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Cytotoxic T Lymphocyte Reactivity to gp100, MelanA/MART-1, and Tyrosinase, in HLA-A2-Positive Vitiligo Patients

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Vitiligo is a common depigmentation disorder thought to result from autoimmune destruction of melanocytes. Recent studies suggest a role for cell-mediated immune responses to melanocyte differentiation antigens, including gp100, MelanA/MART-1, and tyrosinase, in vitiligo pathogenesis. This study investigated T cell reactivity to MelanA/MART-1, tyrosinase, and gp100, in HLA-A2-positive patients with vitiligo. Melanocyte-specific T cell responses were measured *ex vivo* via enzyme-linked immunospot assay following stimulation with MelanA/MART-1, tyrosinase, and modified gp100 epitopes. Antigen-specific T lymphocyte reactivity to gp100 peptides was seen in 15 of 17 (88%) patients, with many demonstrating very high reactivity at levels comparable with those observed with common recall antigens. Reactivity to gp100 was noted to be associated with disease activity. Antigen-specific T lymphocyte reactivity to MelanA/MART-1 and tyrosinase peptides was not observed *ex vivo* in our patients, and only one patient demonstrated responses to MelanA/MART-1 and tyrosinase peptides following *in vitro* re-stimulation. Our findings implicate T cell reactivity to gp100 in patients with active disease and support the concept of an immunopathologic mechanism in vitiligo, in which cell-mediated responses to normal melanocyte antigens play a crucial part. *Key words: CD8$^+$ T cells/enzyme-linked immunospot assay/melanocyte antigens/melanoma/pigmentary disorders. J Invest Dermatol 121:550–556, 2003*

Vitiligo is a common acquired depigmentation disorder characterized by loss of epidermal melanocytes (Kovacs, 1998). Although the precise etiology of vitiligo is unknown, an autoimmune mechanism has been proposed (Kemp *et al*, 2001). This stems from the fact that many vitiligo patients also exhibit other autoimmune disorders, such as autoimmune thyroiditis, Addison's disease, alopecia areata, pernicious anemia, and type I diabetes (Kemp *et al*, 2001). Further support for this hypothesis is provided by the finding that many vitiligo patients have antibodies to melanocytes (Naughton *et al*, 1983; Bystryn and Naughton, 1985; Song *et al*, 1994; Kemp *et al*, 1998), and the extent of depigmentation is correlated with the incidence and level of anti-melanocyte antibodies (Naughton *et al*, 1983). Moreover, patients may respond to immunosuppressive treatments, such as psoralen with ultraviolet A radiation (Parrish *et al*, 1976), topical corticosteroids (Kumari, 1984), and cytotoxic drugs (Tsuji and Hamada, 1983).

Recent evidence has emerged for a role for cell-mediated immunity in vitiligo pathogenesis. Infiltrating activated T lymphocytes have been observed at the periphery of vitiligo lesions (Badri *et al*, 1993). A recent immunopathologic study of lesional skin of vitiligo patients noted a high frequency of cutaneous lymphocyte antigen-positive activated cytotoxic T cells clustered in perilesional skin in the vicinity of disappearing melanocytes (van den *et al*, 2000). Melanocytes in close proximity to activated lymphocytes focally expressed HLA-DR and intercellular adhesion molecule-1, suggesting a major role for skin-homing T cells in melanocyte death (van den *et al*, 2000). An increased level of soluble interleukin (IL)-2 receptor (Caixia *et al*, 1999; Yeo *et al*, 1999), IL-6, and IL-8 (Yu *et al*, 1997), has been observed in vitiligo patients, which also suggests that T cell activation may be a component in vitiligo pathogenesis. Further evidence for a part played by cytotoxic T cells (CTL) in vitiligo stems from studies of melanoma patients. Vitiligo is found more frequently in patients with metastatic melanoma (Nordlund *et al*, 1983; Bystryn *et al*, 1987; Cui and Bystryn, 1995; Cavallari *et al*, 1996) and is associated with an improved prognosis (Nordlund *et al*, 1983; Bystryn *et al*, 1987). Vitiligo-like depigmentation has been observed following successful immunotherapy of melanoma (Rosenberg and White, 1996; Nestle *et al*, 1998; van Elsas *et al*, 1999; Yee *et al*, 2000), including high-dose IL-2 therapy (Rosenberg *et al*, 1996) and infusions of peptide-pulsed dendritic cells (Nestle *et al*, 1998) and MelanA/MART-1-specific CTL clones (Yee *et al*, 2000). Furthermore, cytotoxic T cells generated from melanoma tissue also recognize differentiation antigens expressed by normal melanocytes (Kawakami *et al*, 2000; Yee *et al*, 2000), suggesting a link between autoimmunity and tumor immunity.

