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Recombinant Fowlpox Viruses Encoding the Anchor-modified gp 100 Melanoma Antigen Can Generate Antitumor Immune Responses in Patients with Metastatic Melanoma

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Abstract

Purpose—The purpose of this study was to evaluate the immunological responses and therapeutic effectiveness of immunization with fowlpox vaccines encoding the gp 100 melanoma antigen in patients with metastatic melanoma.

Experimental Design—In three consecutive clinical trials, patients were immunized with recombinant fowlpox viruses encoding three different forms of the melanoma/melanocyte-associated antigen gp100: (*a*) the native, full-length gp100 molecule; (*b*) the gp100 molecule with two amino acids modified to increase binding to HLA-A*0201 molecules; and (*c*) a “minigene” construct encoding a single, modified epitope gp100:209–217(210M) targeted to the endoplasmic reticulum. The immunogenicity of these constructs was studied using peripheral blood mononuclear cells to measure epitope-specific release of IFN-γ.

Results—Reactivity against gp100 was not seen in any patient before receiving fowlpox immunization. Whereas just one of seven patients developed reactivity after receiving fowlpox encoding native gp100, 10 of 14 patients who received fowlpox encoding the anchor modified full-length gp100 exhibited reactivity against the native gp100 molecule, and 12 of 16 patients were successfully immunized after inoculation with the modified minigene construct ($p2 = 0.02$). There was no difference in the latter group between those randomized to vaccination by i.v. or i.m. routes. There was one partial cancer regression in the group of 46 patients receiving virus in the absence of interleukin (IL)-2. Once patients showed evidence of progressive disease, they were eligible for “cross-over” treatment to IL-2 alone or with the fowlpox virus. None of the 13 patients receiving the full-length or modified full-length forms of gp100 responded when receiving IL-2, whereas 6 of 12 patients who received the fowlpox containing the minigene construct and then received IL-2 showed objective cancer regressions, including three patients with complete regression.

Conclusions—These data underscore the importance of modifying anchor residues of nonmutated self-antigen peptides to generate cellular immune responses after immunization and support the further investigation of recombinant fowlpox viruses encoding modified epitopes administered in combination with IL-2.

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INTRODUCTION

The identification and molecular characterization of cancer-associated antigens have opened new opportunities for the development of active immunization strategies in cancer patients (1). Antigens such as gp100, MART-1, TRP-1, TRP-2, and tyrosinase represent one class of tumor antigens with shared expression on melanomas as well as melanocytes, the cell of origin of this cancer. Immunization against these normal non-mutated melanoma-melanocyte antigens represents a unique challenge because of potential T-cell tolerance or anergy that may inhibit immune reactivity against normal self tissues. We have thus explored a variety of methods to raise T cells in patients with melanoma that are reactive against these cancer antigens.

Multiple peptide epitopes from these antigens have been identified that are presented on the surface of MHC class I and class II molecules (reviewed in Ref. 2). In previous studies, we have shown that increased levels of T-cell precursors can be raised against several of these peptides by altering anchor residues that enhance binding to the appropriate MHC class I molecule (3–5). Because of the limitations of immunizing against individual class I peptides, we have explored immunization strategies that involve recombinant viruses containing genes that encode the entire molecule and thus may immunize against multiple class I- and II-restricted epitopes. Fowlpox virus is an attractive vector for such immunizations because of the ease of manipulating its genome to encode large gene fragments (reviewed in Ref. 6). These avipox viruses are extremely safe because of their ability to infect but not multiply in human tissues. In the present studies, we have therefore investigated three separate fowlpox virus constructs encoding either the native gp100 melanoma antigen, a modified gp100 molecule including two amino acid substitutions that increase the binding of two immunodominant epitopes to MHC class I molecules, and a fowlpox virus encoding only a modified peptide epitope preceded by a signal sequence that enhances processing of this epitope by antigen-presenting cells. These studies have demonstrated that fowlpox viruses can be used to effectively immunize against several epitopes in the same protein and have demonstrated the importance of modifying the anchor residues of peptides to improve immunogenicity.

