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IDENTIFICATION OF A NOVEL PEPTIDE DERIVED FROM THE MELANOCYTE-SPECIFIC GP100 ANTIGEN AS THE DOMINANT EPITOPE RECOGNIZED BY AN HLA-A2.1-RESTRICTED ANTI-MELANOMA CTL LINE

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Cytotoxic T lymphocytes (CTL) reactive with human melanoma tumor cells occasionally display cross-reactivity with normal melanocytes. Previously, we identified the melanocyte lineage-specific antigen gp100 that is expressed by both melanoma cells and normal melanocytes, as a target antigen for tumor-infiltrating lymphocytes derived from a melanoma patient (TIL 1200). Here, we demonstrate that the oligoclonal HLA-A2.1-restricted TIL 1200 line is reactive with 2 distinct peptides derived from the gp100 protein. Apart from the peptide corresponding to gp100 amino acids 457–466, we identified the gp100 peptide 154–162 as a second epitope recognized by TIL 1200. A 100-fold lower concentration of this novel gp100 peptide was required for target-cell sensitization compared to peptide 457–466, indicating that the 154–162 peptide is the dominant gp100 epitope for TIL 1200. Together with the recently described gp100 280–288 epitope, 3 distinct CTL epitopes have now been identified in gp100, all presented in the context of HLA-A2.1. Therefore, gp100 is an attractive target antigen in the development of immuno-therapeutic protocols against melanoma.

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CTL that recognize melanoma tumor cells in a T-cell-receptor (TcR)-mediated and MHC-restricted manner have been isolated from tumor-bearing patients (for a review see Knuth *et al.*, 1992). However, the identity of the antigens and epitopes recognized by anti-melanoma CTL remains largely unknown. These CTL often display cross-reactivity with allogeneic HLA-A2.1 matched melanomas, implying that shared melanoma antigens are recognized in the context of HLA-A2.1 (Darrow *et al.*, 1989; Kawakami *et al.*, 1992). Recently, it has been reported that some CTL react not only with melanoma tumor cells but also with normal melanocytes, suggesting recognition of melanocyte differentiation antigens (Anichini *et al.*, 1993).

Using a genetic approach, Boon and colleagues have succeeded in identifying a number of antigens recognized by anti-melanoma CTL. Apart from the tumor-specific MAGE-1 and -3 antigens (Van der Bruggen *et al.*, 1991; Gaugler *et al.*, 1994), they also identified the melanocyte lineage-specific tyrosinase protein as a target for melanoma-specific CTL (Brichard *et al.*, 1993). Similarly, we have identified the gp100 melanocyte-specific protein as a target antigen for melanoma tumor-infiltrating lymphocytes (TIL) (Bakker *et al.*, 1994). Recently, another melanocyte differentiation antigen, Melan A/MART-1, was identified as a target for anti-melanoma CTL (Coulie *et al.*, 1994; Kawakami *et al.*, 1994a). Collectively, these findings demonstrate that melanocyte differentiation antigens can be immunogenic.

The gp100 antigen is an intracellular glycoprotein of approximately 100 kDa and is recognized by MAb NKI-beteb (Venne-goor *et al.*, 1988). Expression of gp100 is restricted to cells of the melanocytic lineage. Recently, we isolated a cDNA clone encoding gp100 (Adema *et al.*, 1993). Characterization of the gp100 cDNA revealed that gp100 is a type-I transmembrane glycoprotein highly homologous to another melanocyte-

specific protein, Pmel17 (Adema *et al.*, 1994). Introduction of the gp100 encoding cDNA into gp100-negative BLM cells conferred susceptibility to lysis by tumor-infiltrating lymphocytes derived from a melanoma patient (TIL 1200) (Bakker *et al.*, 1994). Furthermore, short-term cultures of HLA-A2.1⁺ normal melanocytes were also lysed by TIL 1200, demonstrating that TIL 1200 recognizes the non-mutated gp100 protein.

Recently, it has been demonstrated that a naturally processed gp100 peptide (amino acids 280–288) that was identified by tandem mass spectroscopy, could direct target-cell lysis by HLA-A2.1⁺ restricted anti-melanoma CTL lines (Cox *et al.*, 1994). We have shown that TIL 1200 is reactive with the gp100 peptide 457–466 in the context of HLA-A2.1 (Kawakami *et al.*, 1994b). Here, we report the identification of a novel HLA-A2.1-presented gp100 peptide, corresponding to amino acids 154–162, as the dominant epitope recognized by the oligoclonal TIL 1200 line.