The possibility that melanocyte differentiation antigens represent the molecular targets of a cell-mediated autoimmune response in vitiligo has recently been investigated. These antigens

Manuscript received October 10, 2002; revised February 28, 2003; accepted for publication April 16, 2003

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Abbreviations: ELISPOT, enzyme-linked immunospot; PBMC peripheral blood mononuclear cells; gp100, glycoprotein 100; CTL, cytotoxic T cell.

include melanosomal proteins, such as MelanA/MART-1, tyrosinase, and gp100, involved in biosynthesis of melanin (Norlund *et al*, 1983). Recent studies have noted a specific cellular immune response predominantly directed against the melanosomal protein Melan-A/MART-1 in HLA-A2-positive vitiligo patients (Ogg *et al*, 1998; Lang *et al*, 2001; Palermo *et al*, 2001), where $CD8^+$ T cells displaying MelanA/MART-1-specific reactivity *ex vivo* were demonstrated in the peripheral blood of these patients. In this study we investigated T cell reactivity to MelanA/MART-1, tyrosinase, and gp100, in vitiligo patients, in order to elucidate the role of cell-mediated immunity in disease pathogenesis. Herein we show that T cell reactivity to the modified forms of gp100 epitopes can be readily detected *ex vivo* in vitiligo patients with active disease, suggesting that a component of this cell-mediated autoimmune disease is directed to the melanocyte gp100 protein. We also show that there is an association of anti-gp100 reactivity with the active disease state of individual patients in our cohort of HLA-A2-positive individuals.

MATERIALS AND METHODS

Patients Study subjects consisted of 27 patients with nonsegmental vitiligo each of whom had been seen at the Cosmetic Clinic at the Sunnybrook and Women's College Health Sciences Center Dermatology Unit. Following informed consent, historical data were obtained via direct interview, questionnaire, and chart review. Patients were questioned regarding the progression of their vitiligo as well as their personal and family history of vitiligo and associated autoimmune diseases, including thyroiditis, alopecia areata, pernicious anemia, myasthenia gravis, and type I diabetes. Patients were classified as having progressive disease if they had developed new lesions within the previous 6 mo. A family history was regarded as being positive if there was one or more first, second, or third degree relatives affected. Patients and blood samples were assigned a number to ensure blinding of laboratory investigators to the subjects' clinical characteristics. Immunologic analysis by patient number was completed prior to amalgamation with historical data. This study was approved by the Institutional Review board of Sunnybrook and Women's College Health Sciences Center (SWCHSC), and all patients signed informed consents before giving blood.

HLA-A2 typing HLA-A2-positive patients were identified by flow cytometry using fluorescein isothiocyanate conjugated allele-specific antibody BB7.2 (Parham and Brodsky, 1981). Of the total of 27 patients screened for the study, 18 were found to be HLA-A2 positive and this group constitutes the study population. Eight healthy asymptomatic HLA-A2-positive individuals were also included as a control group.

Lymphocyte preparation Peripheral blood mononuclear cells (PBMC) were isolated from 40 to 50 mL heparinized blood samples by density gradient centrifugation over Ficoll-Hypaque 1.077 (Amersham Pharmacia, Sweden). PBMC were washed with Hank's balanced salt solution (Gibco-BRL Burlington, Ontario, Canada) and frozen in RPMI-1640 medium (Gibco-BRL) containing 20% AB serum (Sigma Oakville, Ontario, Canada) and 10% dimethyl sulfoxide and stored in liquid nitrogen until analysis.

Peptide synthesis The following HLA-A2-restricted peptides were used for stimulation in the ELISPOT assay. For gp100 protein: the modified epitopes 209.2m (IMDQVPSFV) and 280.9v (YLEPGPVTV) (Parkhurst *et al*, 1996); for MelanA/MART-1: AAGIGILTV (Coulie *et al*, 1994); and for tyrosinase: YMDGTMSQV (Skipper *et al*, 1996). Also included were GILGFVFTL (Gotch *et al*, 1987), the dominant epitope from influenza A (Flu) matrix protein (MP), NLVPMVATV (Wills *et al*, 1996) from cytomegalovirus (CMV) p65, and the HIV p17Gag protein derived peptide SLYNTVATL (Parker *et al*, 1992). All peptides were synthesized at Aventis Pasteur (Toronto, Canada), in an automated multiple peptide synthesizer (MPS-396, Advanced ChemTech, Louisville, KY), using standard Fmoc chemistry. The cleavage was performed on-line, using the reagent K TFA/water/thioanisole/phenol/ethanedithiol, 82.5:5:5:5:2.5). The crude peptides were precipitated with diethylethyl ether, washed (3 × diethyl ether), dissolved in 20% acetonitrile and lypholized. The identities of the products were confirmed by ion spray mass spectroscopy.