MATERIALS AND METHODS

Clinical Protocols

These clinical protocols were approved by the Investigational Review Board of the National Cancer Institute and by the United States Food and Drug Administration. All patients signed an informed consent before protocol entry. All patients had histologically confirmed metastatic melanoma and underwent a complete clinical evaluation including measurements and radiological examination of all evaluable tumor sites. All patients were HLA-A*0201 positive as assessed by high-resolution nested sequence PCR subtyping. No patients had received any immunosuppressive drugs or any treatment for their melanoma in the month prior to entering the protocol. Patients underwent leukapheresis, and PBMCs[2] isolated by Ficoll-Hypaque separation were cryopreserved at 10^8 cells/vial and stored at −180°C. Leukaphereses were repeated 2–3 weeks after every other viral administration. At approximately 6 weeks after initiation of treatment, patients were reevaluated at all known tumor sites, and if signs of tumor progression were seen, patients, if eligible, were treated with high-dose IL-2.

Three sequential pilot trials were performed in which patients received $5–6 \times 10^9$ plaque-forming units of fowlpox virus either i.v. over a 20–30-min infusion or i.m. in the anterior thigh. In the first trial, eight patients received fowlpox virus i.v. containing a gene encoding

[2]The abbreviations used are: PBMC, peripheral blood mononuclear cell; IL, interleukin; ER, endoplasmic reticulum; CM, complete medium.

the entire native gp100 molecule (rF-gp100) originally isolated at the National Cancer Institute from a cDNA library derived from the human melanoma cell line 501-mel. In the second trial, 15 patients received a modified fowlpox virus (rF-Mgp100) i.v. containing a gene encoding the entire gp100 molecule modified to substitute a methionine for a threonine amino acid at position 210 and valine for alanine at position 288 to enhance binding of these peptides to cell surface HLA-A*0201 molecules. In these two trials, patients received virus i.v. every 2 or 4 weeks, respectively, with clinical and immunological evaluation performed after the second injection. In the first two trials, patients who progressed were treated with IL-2 alone administered at 720,000 IU/kg every 8 h for up to 12 doses as described previously (7,8), if eligible. The United States Food and Drug Administration would not permit the administration of i.v. virus along with IL-2 in these trials.

In the third trial, 23 patients received fowlpox virus containing a gene encoding the gp100:209–217 peptide with a methionine substituted in the second position preceded by DNA encoding a 17-amino acid ER insertion signal sequence that originated from the adenovirus E3/19k protein design to facilitate the transport of the peptide into the ER (rF-gp100:ER209-2M; Ref. 9). We have previously shown that the addition of this ER signal sequence could significantly enhance the priming of antigen-specific $CD8^+$ T cells *in vivo* in mice (10). In this third trial, patients were prospectively randomized to receive the fowlpox virus either i.v. (11 patients) or i.m. (12 patients) to determine the preferred method of administration for the generation of precursor T cells. Patients with progressive disease, if eligible, then received high-dose IL-2 every 4 weeks in conjunction with the administration of virus the day before beginning the cycle of IL-2 infusion. All patients who received the i.v. injection of virus were hospitalized overnight for observation, and all i.m. injections were administered on an outpatient basis.

The characteristics of the 46 patients in these three sequential protocols are shown in Table 1. The majority of patients were between 40 and 60 years old with good performance status, and most had been heavily pretreated (85% and 46% of patients had received at least two or three prior treatments for their melanoma, respectively).

Construction of Recombinant Fowlpox-based Vaccines

Recombinant fowlpox viruses were constructed and manufactured by Therion Biologics Corp. as described previously (11). In brief, a plaque-purified isolate from the POXVAC-TC vaccine strain of fowlpox virus was used as the parental virus for these recombinant vaccines. The gp100 sequences were inserted into the *Bam* HI J region of the fowlpox virus genome under the control of the vaccinia 40K promoter. In addition, the lacZ gene, under the control of the fowlpox C1 promoter, was included to identify and isolate recombinant viruses using a chromogenic assay for β-galactosidase.

Measurement of IgG Antibody against Fowlpox and Vaccinia Virus

Vaccinia and fowlpox antibody titers were determined by ELISA. Vaccinia antigen was prepared as a clarified cell lysate of the NYCBH strain of vaccinia virus grown on rabbit kidney RK13 cells. Fowlpox antigen was prepared as a sucrose-cushioned cell lysate of the POXVAC-TC strain of fowlpox virus grown on chick embryo dermal cells. Serial dilutions of patient sera were reacted with pox virus antigen coated onto Nunc maxisorp plates. Plates were then treated with horseradish peroxidase-conjugated goat antimouse IgG and then developed with TMB microwell peroxidase substrate (Kirkegaard and Perry Laboratories). The $A_{450\ nm}$ of each sample was determined using an ELISA plate reader, subtracting the $A_{450\ nm}$ of control samples that lacked sera. Titers were defined as in the highest dilution with $A_{450\ nm}$ above back-ground.

