

Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection

(expression cloning/tumor antigen/gp100/HLA-A2.1/immunotherapy)

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Communicated by Henry Metzger, March 16, 1994 (received for review January 12, 1994)

ABSTRACT The cultured T-cell line TIL1200, established from the tumor-infiltrating lymphocytes (TILs) of a patient with advanced metastatic melanoma, recognized an antigen on most HLA-A2⁺ melanomas and on all HLA-A2⁺ cultured neonatal melanocytes in an HLA-A2 restricted manner but not on other types of tissues or cell lines tested. A cDNA encoding an antigen recognized by TIL1200 was isolated by screening an HLA-A2⁺ breast cancer cell line transfected with an expression cDNA library prepared from an HLA-A2⁺ melanoma cell line. The nucleotide and amino acid sequences of this cDNA were almost identical to the genes encoding glycoprotein gp100 or Pmel17 previously registered in the GenBank. Expression of this gene was restricted to melanoma and melanocyte cell lines and retina but was not expressed on other fresh or cultured normal tissues or other types of tumor tested. The cell line transfected with this cDNA also expressed antigen recognized by the melanoma-specific antibody HMB45 that bound to gp100. A synthetic 10-amino acid peptide derived from gp100 was recognized by TIL1200 in the context of HLA-A2.1. Since the administration of TIL1200 plus interleukin 2 resulted in regression of metastatic cancer in the autologous patient, gp100 is a possible tumor rejection antigen and may be useful for the development of immunotherapies for patients with melanoma.

T cells play an important role in the regression of several murine tumors. In humans, the adoptive transfer of tumor-infiltrating lymphocytes (TILs) with interleukin 2 (IL-2) resulted in cancer regression in 35–40% of melanoma patients (1). Thus, characterization of the antigens recognized by TILs may be important for understanding the basis of T-cell recognition of growing tumors and for the development of new immunotherapeutic approaches.

Many melanoma TILs recognize shared antigens on melanoma cell lines established from different patients in a class I major histocompatibility complex (MHC)-restricted fashion *in vitro* (2, 3). Three normal self-proteins encoded by the MAGE-1, tyrosinase, and MART-1 genes have been identified as antigens recognized by melanoma-specific T cells (4–7). The MART-1 gene was isolated using TILs, but the MAGE-1 and tyrosinase genes were identified using T-cell clones established from the peripheral blood of patients who were repetitively immunized *in vivo* with mutagenized tumor cells or whose peripheral blood lymphocytes were sensitized by repetitive *in vitro* stimulation with tumor.

In the present study, we have cloned a gene encoding a second human melanoma tumor antigen recognized by TILs

that were associated with a clinical anti-cancer response when the TILs were used to treat the autologous cancer patient. Analysis of gene sequences in GenBank revealed that the cloned gene was almost identical to the gene encoding melanocyte/melanoma-specific protein Pmel17 or gp100, which was recognized by monoclonal antibody HMB45. We have also identified a 10-amino acid antigenic peptide in gp100, which upon reconstitution in HLA-A2⁺ mutant T2 cells confers cytotoxic T lymphocyte (CTL) lysis. This antigen may be useful for the development of new immunotherapeutic strategies for the treatment of patients with melanoma.

MATERIALS AND METHODS

cDNA Expression Cloning. The cDNA25 clone encoding the melanoma antigen gp100 was cloned by techniques similar to those previously described (6, 8). Briefly, a breast cancer cell line, MDA231, transfected with a cDNA library in λ pCEV27 made from the 501mel melanoma cell line was screened for antigen positivity by measuring interferon γ (IFN- γ) secretion when cocultured with TIL1200. TIL1200 was generated by techniques previously described (9). The integrated cDNA was recovered from the genomic DNA of positive transfectants by PCR and cloned into the mammalian expression plasmid pcDNA3 (Invitrogen). The full-length cDNA for cDNA25 was isolated from the 501mel λ pCEV27 library using the cDNA25 probe. The λ phage containing the full-length cDNA25 was digested with *Xho* I and then self-ligated with T4 DNA ligase to make the plasmid pCEV27-FL25. Alternatively, a full-length cDNA25 isolated by PCR using the specific primers designed for gp100 was cloned in pCRII (Invitrogen) and then cloned into pcDNA3 (pcDNA3-FL25). To test whether this cDNA encoded a melanoma antigen it was retransfected into COS7, A375, or MDA231 and the resulting transfectants were tested for stimulation of TIL1200. DNA sequences of the plasmid clones were determined with an automated DNA sequencer (model 373A; Applied Biosystems), using the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) using the manufacturer's instructions.