ELISPOT assay Nitrocellulose 96 well plates (MAHA 45, Millipore, Bedford, Massachusetts) were coated with 100 μL per well of human interferon (IFN)-γ specific antibody 5 μg per mL (clone 1-D1K, MABTECH, Stockholm, Sweden) in coating buffer (0.1 M Na_2HPO_4, pH 9.0) overnight at 4°C. Unbound antibody was removed by washing three times with phosphate-buffered saline. Nonspecific sites on the plates were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Plates were washed three times with phosphate-buffered saline and the appropriate number of responder PBMC were added. Plates were incubated overnight at 37°C, 5% CO_2. Cells were discarded and plates were washed three times with deionized water, and five times with phosphate-buffered saline/Tween. Biotinylated anti-IFN-γ antibody (clone 7-B6-1, MABTECH) was added in 100 μL per well (1 μg per mL). Plates were then incubated for 3.5 h at room temperature. This was followed by the addition of a 1:500 dilution of extra avidin–alkaline phosphatase (Sigma) in 100 μL blocking buffer/Tween added per well and plates were incubated for 1 h at room temperature. The plates were developed using NBT/BCIP phosphatase substrate solution (Sigma) and blue spots were counted using an AID Inc. ELISPOT Image Analyzer and software (Cell Technology Inc. Jessup, MD).

***Ex vivo* ELISPOT assays to detect IFN-γ secretion** PBMC were thawed, washed, and incubated overnight in AIM-V medium (Gibco-BRL) containing 50 μM 2-mercaptoethanol (Sigma) at 37°C in 5% CO_2. For the direct detection of IFN-γ secretion, 1×10^5 PBMC were added directly to the ELISPOT plate with the appropriate peptides listed in the figure legends. Plates were developed 18 h later. To attempt to allow peptide specific T cells to increase in numbers, PBMC were plated in complete AIM-V medium (10% AB serum, Valley Biomedical, [Winchester, VA] 50 μm 2-mercaptoethanol), at $1–2 \times 10^6$ cells per well in a 48 well plate. The next day, 10 μg per mL of peptides were added plus 10 ng per mL recombinant IL-7 (R&D Systems, Minneapolis, Minnesota). Recombinant IL-2 (Chiron, Emeryville, California) was added on days 2 and 6 at 20 IU per mL. Cells were harvested 9 d after stimulation and added to the ELISPOT plate at 1×10^5 cells per well. For each peptide-stimulated group, the same melanocyte-derived peptide was added to wells in triplicate. To control for nonspecific cell culture responses, a known HLA-A2 binding peptide from the HIV GAG protein was added to samples from each group. This served as a control from nonspecific responses. All peptides were added at a final concentration of 10 μg per mL.

Statistical testing The Wilcoxon signed-rank test was used to compare responses between paired data. The Wilcoxon sum-of-ranks test for comparing two unmatched samples was used for testing differences in ELISPOT ratios between different subgroups of study subjects. $p < 0.05$ was considered statistically significant.

RESULTS

Peptide-specific $CD8^+$ T lymphocyte reactivity to gp100 detected by ELISPOT assay in vitiligo patients The *ex vivo* recall response to melanocyte peptide epitopes was measured in vitiligo patient PBMC by stimulating cultures with the HLA-A2-restricted peptides from MelanA/MART-1, tyrosinase, and gp100. To optimize the detection of cell-mediated anti-melanocyte activity to gp100, an admixture of both gp100 modified epitopes were added to the same culture wells. Modification of the gp100 epitopes 209 to 217 and 280 to 288, by substituting amino acids at the primary anchor residues for peptide/major histocompatibility complex binding, has been shown to increase major histocompatibility complex binding and improve CD8 T cell specific responses (Parkhurst *et al*, 1996). Previous reports have shown that the use of the modified gp100 epitopes can reveal CD8 T cell responses in the peripheral blood of patients with vitiligo (Palermo *et al*, 2001) and melanoma (Nielsen *et al*, 2000). We chose to assay for melanocyte-specific immunity using a short-term *ex vivo* assay designed to optimize the detection of cell-mediated anti-melanocyte activity without the need of an *in vitro* re-stimulation step. *In vitro* re-stimulation of PBMC cultures can result in the introduction of nonspecific T cell activation, which can mask peptide specific responses. In addition, culturing PBMC with peptides may drive primary responses instead of measuring true recall responses to previously seen antigen. Measurement of peptide-specific $CD8^+$ T cell activity immediately *ex vivo* may more realistically

reflect the activation state of peptide-specific CTL present in the peripheral blood. As a measure of antigen-specific T cell effector function, secretion of IFN-γ was monitored to determine the frequency of responding $CD8^+$ T cells using the ELISPOT assay. To provide a reference point for the magnitude of the T cell response to melanocyte peptides, patient samples were also screened for peptide-specific responses to a mixture of CMV and Flu peptides representing the immunodominant HLA-A2 epitopes. The admixture of both Flu and CMV epitopes was done to gauge the overall capacity of T cells in the periphery to produce IFN-γ upon peptide stimulation immediately *ex vivo*. We reasoned that as viral infection with either Flu or CMV generates strong recall responses, their dominant epitopes would be good markers to measure IFN-γ produced by antigen-experienced CTL.