Peptide

The synthetic peptides from gp100 used for *in vitro* testing in this study are shown in Table 2. The identity of each peptide was confirmed by mass spectral analysis, and each peptide was >98% pure as assessed by high-pressure liquid chromatography analysis.

Cultured Cell Lines

The melanoma cell lines 624-mel (HLA-$A2^+$), 624-38-mel (HLA-2^+), 526-mel (HLA-$A2^+$), 888-mel (HLA-$A2^-$), 624-28-mel (HLA-$A2^-$), and 938-mel (HLA-$A2^-$) were established in the Surgery Branch, National Cancer Institute. These tumor lines and the T2 cell line (HLA-$A2^+$, TAP-deficient T-B-cell hybrid) were maintained in continuous culture in medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin.

Evaluation of Clinical Response to Treatment

The response was considered complete if all tumors disappeared. A partial response was defined as a 50% or greater decrease of the sum of the products of the longest perpendicular diameters of all lesions lasting at least 1 month and without growth of any tumors or the appearance of any new tumors. Any patient not achieving a complete or partial response was considered a nonresponder.

In Vitro Assessment of Melanoma-specific Immunological Activities

Cryopreserved PBMCs were thawed into CM consisting of Iscove's modified DMEM (Gibco, Grand Island, NY) with 25 mM HEPES buffer, 10% heat-inactivated human AB serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD) and assayed as described previously (5). Cells were washed once and resuspended at 1.5×10^6 cells/ml in 2 ml of CM containing 1 μM peptide. Two days later, 300 IU/ml recombinant IL-2 (Chiron Corp.) was added to the culture. On day 5 and whenever the medium became acidic, CM (1 ml) was withdrawn and replaced with fresh CM containing IL-2. Cells were harvested between days 11 and 13 after initiation of the culture and washed once in HBSS, and 10^5 cells were added in 0.1 ml CM to wells of flat-bottomed 96-well plates. Stimulator cells consisting of 10^5 melanoma cells or of T2 cells pulsed with peptide were added in 0.1 ml. To pulse T2 cells with peptide, 10^{-4} to 1 μM peptide was incubated with 6×10^6 T2 cells in 3 ml CM for 3 h at 37°C in 5% CO_2. In all assays, only the native peptides were used in the final coculture assay. IFN-γ release into the supernatant was measured after overnight incubation using a standard ELISA assay. The release of other cytokines was not tested. Underlined values are ≥100 pg/ml interferon and at least twice background.

RESULTS

Antibody Titers against Fowlpox Virus and Vaccinia Virus in Melanoma Patients

We tested antibody titers to fowlpox virus in the sera from patients with metastatic melanoma before they had received any immunotherapy (Fig. 1). Only 1 of 38 (2.6%) patients had detectable (>100 titer) levels of antibody against fowlpox virus. However, after two doses of fowlpox virus, 35 of 36 patients developed detectable levels of antifowlpox antibody, with a median titer of 1600. After four and eight doses, the median titer rose to 3200 in 14 and 5 patients, respectively (Fig. 1). Titers were similar in patients receiving fowlpox virus i.m. or i.v. The low number of clinical responses prevented a reliable assessment of the impact of postimmunization antibody titer on tumor regression. In an earlier series of patients, before any immunization, anti-vaccinia virus antibody titers greater than 1000 were seen in 41 of the 46 (89%) patients ($P < 0.0001$). Based on these measurements, we investigated immunization of melanoma patients using recombinant fowlpox viruses.

Immunological Assessments after Fowlpox Immunization

All immunological assessments comparing reactivity before and after vaccination were performed in the same assay on cryopreserved lymphocytes obtained before and after two immunizations. PBMCs from patients who were immunized with either of the two full-length gp100 fowlpox viruses were *in vitro* sensitized using the modified and unmodified gp100:209–217 and gp100:280–288 peptides as well as the native gp100:154–162 peptide, whereas PBMCs from patients who were immunized with the rF-gp100:ER209-2M construct were *in vitro* sensitized against just the native and modified gp100:209–217 peptides. In all cases, the final assay of lymphocyte reactivity was tested against the native unmodified peptides presented on the melanoma cell surface as well as HLA-A*0201-positive and -negative melanomas. Assays were performed on all patients for whom cells were available before and after two immunizations.

