradermal</i>		
2	46/F	S, C, I	Lymph nodes, intraperitoneal	2	NR
3	67/M	S, I	Lung	2	NR
4	42/M	S, C, I	Lung, intestine	8	NR
5	42/M	S, C, R	Liver, lung	2	NR
6	67/F	S, C, I	Lymph nodes	2	NR
7	51/M	S, I	Cutaneous	4	NR
8	59/M	S, C, I, R	Subcutaneous, lymph nodes	1	NR
9	75/M	S, C, I, R	Lung, adrenal	1	NR
10	58/F	S, I, R	Lung, cutaneous, subcutaneous	2	NR
11	49/F	S, C, I, R	Lung, subcutaneous	1	NR
12	36/F	S, C	Lung, subcutaneous Pancreas	2	NR

^a S, Surgery; I, immunotherapy; C, chemotherapy; R, radiotherapy.

^b PR, Partial response; NR, no response.

TABLE 2

In vitro ASSAY OF PBMCs

Patient	Vaccine	In vitro peptide boost	Target ^a : T2 cells pulsed with:			
			T2	gp100:280–288	gp10:209–217	Flu peptide
1	DNA, intramuscular	gp100:280–288	81	136	38	82
		gp100:209–217	164	142	119	588
		Flu	160	140	145	292,100
2	DNA, intramuscular	gp10:280–288	446	96	110	121
		gp100:209–217	172	79	164	101
		Flu	101	130	106	2,175
3	DNA, intradermal	gp100:280–288	91	94	53	76
		gp100:209–217	158	117	120	560
		Flu	85	49	118	18,810
4 ^b	Peptide	gp10:280–288	38	42	592	24
		gp100:209–217	34	28	11,860	0
		Flu	0	51	2,023	26,210

^aTargets were T2 cells unpulsed or pulsed with gp100:280–288, gp100:209–217, or flu peptide. Results expressed as picograms of IFN- γ per milliliter.

^bPBMCs from a patient who received the gp100:209–217 (210M) peptide in Freund's incomplete adjuvant.

TABLE 3
IMMUNIZATION OF MELANOMA PATIENTS WITH THE gp100 ANTIGEN

Vaccine	Reactivity against ^a :		
	gp100:209–217	gp100:280–288	gp100:254–162
gp100 DNA, intramuscular	0/5	0/5	0/3
gp100 DNA, intradermal	0/8	0/8	0/6
Fowl poxvirus gp100	4/14	7/14	1/14
gp100:209–217 (210M) peptide in Freund's incomplete adjuvant	9/10	—	—

^aNo. of patients positive/total.