To delineate reactivity of antigen-specific $CD8^+$ T cells to melanocyte antigens in vitiligo patients, the number of spots generated by ELISPOT assay to test peptides was compared with the number of spots generated to control peptide for each patient. The control peptide was tested as a measure of overall background reactivity. To quantify reactivity to the melanocyte peptides, a ratio was determined by dividing the number of spots generated to test peptides on ELISPOT assay by the number of spots generated to control peptide. A ratio of more than 1.0 was indicative of reactivity to melanocyte peptides, whereas a ratio of 1.0 or less indicated no peptide reactivity (**Table I**). Significant reactivity to gp100 peptides relative to control peptide was demonstrated in HLA-A2-positive vitiligo patients (median ratio 3.33, $\text{percentile}_{25-75}$ (1.5, 20.0), 95% confidence interval (2.3812, 9.1057); $p = 0.0001$, one-sided signed-rank test (**Table I**). Fifteen of 17 patients (88%) demonstrated reactivity to gp100, with ratios greater than 1.0. Eight of 17 patients had at least a 4-fold greater reactivity above background where the magnitude of the anti-gp100 response approached or surpassed the level seen with the Flu/CMV combination.

Figure 1 depicts melanocyte peptide reactivity in a sample of patients and is representative of the type of results generated in our assay. Reactivity to gp100 was not demonstrated among eight healthy HLA-A2-positive control donor PBMC (**Fig 2**).

Lack of peptide-specific responses to MelanA/MART-1 and tyrosinase In contrast to previous results (Ogg *et al*, 1998; Lang *et al*, 2001; Palermo *et al*, 2001), we were unable to detect significant *ex vivo* reactivity to the MelanA/MART-1 (mean of ratios = 0.82, 95% confidence interval (0.50, 1.13); one-sided signed-rank test, $p = 0.1108$) or tyrosinase (mean of ratios = 0.82, 95% confidence interval for ratio is (0.55, 1.09); one-sided signed-rank test, $p = 0.0858$) peptides (**Fig 1**, **Table I**) among vitiligo patients. Ratios of greater than 1.0 for both MelanA/MART-1 and tyrosinase peptides were seen in 28% (five of 18) of patients, suggesting that some low-level reactivity might be present. In an attempt to enhance the reactivity to MelanA/MART-1 and tyrosinase epitopes, four patients' PBMC were cultured *in vitro* for 9 d with IL-2 in hopes of expanding out low frequencies of responding lymphocytes. As is shown in **Fig 3** and **Table II**, upon re-stimulation only patient 49 exhibited responses to MelanA/MART-1 and tyrosinase, with ratios of 13.58 and 8.67, respectively. In three of the four patients (patients 37, 40, 55), the nonspecific response was equal to or greater than the peptide-specific response. This suggests that some reactivity to MART-1 and tyrosinase may exist but at low levels and in a minority of patients. No reactivity to MelanA/MART-1 or tyrosinase was seen in eight healthy HLA-A2-positive donors (data not shown).

Association of disease activity and T cell reactivity to gp100 peptides We next tested to see if there was a positive association between disease activity and T cell reactivity to melanocyte antigens. Twelve of 18 (67%) HLA-A2-positive vitiligo patients were classified as having "active" disease (i.e., new lesions within the previous 6 mo) at the time of assessment. The remainder of

Table I. Clinical characteristics and melanocyte peptide-specific CTL reactivity *ex vivo* in HLA-$A2^+$ vitiligo patients

	Patient characteristics			Peptide-specific CTL reactivity[a]									
Patient	Sex	Active disease?	Other autoimmune disease?[b]	Family history?	HIV control peptide	Gp100 peptides 209.2m/ 280.9v	Ratio[c] for Gp100	Tyrosinase Peptide	Ratio[c] for tyrosinase	MART-1 peptide	Ratio[c] for MART-1	CMV/Flu peptides	Ratio[c] for CMV/Flu
2	F	No	Yes	No	53	45	0.85	39	0.74	50	0.94	57	1.08
4	M	No	No	No	3	4	1.33	4	1.33	1	0.33	0	0
5	F	Yes	No	Yes	2	3	1.50	1	0.50	1	0.50	0	0
35	M	No	Yes	Yes	3	7	2.33	6	2.00	5	1.67	5	1.67
37	M	No	No	Yes	3	10	3.33	5	1.67	5	1.67	10	3.33
39	F	Yes	No	Yes	5	7	1.40	3	0.60	3	0.60	91	18.2
40	F	Yes	No	Yes	3	9	3.00	4	1.33	5	1.67	6	2.00
42	M	Yes	No	No	16	ND[d]	ND[d]	12	0.75	16	1.00	29	1.81
44	F	Yes	No	Yes	18	90	5.00	8	0.44	18	1.00	163	9.06
45	M	No	Yes	Yes	1	8	8.00	0	0.00	1	1.00	20	20.00
46	F	Yes	No	Yes	2	45	22.50	2	1.00	0	0.00	46	23.00
47	F	Yes	No	Yes	4	80	20.00	1	0.25	2	0.50	2	0.5
48	M	Yes	Yes	Yes	2	64	32.00	1	0.50	0	0.00	1	0.5
49	F	Yes	No	No	27	47	1.74	34	1.26	52	1.93	79	2.93
50	F	Yes	No	Yes	3	67	22.33	1	0.33	1	0.33	89	29.67
53	F	Yes	No	Yes	1	42	42.00	1	1.00	0	0.00	46	46.00
54	F	Yes	No	Yes	14	61	4.36	3	0.21	3	0.21	3	0.21
55	M	No	Yes	Yes	6	6	1.00	5	0.83	8	1.33	4	0.67