An example of an assay performed on a patient who received the rFgp100:ER209-2M virus is shown in Table 3. Several characteristic findings are shown in this assay. Reactivity was not seen in lymphocytes obtained before vaccination, but after vaccination strong reactivity was seen against the native gp100:209–217 peptide [T2 (209)] but not the control gp100:280–288 peptide [T2 (280)] that was not incorporated into the virus. As seen in this assay, highly avid cells could be generated that recognized peptide at concentrations as low as 0.1 n_M, and these cells could recognize HLA-A*0201-positive but not HLA-A*0201-negative melanomas. Examples of assays from PBMCs of two patients who received the fowlpox virus encoding the modified full-length gp100 antigen are shown in Table 4. Prevaccination PBMCs showed no reactivity (data not shown). Evidence of sensitization was present in postvaccination PBMCs against the gp100:209–217 peptide and the gp100:280–288 peptide but not the gp100:154–162 peptide. In these two patients, stronger reactivity was seen against the gp100:280–288 peptide, which is also reflected in the recognition of two HLA-A*0201-positive melanomas and not two HLA-A*0201-negative melanomas.

A summary of the immunological responses of patients who received two fowlpox immunizations in these three pilot studies is shown in Table 5. Sufficient cells for performing assays both before and after fowlpox virus immunizations were available in 7 of 8 patients receiving rF-gp100, in 14 of 15 patients immunized with the rf-Mgp100, and in 16 of 23 patients immunized with the rF-gp100:ER209-2M.

The rF-gp100 fowlpox virus encoding native gp100 was poorly immunogenic, and only one of the seven patients tested exhibited peptide reactivity. All seven patients had vigorous reactivity against a control influenza peptide attesting to the health of the cells used in the assay. In contrast, when stimulated with the modified peptide *in vitro*, 8 of 14 and 10 of 14 patients who received the rF-Mgp100 (modified gp100) in the second trial exhibited reactivity against the native gp100:209–217 peptide and the native gp100:280–288 peptides, respectively ($p2 = 0.02$ when comparing the native with the modified gp100 fowl-pox virus). Only rare reactivity was seen against the high avidity immundominant gp100:154–162 peptide (1 of 14 patients), consistent with our prior immunization studies with this peptide in adjuvant (12).

Although the three immunodominant gp100 peptides (gp100:209–217, gp100:280–288, and gp100:154–162) were identified by tumor-infiltrating lymphocytes from melanoma patients, seven additional class I-restricted gp100 peptides have been identified as reactive with T cells generated after multiple *in vitro* sensitizations (13–15). We thus performed the *in vitro* sensitization assay using all 10 of the described gp100 peptides plus the modified gp100:209–217(210M) peptide and the modified gp100:280–288(288V) peptide in four patients. After two immunizations with the fowlpox virus encoding the modified full-length gp100, two of the four patients tested generated reactivity against the native gp100:209–217 peptide, and all four generated reactivity against the gp100:280–288 peptide. None of the other eight native peptides

were recognized (data not shown). Thus, despite the expression of the full-length protein in the immunizing fowlpox, only reactivity against the gp100:209–217 and gp100:280–288 peptides was seen.

The rF-gp100:ER209-2M fowlpox virus encoding the modified gp100:209–217(210M) peptide preceded by the ER signal sequence induced T-cell responses against the native gp100:209–217 peptide in 12 of 16 patients, and there was little difference seen when injecting this virus i.v. or i.m. (Table 5).

Clinical Response in Patients Receiving Fowlpox Viruses

Transient grade 1 fever, chills, nausea, and headache, generally lasting for several hours after i.v. injection of fowlpox, were common and promptly resolved. Five patients receiving i.v. fowlpox experienced transient grade 2 hypertension, and one patient experienced a grade 3 rash with desquamation fever and chills. There were no clinical responses in the eight patients receiving the fowlpox virus encoding the native gp100 antigen or in the 23 patients who received fowlpox encoding the ER minigene construct (Table 6). There was one partial response (ongoing at 10 months) in the 15 patients who received the fowlpox virus encoding the modified gp100 antigen. Patients who were eligible to receive high-dose IL-2 were crossed over to receive high-dose IL-2 when disease progressed. There were no responses in two patients who received IL-2 as a single agent after receiving the native gp100 and no responses in 11 patients who received IL-2 after receiving the modified gp100 virus. However, 6 of 12 patients who received the fowlpox virus encoding the ER minigene had objective responses to virus plus IL-2 including, three complete responders currently free of disease at 16, 18, and 26 months, respectively. The latter patient had complete resolution of lung metastases but had a bone recurrence at 2 months that completely resolved after radiation therapy, and he remains disease free. Three patients had partial responses, two of which are ongoing. The characteristics of the patients achieving an objective response to the rF-gp100:ER209-2M virus plus IL-2 are shown in Table 7. Six and four responding patients, respectively, exhibited evidence of immunization against the native gp100:209–217 epitope when stimulated *in vitro* with the modified and native peptide, respectively.