Peptide Synthesis and Identification of Antigenic Peptides. Peptides were synthesized by a solid-phase method using a Gilson AMS 422 multiple peptide synthesizer. The peptides were purified by HPLC on a Vydac C-4 column with 0.05%

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Abbreviations: IFN- γ , interferon γ ; IL-2, interleukin 2; HLA, human leukocyte antigen; TIL, tumor-infiltrating lymphocyte; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte.
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trifluoroacetic acid/water/acetonitrile. To identify antigenic peptides, TIL lysis of T2 cells preincubated with peptides for 2 hr was measured using a ^{51}Cr release cytotoxicity assay.

RESULTS

Treatment of a Patient with Metastatic Melanoma using TIL1200. A 29-year-old male patient, 1200, with a widely metastatic melanoma who had previously failed chemotherapy and radiation therapy was treated with a single preparatory dose of 25 mg of cyclophosphamide per kg followed by the i.v. infusion of 1.6×10^{11} TILs (including 9.1×10^9 ^{111}In -labeled TILs) plus seven doses of IL-2 at 720,000 international units/kg given every 8 hr. A second cycle of treatment with TILs and IL-2 was given 3 weeks later. Radionuclide scans showed localization of TILs in tumor deposits (Fig. 1A). Biopsy of s.c. tumors on days 8 and 11 after treatment showed significant localization of TILs to tumor (ratios of injectate per g in tumor compared to normal tissue were 14.9

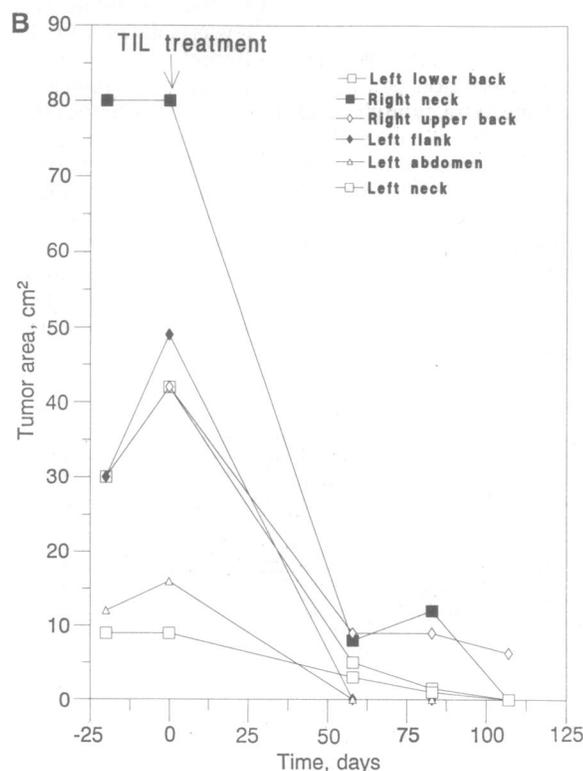
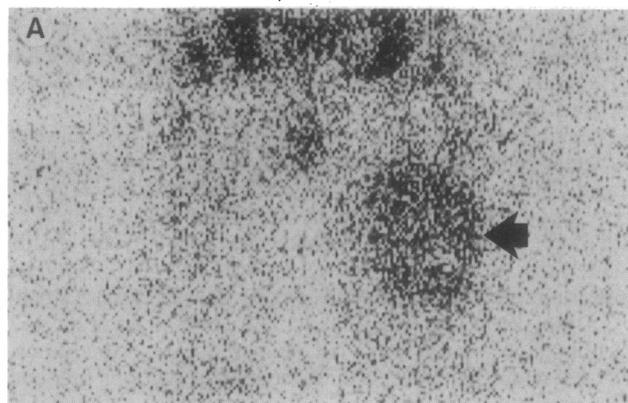


FIG. 1. (A) Radionuclide scan of patient 1200 with metastatic melanoma after receiving the adoptive transfer of autologous ^{111}In -labeled TIL1200. The arrow indicates one of the areas of TIL accumulation corresponding to a metastatic lesion in the left thigh. (B) Regression of s.c. metastatic tumors following treatment with TIL1200 plus IL-2. Treatment began on day 0.

and 14.0, respectively). The patient's cancer regressed rapidly following the first course of treatment. By 3 months after treatment, two of three liver lesions had disappeared and a third lesion shrank by 50%. Multiple s.c. metastases regressed completely as shown in Fig. 1B (the product of perpendicular diameters of individual lesions is shown).