[a]Shown is the number of spots seen on ELISPOT assay/1×10^5 PBMC after *ex vivo* stimulation with melanocyte, HIV, and CMV/Flu peptide antigens.
[b]Including thyroiditis, alopecia areata, pernicious anemia, myasthenia gravis, and type I diabetes.
[c]Peptide ratios were derived by dividing the number of spots generated to test peptides on ELISPOT assay by the number of spots generated to HIV control peptide. Note that 15 of 17 patients (88%) had gp100 reactivity ratios of greater than 1.0, and eight of 17 patients (47%) displayed reactivity to gp100 of at least 4-fold above control at levels comparable with those seen with the Flu/CMV combination, corresponding to a high level of CTL reactivity to gp100 in HLA-$A2^+$ vitiligo patients ($p < 0.0001$). In contrast, reactivity ratios of 1.0 to 2.0 to MART-1 and tyrosinase peptides were seen in only five of 18 patients (28%), and no patients displayed ratios of greater than 2.0, corresponding to an absence of CTL reactivity to MART-1 and tyrosinase antigens in our patient population.
[d]Not determined. Reactivity of patient 42 to gp100 peptides could not be determined due to inadequate serum sample; therefore, only reactivity to MART-1 and tyrosinase is depicted for this patient.

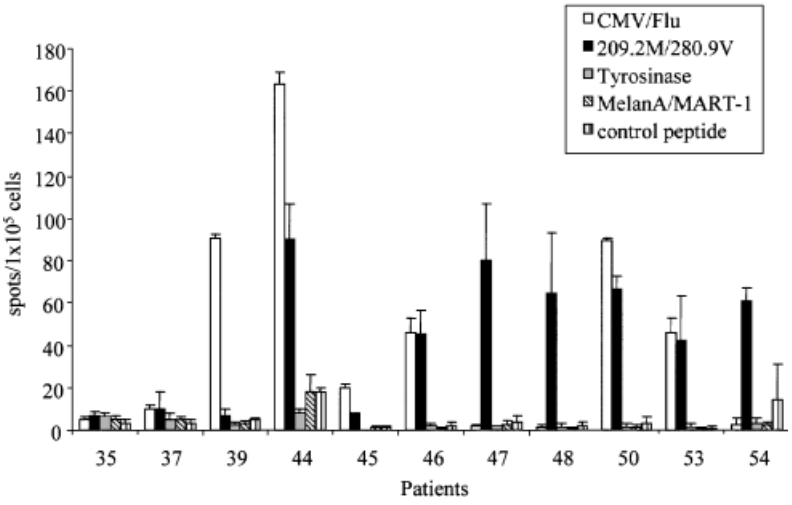

Figure 1. *Ex vivo* ELISPOT assay for IFN-γ specific T cell responses in vitiligo patients. Patient PBMC (1×10^5 per well) were cultured on ELISPOT plates with 10 μg per mL of each peptide overnight and developed the next day. A combination of the peptides from CMV and Flu, combinations of the modified gp100 peptides, 209.2m and 280.9v, or MelanA-MART-1, or tyrosinase, or HIV peptide as a control were added to each of the appropriate wells. Each group was set up in triplicate.

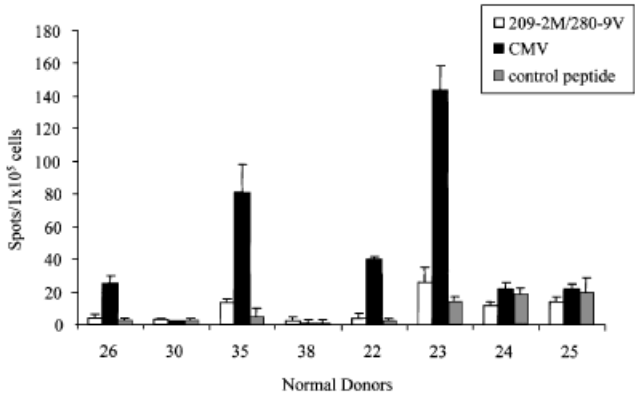

Figure 2. *Ex vivo* gp100 responses in normal donor PBMC. PBMC (1×10^5 per well) from healthy donors were incubated overnight with the combination of the modified gp100 peptides, 209.2m and 280.9v, or CMV peptide or HIV peptide as a control. IFN-γ spots were detected as described in *Materials and Methods*. Each group was set up in triplicate.