DISCUSSION

Active immunization against cancer antigens is aimed at the development of strong cellular immune responses. Immunization against nonmutated self-antigens such as the melanoma-melanocyte antigens gp100 and MART-1 poses unique problems because of the potential tolerogenic mechanisms that limit reactivity against these self proteins. Although T cells reactive against self-antigens can be induced in patients using individual class I-restricted peptides in adjuvant, it has been hypothesized that simultaneous immunization against multiple class I and II-restricted epitopes using recombinant viruses encoding the entire protein may provide more effective antitumor immune responses.

The two major viral families used for immunization against human cancer antigens are the adenoviruses and the poxviridae viruses. In prior studies, adenoviruses were unsuccessful in immunizing against the gp100 or MART-1 antigens, probably because of the high titers of preexisting neutralizing antiadeno-viral antibodies present in the great majority of adults (16). Recombinant pox viruses are particularly attractive as immunizing vectors because they have a wide host and cell type range, can incorporate large amounts of foreign DNA without loss of activity, are highly stable, can be readily grown to high titers under Good Manufacturing Practice conditions, and, after infecting mammalian cells, can undergo efficient posttranslational processing of the inserted transgene (reviewed in Ref. 6). Recombinant vaccinia viruses incorporating genes encoding tumor antigens have been used in several clinical studies; however, the replication-competent nature of this virus limits its use in

immunocompromised individuals. The high titer of cross-reactive antibodies in most humans born before 1970 who received vaccinations against smallpox may diminish the effectiveness of this virus (Table 3). Fowlpox virus, a member of the avipox family that includes canarypox virus, is replication defective in mammalian cells and can express transgenes in infected cells for up to 3 weeks, in contrast to vaccinia virus-infected cells that express antigens from 1 to 2 days before cell death. Because of these advantages and the absence of antibody against fowlpox viruses in most humans (Fig. 1), we have emphasized the use of fowlpox viruses to attempt to immunize against the gp100 self-antigen. Earlier studies using peptide immunization emphasized the value of modifying the anchor residues of peptides to enable more avid binding to cell surface MHC molecules (3,17). Most peptide antigens from nonmutated, self proteins are poorly immunogenic and have intermediate to low binding affinity to cell surface MHC molecules, a finding consistent with the hypothesis that T cells recognizing high-affinity self peptides would be eliminated by negative selection in the thymus (13).

Based on these considerations, we selected fowlpox as an immunizing vector and developed recombinant viruses encoding either (*a*) the native gp100 molecule, (*b*) the gp100 molecule containing two amino acid substitutions that increased the binding avidity of two immunodominant peptides to HLA-A*0201 molecules, or (*c*) the gp100:209–217(210M) peptide preceded by an ER insertion signal sequence to facilitate the processing and presentation of the peptide on the cell surface.

The gp100 melanoma antigen is a 661-amino acid melanosomal matrix protein involved in melanin synthesis and was originally identified as a potential tumor rejection antigen recognized by T cells because of its recognition by tumor-infiltrating lymphocytes associated with cancer regression in melanoma patients (14). A significant correlation was observed between clinical cancer regression and adoptive transfer of lymphocytes reactive with the gp100 but not the MART-1 tumor antigen (15). Ten different gp100 peptide epitopes presented on HLA-A* 0201 have been identified, although only three of these peptides (gp100:209–217, gp100:280–288, and gp100:154–162) were recognized by tumor-infiltrating lymphocytes (15,18). The correlation of tumor regression and vitiligo in melanoma patients treated with immunotherapy lends further credence to the use of this melanoma-melanocyte antigen for the immunotherapy of patients (19). The i.v. route for immunization was selected in the first two studies because studies in mice using model antigens encoded in poxvirus showed the i.v. route to be the most effective means for generating cellular immune responses (20). These were sequential, not randomized, trials, and although the same assays were used in all three trials conclusions, comparing results using these viruses should be done with caution.