Characterization of *in Vitro* Function of TIL1200. A number of TIL lines established from HLA-A2⁺ melanoma patients lysed melanoma cell lines in a class I MHC-restricted fashion (2) and were shown to release IFN- γ , tumor necrosis factor α , or granulocyte/macrophage colony-stimulating factor when cocultured with the same tumor cell lines (3). A CD8⁺ CTL line, TIL1200, established from a metastatic s.c. tumor mass of patient 1200, lysed fresh autologous melanoma cells as well as 10 of 15 HLA-A2⁺ allogeneic melanoma cell lines but did not lyse 16 of 18 HLA-A2⁻ melanoma cell lines or 6 of 8 HLA-A2⁺ nonmelanoma cell lines (ref. 10; unpublished data). Table 1 shows a cytotoxicity assay against 5 representative HLA-A2⁺ melanoma cell lines that were lysed by TIL1200, 4 representative HLA-A2⁺ melanoma cell lines that were not lysed by TIL1200, and 1 HLA-A2⁻ melanoma cell line. TIL1200 also secreted IFN- γ when cocultured with HLA-A2⁺ normal cultured melanocytes established from neonatal foreskin as well as HLA-A2⁺ melanoma cell lines (Table 2). Therefore, TIL1200 appeared to recognize a non-mutated self-peptide expressed in most melanomas and cultured neonatal melanocytes in an HLA-A2 restricted fashion.

Cloning of the cDNA Coding for a Melanoma Antigen Recognized by T Cells. A cDNA library in λ pCEV27 from the HLA-A2⁺ 501mel melanoma cell line, which was lysed by most HLA-A2 restricted melanoma-specific TILs, was stably transfected into the highly transfectable HLA-A2⁺ melanoma antigen-negative MDA231 clone 7 or A375 clone 1-4. G418-resistant cells were selected and ≈ 6700 individual transfectants from each cell line were isolated and screened based on their ability to stimulate IFN- γ secretion from TIL1200. Six DNA fragments were isolated by PCR using SP6/T7 primers flanking the integrated DNA from four MDA231 and one A375 transfectants that were positive in a second screening and were cloned into the mammalian expression vector pcDNA3.

These fragments in the pcDNA3 vector were transiently expressed in the COS7 cells with or without pcDNA3-HLA-A2.1. Transfection into COS7 of one of the cDNAs tested,

Table 1. Specificity of antigen recognition by TIL1200: Lysis of HLA-A2⁺, gp100⁺ melanoma cell lines

Target	HLA-A2	gp100		% specific lysis	
		FACS	Northern blot	TIL1200	LAK
501mel	+	+	+	46	78
526mel	+	+	+	39	74
624mel	+	+	+	33	76
952mel	+	+	+	25	76
Malme3M	+	+	+	43	70
C32	+	-	-/+*	6	82
RPMI7951	+	-	-	9	67
WM115	+	-	-	5	68
HS695T	+	-	-	3	87
397mel	-	+	+	0	70

A 5-hr ^{51}Cr release assay was performed to measure cellular cytotoxicity at an effector:target ratio of 40:1 as described (9). Expression of HLA-A2 and gp100 recognized by monoclonal antibody HMB45 (Enzo Diagnostics) was measured by flow cytometry [fluorescence-activated cell sorting (FACS)]. Expression of gp100 RNA was analyzed by Northern blot with a cDNA25 probe. LAK, lymphokine-activated killer cells.

*-/+ indicates very weak positive.

Since gp100 was known to be recognized by monoclonal antibody HMB45 (14) the expression of gp100 on COS7 cells transfected with the full-length cDNA25 was evaluated by flow cytometry using this monoclonal antibody. After transient expression of either pCEV27-FL25 or pcDNA3-FL25, COS7 expressed the antigen detected by HMB45 (data not shown).

Expression of RNA for cDNA25. Northern blot analysis was performed with the cDNA25 probe to evaluate the tissue-specific expression of this gene. Ten of 15 melanoma cell lines and 6 of 6 melanocyte cell lines were positive for cDNA25. Of many normal tissues tested, only retina was positive (Fig. 3). Seven cell lines from T-cell (TILA, TILB), B-cell (501EBVB, 836EBVB), and fibroblast (M1) and 20 nonmelanoma tumor cell lines (colon cancer, Colo, SW480, WiDr; breast cancer, MDA231, MCF7, HS578, ZR75; neuroblastoma, SK-N-AS, SK-N-SH; Ewing sarcoma, TC75, RD-ES, 6647; sarcoma, 143B; glioma, U138MG, HS683; renal cell cancer, UOK108, UOK117; small cell lung cancer, H1092; Burkitt lymphoma, Daudi; myeloma, HMY) were all negative for cDNA25 (data not shown). Therefore, this gene appeared to be specifically expressed in melanocyte lineage cells, consistent with the expression pattern of gp100 when analyzed using monoclonal antibody HMB45, NKI/betab, or HMB-50 (14–18). The levels of expression of gp100 RNA in cultured neonatal melanocyte cell lines were significantly lower than that in melanoma cell lines. There was a perfect correlation between gp100 expression tested by Northern blot analysis with cDNA25 and flow cytometry using HMB45 antibody and melanoma lysis by TIL1200 in the 10 HLA-A2⁺ melanoma cell lines as shown in Table 1.