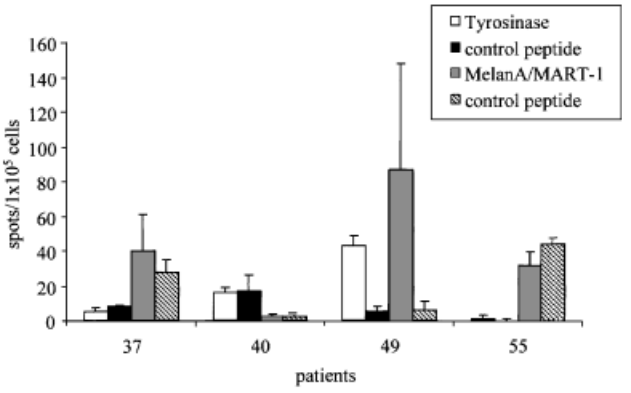

Figure 3. *In vitro* re-stimulation of PBMC from vitiligo patients. PBMC were harvested from bulk cultures at day 9 and reseeded on IFN-γ coated ELISPOT plates at 1×10^5 cells per well. Bulk cultures that have been stimulated with tyrosinase peptide were restimulated in triplicate wells with tyrosinase peptide and HIV peptide as a control. Bulk cultures stimulated with MelanA/MART-1 were restimulated in triplicate wells with MelanA/MART-1 peptide and HIV peptide as a control. Peptides were added at a final concentration of 10 μg per mL to the appropriate wells. Plates were incubated overnight and then developed as described in the *Materials and Methods* section.

patients was classified as "inactive". A positive association was noted between disease activity and T cell reactivity to gp100 peptides (one-sided Wilcoxon rank-sum test, $p = 0.0238$). The significance of this finding is unclear, given that the number of patients with inactive disease was small (six), and two of these patients were noted to have ratios of greater than 3; however, the fact that the p-value was significant despite the small sample size makes the result noteworthy and suggests that this may be a true effect.

Other autoimmune disease and T cell reactivity to melanocyte peptides Five of 18 (28%) HLA-A2 vitiligo patients were classified as having comorbid autoimmune disease at the time of assessment. No association was noted between the presence of comorbid autoimmune disease and T cell reactivity to gp100 (two-sided Wilcoxon rank-sum test, $p = 0.5058$). Similarly, no association was noted between disease activity and T cell reactivity to MelanA/MART-1 (two-sided Wilcoxon rank-sum test, $p = 0.1266$) or tyrosinase (two-sided Wilcoxon rank-sum test, $p = 0.1876$) peptides.

DISCUSSION

In this study we have demonstrated an increase in the frequency of antigen-specific $CD8^+$ T cells present in the circulation of HLA-A2-positive vitiligo patients exhibiting reactivity to the modified gp100 peptide epitopes. This reactivity appears to correlate with the patient's active disease state and further supports the notion that there is a cell-mediated component of disease progression. Reactivity to gp100 was detected *ex vivo* in 88% of study patients and, in some instances, was marked, with T cell IFN-γ response reaching 42 times control, at levels comparable with those seen with the common recall antigens, CMV and Flu. The ability to detect peptide-specific responses *ex vivo* without an *in vitro* culture step more closely reflects a truer measurement of an active recall response to previous encountered antigen. Overall, these findings indicate a clear association between $CD8^+$ T lymphocyte reactivity to melanocyte gp100 and vitiligo, suggesting a pathogenic role for gp100-specific T cells in this disease.

$CD8^+$ T lymphocyte reactivity to MelanA/MART-1 peptide *ex vivo* was not seen in this vitiligo patient population, although 28% of patients did demonstrate low levels of reactivity, with ratios between 1.0 and 2.0. Moreover, significant reactivity to MelanA/MART-1 was seen in only a single patient (patient 49) after *in vitro* re-stimulation. These findings suggest that, whereas

Table II. *In vitro* re-stimulation of HLA-A2$^+$ vitiligo patients' PBMC with MelanA/MART-1 and tyrosinase peptides[a]

	Peptide-specific CTL reactivity					
	Spots/1 × 10^5 PBMC			Spots/1 × 10^5 PBMC		
Patient	MART-1	Control	MART-1 ratio[b]	Tyrosinase	Control	Tyrosinase ratio[b]
37	39.7	28.3	1.4	5.3	7.7	0.7
40	2	1.7	1.2	16.3	17.3	0.94
49[c]	86	6.3	13.58	43.3	5.0	8.67
55	31	44.3	0.7	1.3	0.3	4.3

[a]*In vitro* re-stimulation of PBMC from vitiligo patients. PBMC were cultured for 9 d with either tyrosinase$_{369–377}$ or MelanA/MART$_{127–35}$. Cells were harvested and added to ELISPOT plates with either stimulating peptide or HIV control peptide. Plates were developed the next day.
[b]Ratios were derived by dividing the number of spots generated to test peptides on ELISPOT assay by the number of spots generated to HIV control peptide.
[c]Note the emergence of CTL reactivity in patient 49 to both MART-1 and tyrosinase peptides with *in vitro* restimulation.