As shown in Table 5, only one of seven patients who received recombinant fowlpox encoding native gp100 generated immune reactivity as measured by IFN-γ secretion against gp100 peptides, in contrast to up to 10 of 14 patients who generated reactivity against native peptides when receiving fowlpox encoding gp100 with modified amino acids at peptide anchor binding residues ($p_2 = 0.02$). Thus, as was the case when using peptide immunization, modifying individual anchor amino acids to create epitopes with higher binding affinity to cell surface MHC can substantially increase the immunogenicity of gp100 when incorporated into recombinant poxvirus (3). The dominance of reactivity to the gp100:209–217 and gp100:280–288 peptides was further demonstrated by the relative inability to immunize against the nonmodified gp100:154–162 peptide (only 2 of 21 patients could be immunized). Difficulty in immunizing against the gp100:154–162 peptide was also seen in studies of peptide immunization and may be due to the high binding affinity of this peptide to HLA-A*0201, which can lead to control elimination of reactive precursors in the thymus (17). When testing four patients who received fowlpox encoding the rF-Mgp100 for reactivity against all 10 known gp100 peptides, reactivity was seen only against the gp100:209–217 and gp100:280–288 peptides.

Because of the high degree of reactivity against the modified gp100 molecule, we next tested a recombinant fowlpox virus encoding only the anchor-modified gp100:209–217(210M) peptide. To further explore possible improvements in immunization, this DNA was preceded by a short sequence encoding a 17-amino acid peptide from the adenovirus E3/19k protein to facilitate transport of the gp100 peptide into the ER where MHC class I loading occurs (9). In this latter study, we also randomized patients to receive this same dose of fowlpox virus either i.v. or i.m. Effective immunization was seen when virus was given by either route, and 12 of the 16 patients evaluable for immunological response in this trial showed evidence of immunization against the native peptide (Table 3 and Table 5). It is important to emphasize that lymphocytes that react strongly against the gp100 peptides can recognize and attack tumor cells (Table 3 and Table 4).

The paradoxical paucity of clinical responses seen in patients, even when generating antitumor cellular responses (Table 6), is a common theme in studies of active immunization against cancer antigens and is the subject of considerable study. Immunological assays do not predict clinical antitumor efficacy. Only a single patient of the 46 patients in this study achieved an objective partial response when treated with one of the gp100 recombinant fowlpox constructs alone, *i.e.*, without IL-2. Of interest was the finding that 6 of 12 patients who received fowlpox virus encoding the ERgp100:209–217(210M) peptide and then received IL-2 in conjunction with additional immunization with fowlpox experienced objective cancer responses, including three patients with partial responses and three patients with complete responses who are now without evidence of disease at 12–24 months (Table 7). The objective responses in half of the patients in this group compares favorably with the 15% response rate seen in 183 melanoma patients treated with the same dose of IL-2 without vaccine ($p2 = 0.0008$), although this may simply be a reflection of the small number of patients treated with the recombinant fowlpox encoding the minigene and the vagaries involved in comparison with historical controls (7,8). It is possible, however, that the combination of stronger immunization against the gp100 peptide resulted from the ER targeting sequence along with helper factors resulting from fowlpox immunization contributed to a higher response rate to IL-2 in patients previously immunized with this virus. New clinical protocols exploring this possibility are in progress.

Thus it appears that administration of recombinant fowlpox viruses can generate T-cell responses against several epitopes in a nonmutated self-antigen expressed on tumor cells. Additional studies to enhance immune reactivity such as the use of recombinant vaccinia and fowlpox viruses in prime boost regiments, the administration of viruses along with adjuvants, incorporation of costimulatory molecules or multiple antigens in the same poxvirus as well as attempts to generate both class I- and class II-restricted responses after immunization are in progress.