Identification of the Epitope in gp100. Based on a comparison of the amino acid sequence of the truncated form of cDNA25 to known binding motifs of HLA-A2.1 (19–21), 30

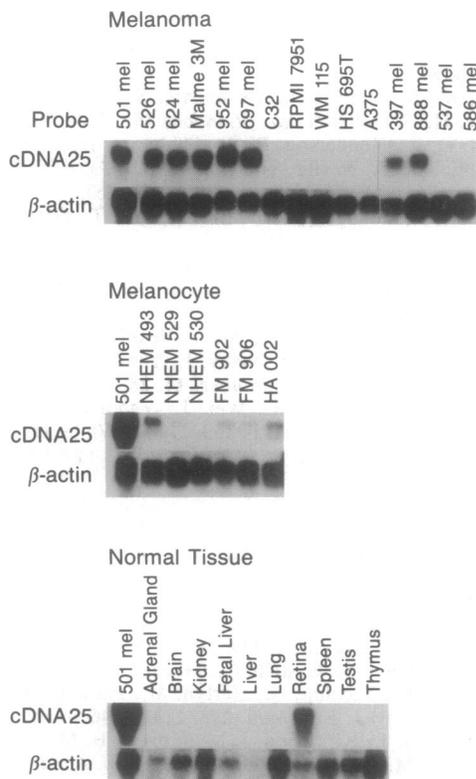


FIG. 3. Northern blot analysis of melanoma and neonatal melanocyte cell lines and various fresh tissues (10–20 μ g of total RNA) with a cDNA25 probe (the *Sal* I-digested fragment of pCRII-cDNA25) and the β -actin probe (Clontech). C32 and 586mel melanoma cell lines and NHEM529 and NHEM530 neonatal melanocyte cell lines were very weak positive.

Table 4. TIL1200 lysis of the T2 HLA-A2⁺ cell line pulsed with the peptide LLDGTATLRL

Target	HLA-A2	Peptide,* μ g/ml	% specific lysis [†]	
			TIL1200	TIL1235 [‡]
501mel	+	0	66	51
397mel	–	0	1	0
T2	+	0	2	1
T2	+	40	28	ND
T2	+	10	32	0
T2	+	1	24	ND
T2	+	0.1	6	ND
T2	+	0.01	0	ND
T2	+	0.001	2	ND

ND, not done.

*TIL1200 lysed T2 cells pulsed with the 10-mer peptide, LLDG-TATLRL (457–466), but not other 29 peptides (residues 273–281, 297–306, 373–381, 399–407, 399–408, 409–418, 456–464, 463–471, 465–473, 476–485, 511–520, 519–528, 544–552, 544–553, 570–579, 576–584, 576–585, 585–593, 592–600, 597–605, 597–606, 602–610, 602–611, 603–611, 605–614, 606–614, 606–615, 619–627, 629–638).

[†]Effector:target ratio of 50:1.

[‡]TIL1235 is an HLA-A2 restricted melanoma-specific CTL that does not recognize gp100.

peptides of 9 or 10 amino acids in length from cDNA25 were synthesized. TIL1200 lysed the HLA-A2⁺ cell line, T2, only when incubated with the peptide LLDGTATLRL (residues 457–486) but not when incubated with the other 29 peptides (Table 4, Fig. 2A). Only peptide LLDGTATLRL was able to also stimulate IFN- γ secretion by TIL1200 (data not shown).

DISCUSSION

Many melanoma-specific CTLs derived from TILs appear to recognize nonmutated self peptides derived from melanocyte/melanoma lineage-specific proteins, since these TILs recognize most melanoma cell lines and normal cultured melanocytes sharing the appropriate restriction element (22, 23). In an attempt to isolate and identify melanoma antigens of possible value in the immunotherapy of melanoma patients, we used TILs, TIL1200, which, when transferred into a patient with metastatic cancer, localized to the tumor site and was associated with a dramatic tumor regression. We have previously shown that, in contrast to nonactivated lymphocytes and lymphokine-activated killer cells, autologous TILs localize to tumor sites. This localization correlated with the ability of these TILs to mediate tumor regression (24). TIL1200, which is a TIL line containing multiple CTL species, recognized a tumor antigen in the context of HLA-A2, which is the most frequently expressed class I MHC antigen (about 50% of individuals) and has been shown to be a dominant restriction element for the induction of melanoma-specific CTLs (25).