reactivity to MelanA/MART-1 may exist in vitiligo patients, it occurs at low levels and in a minority of our cohort of patients. In addition, previous studies have demonstrated that through repeated antigen-specific stimulation *in vitro*, tyrosinase- and MelanA/MART-1-specific CTL may be generated from normal controls (Visseren *et al*, 1995). These CTL have the functional capacity to lyze melanoma target cells (Visseren *et al*, 1995). Our findings stand in contrast with those of previous studies, which have implicated MelanA/MART-1 as an important CTL target in vitiligo and found little evidence of T cell reactivity to gp100 in the disease. Ogg *et al* (1998) demonstrated high frequencies of circulating MelanA/MART-1-specific cytotoxic T lymphocytes in seven of nine HLA-A2-positive individuals with vitiligo using HLA-peptide tetrameric complexes. Palermo *et al* (2001) also used HLA-melanocyte peptide tetramers to stain PBMC *ex vivo* from nine HLA-A2-positive vitiligo patients and noted high numbers of both MelanA/MART-1- and tyrosinase-specific T cells in all patients. In contrast, no gp100-specific T cells were detected *ex vivo* in any patient, and enrichment of gp100 CD8$^+$/tetramer$^+$ T cells was achieved after *in vitro* re-stimulation in cell lines derived from only one of four patients. Lang *et al* (2001) used the ELISPOT assay to identify reactive CD8$^+$ T cells with five peptides from gp100, four peptides from MelanA/MART-1, and two peptides from tyrosinase. In these studies melanocyte-specific reactive CD8$^+$ T lymphocytes were found in 11 of 32 HLA-A2 vitiligo patients (43%). T cells were predominantly directed against MelanA/MART-1 epitopes (34% of patients) with reactivity to gp100 epitopes occurring in only three of 32 (9%) of patients.

A number of factors may account for the discrepancy between our findings and those of the studies previously described. First, with regards to the higher level of reactivity to MelanA/MART-1 noted in prior studies, both Ogg *et al* (1998) and Palermo *et al* (2001) used an analog of the MelanA/MART-1$_{26–35}$ peptide modified to increase binding affinity to HLA-A2 (ELAGIGILTV (Valmori *et al*, 1998)) in constructing tetramers, whereas we used an unmodified epitope AAGIGILTV (Coulie *et al*, 1994). Moreover, Ogg *et al* (1998) included a round of *in vitro* re-stimulation to aid in the expansion of potential peptide-specific CD8 T cells. Lang *et al* (2001) in their study tested both modified and unmodified MelanA/MART-1 peptides. Reactivity to ELAGIGILTV was seen in 34% of patients, whereas AAGIGILTV elicited a response in only two of 32 (6%) of patients, which is in keeping with our findings. The lower level of reactivity to MelanA/MART-1 noted in this study and in Lang *et al* (2001) may relate to the possible presence of nonreactive melanocyte-specific T cells in vitiligo patients. Such cells have been demonstrated in melanoma patients (Lee *et al*, 1999) and would be identifiable with tetramer staining but not in IFN-γ ELISPOT assay. We employed the modified gp100 peptides 209.2m and 280.9v (IMDQVPSFV and YLEPGPVTV, respectively) (Parkhurst *et al*, 1996), to test the CD8$^+$ T lymphocyte response, which may explain the high level of T cell reactivity to gp100 in our study. A recent study (Lang *et al*, 2001) tested reactivity to five unmodified gp100 peptides, which would be expected to generate a lesser T cell response, whereas another study (Palermo *et al*, 2001) used the modified gp100 209.2m peptide and noted only occasional presence of gp100-specific cytotoxic T lymphocytes in vitiligo patients; however, the majority of these patients had stable vitiligo, unlike our study wherein most patients had active disease. In addition, the combination of gp100 peptides in our study may have resulted in additive effects and higher ratios than might be seen with assays involving only single peptides. This is a potential limitation of this study, as it is possible that the inclusion of other identified peptide epitopes from MelanA/MART-1 or tyrosinase may have revealed higher ratios of reactivity; however, given that the majority of gp100 ratios that we derived were many times greater than those found for MelanA/MART-1 and tyrosinase, and given that the overall reactivity to MelanA-MART-1 and tyrosinase was undetectable in most instances, we feel that the observed reactivity to gp100 is a true phenomenon. Finally, a variable response to different epitopes of a given antigen may occur among patients in general, as is seen in previous work (Lang *et al*, 2001). If alternative MelanA/MART-1 and tyrosinase epitopes had been included, we might have found reactivity to these antigens. Taken together, our results and those described using modified epitopes may reveal previous unseen reactivity to defined melanocyte antigens. This raises the possibility of using the modified forms of the dominant T cell epitopes to monitor anti-melanocyte reactivity in melanoma patients receiving antigen-specific vaccines.

CD8$^+$ T lymphocyte reactivity to tyrosinase was not demonstrated *ex vivo* in our patient population and was seen in only one patient following *in vitro* re-stimulation. This is similar to what was reported previously (Ogg *et al*, 1998), in studies that noted no tyrosinase-specific cytotoxic T lymphocytes in nine of nine vitiligo patients using the same epitope of tyrosinase (YMDGTMSQV (Skipper *et al*, 1996)) tetramers. One report (Palermo *et al*, 2001), however, utilizing a different epitope (MLLAVLYCL), found high frequencies of tyrosinase-specific T cells in nine of nine HLA-A2-positive vitiligo patients studied. Studies (Lang *et al*, 2001) that examined two epitopes of tyrosinase (MLLAVLYCL and YMNGTMSQV (Wolfel *et al*, 1994) and noted weak reactivity in one of 32 patients to the former and variable reactivity in four of 32 patients to the latter, again suggesting that the CD8$^+$ T lymphocyte response to tyrosinase is heterogeneous.