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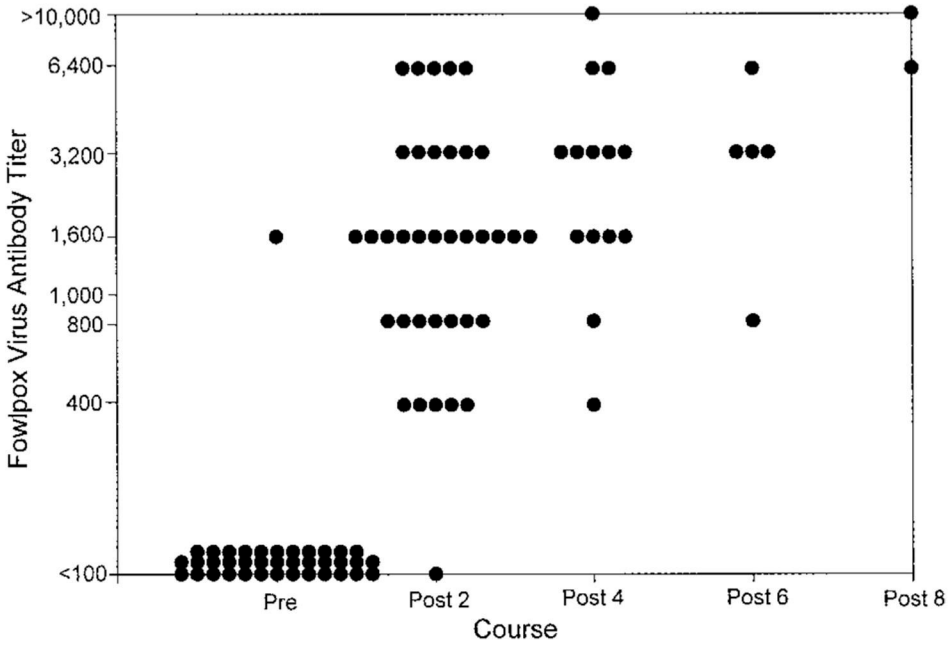

Fig. 1.
Antifowlpox antibody titers in patients receiving immunization with recombinant fowlpox virus encoding gp100.

Table 1

Characteristics of patients receiving fowlpox virus immunization

	No. of patients receiving				
	rF-gp100	rF-Mgp100	rF-gp100:ER209-2M		
	i.v.	i.v.	i.v.	i.m.	Total (%)
Total	8	15	11	12	46 (100%)
Sex					
Male	6	10	10	9	35 (76%)
Female	2	5	1	3	11 (24%)
Age (yrs)					
21–30	0	1	2	0	3 (7%)
31–40	1	4	2	0	7 (15%)
41–50	1	4	2	5	12 (26%)
51–60	5	4	4	5	18 (39%)
61–70	1	2	1	2	6 (13%)
Performance (ECOG)[a]					
0	8	11	11	10	40 (87%)
1	0	3	0	2	5 (11%)
2	0	1	0	0	1 (2%)
Prior therapy					
Surgery	8	15	11	12	46 (100%)
Chemotherapy	4	6	3	7	20 (43%)
Radiotherapy	3	3	3	6	15 (33%)
Hormonal	1	1	0	3	5 (11%)
Immunotherapy	7	12	8	9	36 (78%)
Any 2 or more	8	13	8	10	39 (85%)
Any 3 or more	3	6	3	9	21 (46%)

[a] ECOG, Eastern Cooperative Oncology Group.

Table 2

Multiple epitopes in the gp100 antigen recognized by $CB8^+$ T cells

Peptide	Sequence
gp100:154–162	KTWGQYWQV
gp100:177–186	AMLGTHTMEV
gp100:178–186	MLGTHTMEV
gp100:209–217	ITDQVPFSV
gp100:209–217 (210M)	IMDQVPFSV
gp100:280–288	YLEPGPVTA
gp100:280–288 (288V)	YLEPGPVTV
gp100:457–466	LLDGTATLRL
gp100:476–485	VLYRYGSFSV
gp100:570–579	SLADTNSLAV
gp100:619–627	RLMKQDFSV
gp100:639–647	RLPRIFCSC

Table 3

Assay for antipeptide and antitumor reactivity in PBMCs from a patient immunized with fowlpox virus encoding gp100:ER209-217 (210M)[a]

	In vitro sensitization with[b]			
	gp100:209–217		gp100:209–217 (210M)	
	Before immunization	After immunization	Before immunization	After immunization
T2	28[c]	58	29	64
T2 (280–288) 1 μM	18	52	35	48
T2 (209–217) 1 μM	14	3997[d]	22	21080
0.1 μM	25	4751	23	21480
0.01 μM	22	3045	27	6548
0.001 μM	28	434	20	2514
0.0001 μM	22	106	23	180
$A2^{+}$624-mel	11	2448	13	4222
$A2^{-}$624.28-mel	12	44	14	54

[a] Fowlpox virus encoding the gp100:209–217 (210M) epitope preceded by the adenoviral insertion signal sequence (rF-gp100:ER209-2M).

[b] PBMCs obtained before and after two fowlpox immunizations cultured with each of the two peptides for 12 days as described in "Materials and Methods."