By cDNA expression cloning using T-cell recognition for screening, we have isolated a cDNA encoding an antigen recognized by TIL1200 and identified it as gp100, a membrane glycoprotein also recognized by monoclonal antibody HMB45, HMB50, or NKI/betab (14–18). These antibodies are highly specific for melanocyte lineage tissues and strongly stain most melanoma cells. NKI/betab also reacts with adult melanocytes in normal skin (17). Immunoelectron-microscopic studies using either HMB45 or NKI/betab antibody revealed that gp100 was mainly located in a membrane and filamentous matrix of stage I and II melanosomes in the cytoplasm (17, 26). The function of gp100 is unknown. By a completely independent procedure, the cDNA encoding gp100 was also isolated by screening with a rabbit polyclonal antiserum against gp100 (ref. 14; G.J.A., A. J. de Boer, A. M. Vogel, W. A. M. Loenen, and C. G. Figdor, unpublished data) and TIL1200 also lysed HLA-A2⁺ cell lines transfected with this cDNA clone (27).

The existence of T cells reactive to the self-antigen gp100 in tumors and the possible enrichment of these T cells at the tumor site as a possible consequence of the specific accumulation and expansion of antigen-reactive cells (28) raise important questions about the nature of the immune response to self-antigens on growing cancers and about the mechanisms of immunologic tolerance to self-antigens. The increased expression of gp100 on melanoma cells relative to that in melanocytes demonstrated by Northern blot analysis or the unique inflammatory conditions that might exist at the tumor site, which may be associated with the secretion of cytokines and expression of costimulatory molecules on the cell surface, could break tolerance to gp100. Depigmentation has been reported to be associated with a good prognosis (29, 30) and with clinical response to chemoimmunotherapy (31) in melanoma patients. Although we have seen sporadic vitiligo in the patients receiving melanoma-specific TILs we have not observed any adverse ophthalmologic effects that might be related to melanocyte destruction. Patient 1200 did not develop vitiligo or any ophthalmologic side effects. The surface expression of the gp100 peptide in the context of HLA-A2 on adult melanocytes in normal skin and retina remains to be evaluated.

The gp100 protein and the 10-amino acid peptide that we have identified may represent a human tumor rejection antigen since the transfer into patient 1200 of TIL1200 plus IL-2 was associated with cancer regression. The traffic of TIL1200 to tumor deposits *in vivo* and the rapidity of the antitumor response are characteristics of the response to TIL therapy, although IL-2 may also have been involved in the tumor rejection. Adoptive transfer of three other TIL lines that recognized gp100 as well as MART-1 also mediated tumor regression (7).

Tyrosinase (5) and MART-1 (6) have previously been identified as melanoma antigens recognized by HLA-A2 restricted CTLs. Another antigen, MAGE-1, is recognized by HLA-A1 restricted melanoma-specific CTLs and is expressed on a variety of cancer cells as well as testis (4). However, none of the 10 HLA-A2 restricted TILs recently developed in our laboratory appeared to recognize MAGE-1 or tyrosinase (ref. 32; unpublished data). Since the CTLs used to identify MAGE-1 and tyrosinase were derived from the peripheral blood lymphocytes of patients following repetitive *in vivo* or *in vitro* immunization (4, 5), this may be attributed to the different methods used to generate anti-melanoma CTLs.

The wide expression of gp100 in melanomas, the recognition of a peptide by T cells infiltrating into tumor, its restriction by HLA-A2, present in 50% of individuals, and the association of anti-gp100 reactivity with cancer regression in patient 1200 imply that the gp100 antigen may be particularly useful for the development of immunotherapies for patients with melanoma.

We thank O. M. El Badry, R. Halaban, J. Hazen-Martin, M. Herlyn, M. Linehan, F. M. Marincola, J. D. Minna, M. K. Sgagias, D. A. Sens, S. L. Topalian, and M. Tsokos for providing many cell lines, M. C. Custer for technical assistance, A. Mixon and E. B. Fitzgerald for flow cytometric analysis, M. Gonda and D. Huchison for DNA sequencing, and A. B. H. Bakker and C. G. Figdor for stimulating discussion and critical reading of the manuscript.

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