We have noted a positive correlation between vitiligo disease activity and reactivity to gp100. The fact that our finding was statistically significant despite the small sample size supports the notion that this is a true effect. Our finding is in keeping with that of Lang *et al* (2001), who evaluated patients who had had progression of vitiligo within the previous 6 mo and who also found a higher level of reactivity among patients with "actively progressive" disease (i.e., 10% or higher increase in vitiliginous lesions in the last 3 mo) than among those with "moderately progressive"

disease (i.e., less increase but signs of progression in the last 6 mo). Moreover, they observed a decrease in T cell reactivity in patients whose disease became stable. Similar results were observed revealing the lowest frequencies of MelanA/MART-1-specific T lymphocytes among patients with stable disease (Ogg *et al*, 1998). The relationship between disease activity and melanocyte-specific T lymphocyte frequency and reactivity lends further support to the hypothesis that these cytotoxic lymphocytes play an important part in causing melanocyte damage in vitiligo.

A family history of the disease was seen in 78% (14 of 18) of our patients, which greatly exceeds the prevalence of 25 to 33% reported in the literature (Majumder *et al*, 1993; Kovacs, 1998). This is interesting in light of the fact that relatives of vitiligo patients have been shown to have an increased frequency of serum autoantibodies and autoimmune reactivity (Mandry *et al*, 1996). It is possible that the high level of reactivity to gp100 noted in this study may relate to the relatively large number of our patients with a positive family history of vitiligo; such patients may represent a subset of vitiligo patients with a higher level of immunoreactivity to certain melanocyte antigens. Another possible explanation for the high prevalence of a positive family history of vitiligo in our patients is our inclusion of second and third degree relatives in the tally. Studies indicate that vitiligo is not transmitted as an autosomal dominant or recessive trait but rather as a polygenic trait (Majumder *et al*, 1993); thus, the inclusion of more distant relatives in the definition of family history is appropriate.

It is noteworthy that 67% (18 of 27) of patients initially screened were HLA-A2 positive. This HLA-A subtype is generally expressed in 35 to 45% of the population (Ellis *et al*, 2000). Whereas our sample size is small and the noted higher prevalence may have occurred due to chance, other studies have also observed an increase in HLA-A2 expression among vitiligo patients (Schallreuter *et al*, 1993; Buc *et al*, 1996). Associations with other HLA class I and II subtypes have also been identified (Kemp *et al*, 2001; Kovacs, 1998), and comparative analysis of published data on a possible association of HLA with vitiligo does not support a definitive linkage thus far (Schallreuter *et al*, 1993); however, the observed overrepresentation of the HLA-A2 haplotype among vitiligo patients suggests that antigen-specific T cell reactivity to HLA-A2-restricted melanocyte epitopes such as gp100 may serve as the predominant mechanism in vitiligo pathogenesis. In this regard, HLA-A2 expression may predispose to autoimmune disorders. It may also confer resistance to certain diseases, as is the case with melanoma, where the anti-melanocyte response of vitiligo is associated with improved prognosis (Nordlund *et al*, 1983; Bystryn *et al*, 1987).

Our findings may have implications for the development of immunotherapies for vitiligo and melanoma. If the dominant epitopes that mediate autoimmune diseases such as vitiligo are identified, it is possible to design therapies to treat this disease with antagonist peptide ligands that could block specific T cell responses. Similarly, induction or augmentation of T cell responses to dominant melanocyte peptide antigens to produce vitiligo might constitute an effective therapy for melanoma. We have demonstrated that by using the modified forms of the immunodominant melanocyte epitopes, responses can be detected that reach levels of reactivity seen with common recall antigens. Previous studies have shown that among melanoma patients receiving immunotherapy, definitive anti-tumor responses occur with high levels of circulating antigen-specific $CD8^+$ T cells (Rosenberg *et al*, 1996; Pittet *et al*, 1999).

In summary, we have found evidence of an association between $CD8^+$ T lymphocyte reactivity to the melanocyte antigen gp100, and to a lesser extent MelanA/MART-1, and vitiligo. Furthermore, we have noted that disease activity appears to correlate with reactivity to gp100. Our findings support the concept of an immunopathologic mechanism in vitiligo, in which cell-mediated responses to normal melanocyte antigens play a crucial part. Further studies are needed to delineate the specificities of CTL and their precise role in vitiligo and to advance our understanding and improve our ability to manage this disease.

We thank Lee Graff, Anita Naumoff, Nina Mandel, Mirka Adamczewski, and the staff of the Cosmedic Clinic at the Department of Dermatology, Sunnybrook and Women's College Health Sciences Center, for their assistance with this project and Bob Uger for critical review of the manuscript. We are also grateful to the vitiligo patients for their generous participation.

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