[c] Secretion of IFN-γ in an 18-h coculture assay. Values are given as pg IFN-γ/ml.

[d] Underlined values are those judged to be positive results defined as greater than 100 pg/ml and at least twice greater than background (*i.e.* T2 alone).

Table 4

Assay for antipeptide and antitumor reactivity in PBMCs from a patient immunized with fowlpox virus encoding modified full-length gp100 (rF-Mgp100) [a]

Peptide used for *In vitro* sensitization[b]	Stimulator in assay							
	T2 *In vitro* sensitization[b]	T2	T2 (209–217)	T2 (280–288)	526-mel (154–262)	624-mel ($A2^+$)	888-mel ($A2^+$)	938-mel ($A2^-$)
Patient A								
gp100:209–217	150[c]	382[d]	140	142	148	199	160	82
gp100:209–217 (210M)	71	711	53	57	56	67	60	42
gp100:280–288	76	68	3836	72	217	310	74	52
gp100:280–288 (288V)	105	87	4482	98	210	319	97	69
gp100:154–162	96	77	82	82	63	66	84	53
Patient B								
gp100:209–217	85	683	64	51	13	24	21	14
gp100:209–217 (210M)	810	1677	604	519	35	54	23	13
gp100:280–288	226	217	2147	213	146	150	27	37
gp100:280–288 (288V)	569	562	6803	604	175	203	42	31
gp100:154–162	66	72	74	72	6	4	15	9

[a] Fowlpox virus encoding gp100 with amino acid modifications 210M and 288V.

[b] PBMCs cultured with 1 μM of each of the five peptides for 12 days as described in "Materials and Methods."

[c] Secretion of IFN-γ in an 18-h coculture assay. Values are given as pg IFN-γ/ml.

[d] Underlined values are those judged to be positive results defined as greater than 100 pg/ml and at least twice greater than background (i.e. T2 alone).

Table 5

Immunologic response of patients receiving fowlpox immunization

Fowlpox administration[a]	*In vitro* sensitization assay (day 12) using the following peptide					
	209–217	209–217 (210M)	280–288	280–288 (288V)	154–162	FluM1: 58–66
Native rF-gp100 i.v.	1/7[b]	1/7	1/7	1/7	1/7	7/7
Modified rF-Mgp100 i.v.	4/14	8/14	7/14	10/14	1/14	13/14
rF-gp100:ER209-2M						
i.v.	5/8	5/8	N.A.[c]	N.A.	N.A.	7/8
im.	7/8	6/8	N.A.	N.A.	N.A.	7/8

[a] $5–6 \times 10^9$ plaque-forming units every 2–4 weeks; lymphocytes were tested 2–3 weeks after two immunizations.

[b] All assays tested against T2 cells pulsed with 1 μM native (unmodified) peptide; a positive response is defined as secretion of IFN-γ by PBMCs stimulated by the specific peptide at least twice that stimulated by a control peptide. Values represent number of patients positive/total tested.

[c] N.A., not applicable.

Table 6

Clinical response of patients receiving fowlpox virus immunization[a]

	Fowlpox virus encoding			
			rF-gp100: ER209-2M	
	rF-gp100 i.v.	rF-Mgp100 i.v.	i.v.	i.m
Virus alone				
Total	8[b]	15	11	12
Responder	0	1	0	0
Cross-over to IL-2				
Total	2	11	7	5
Responder	0	0	3	3
Complete	0	0	2	1
Partial	0	0	1	2

[a] Patients received 5–6 × 10^9 plaque-forming units of fowlpox virus i.v. or i.m. every 2–4 weeks; followup as of 4/1/02; no prior gp100 immunization in any patient.

[b] Values represent number of patients.

Table 7

Characteristics of the patients responding to rF-gp100:ER209-2M virus + High-dose IL-2

Patient no.	Age (yrs)/sex	Sites of disease	Response (duration in months)	Comments
1	60/M	Lung	CR (2)[a]	Bone recurrence at 2 months irradiated; now disease-free at 26 months
2	44/M	Hilum, liver, lung	PR (9)	Recurred in stomach
3	45/M	S.C., liver	CR (16+)	Ongoing response
4	60/F	Liver	CR (18+)	Ongoing response
5	56/M	Lymph nodes, S.C.	PR (11+)	Ongoing response
6	46/M	Brain, S.C., lung	PR (3+)	Ongoing response

[a]CR, complete response; PR, partial response.