MATERIAL AND METHODS

Cell culture

TIL 1200 lymphocytes were isolated from metastatic melanoma and were grown as was described previously (Kawakami *et al.*, 1992). HLA-A2.1⁺ melanoma lines BLM and BLM gp100 H2.3 were cultured as described previously (Bakker *et al.*, 1994). COS-7 cells were grown in DMEM (GIBCO, Paisley, UK) supplemented with 7.5% FCS. TAP-deficient TxB cell hybrid T2 cells (Salter *et al.*, 1985) were maintained in Iscove's medium (GIBCO) + 7.5% FCS.

Peptides

Peptides were synthesized with a free carboxy-terminus either by f-moc peptide chemistry using an ABIMED Multiple Synthesizer 422 (Abimed, Langenfeld, Germany) or by t-boc chemistry on a Biosearch 9500 peptide synthesizer (New Brunswick, San Rafael, CA). Peptides were dissolved in DMSO and stored at –20°C.

DNA construction and transfection

Isolation of the gp100 cDNA clone c1 has been described elsewhere (Adema *et al.*, 1993). The gp100 cDNA was inserted into expression vectors pBJ1neo (Lin *et al.*, 1990), pCMVneo (Baker *et al.*, 1990) and pSVL (Pharmacia, Woerden, The Netherlands). To generate a gp100 cDNA lacking the coding sequences for the peptide 457–466, PCR reactions were performed with the following combinations of oligonucleo-

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tides: 5'-CATGGAAGTGACTGTCTACC-3'/5'-CTGAGC-GAATTCGGAACCTGTAATACTTTCCG-3' and 5'-CT-GAGCGAATTCGTGAAGAGACAAGTCCCC-3'/5'-TCACAGCATCATATGAGAGTAC-3' using the full-length gp100 cDNA as a template. PCR products were digested with Eco RI, ligated and served as a template for a nested PCR using the following primers: 5'-GCACAGGCCAACTGCAGA-3'/5'-TTCAGTATCTGGTGCAGAAC-3'. The Kpn I-Cla I fragment from this PCR product was then replaced by the corresponding fragment in pCMVgp100neo to generate pCMVgp100DEL454-481neo. Gp100 cDNA mutants DEL149-661 and DEL454-661 were obtained by deletion of the 1.7-kb Hind III and the 0.8-kb Eco RI fragments from pBJ1gp100DEL454-481neo respectively. Gp100 cDNA mutants DEL100-661, DEL194-528 and DEL167-508 were obtained by deletion of the Bgl I-Sac I, Bam HI-Bgl II and Apa I-Nsi I fragments from pSVLgp100 respectively.

BLM cells were transfected with 20 μ g of pCMV-gp100DEL454-481neo DNA according to the calcium phosphate co-precipitation procedure (Graham and van der Eb, 1973) using calcium phosphate transfection systems (BRL, Gaithersburg, MD) and were selected with 1 mg/ml G418 (GIBCO).

COS-7 cells were co-transfected with 5 μ g of pBJ1HLA-A2.1neo and 5 μ g of pBJ1 or pSVL plasmids containing either full-length or deleted gp100 cDNAs using the DEAE-dextran/chloroquine method (Seed and Aruffo, 1987) (the HLA-A2.1 cDNA was kindly provided by J.J. Neefjes, The Netherlands Cancer Institute, Division of Biochemistry, Amsterdam, The Netherlands). After 48 hr of transfection, COS-7 cells were used as stimulator cells in IFN γ release experiments.

Chromium-release assay

Chromium-release assays were performed as described previously (Bakker *et al.*, 1994). Briefly, 10^6 target cells were incubated with 100 μ Ci Na 51 CrO $_4$ (Amersham, Aylesbury, UK) for 1 hr. Various amounts of effector cells were then added to 2×10^3 target cells in triplicate wells of U-bottomed microtiter plates (Costar, Badhoevedorp, The Netherlands) in a final volume of 150 μ l. In peptide recognition assays, target cells were pre-incubated with various amounts of peptide for 90 min at 37°C in a volume of 50 μ l prior to the addition of effector cells. After 5 hr of incubation, part of the supernatant was harvested and its radioactive content was measured.

IFN γ -release assay

TIL 1200 responder cells (10^5) were incubated together with 5×10^4 transiently transfected COS-7 stimulator cells in 300 μ l medium in the presence of 100 U/ml IL-2 in a flat-bottomed 96-well microtiter plate. After 24 hr of incubation, 100 μ l of supernatant were harvested and screened for the presence of IFN γ using an hIFN γ -IRMA immunoradiometric assay kit (Medgenix, Fleurus, Belgium).

HLA-A2.1 stabilization on T2 cells

Peptide-induced HLA-A2.1 stabilization on T2 cells was performed as described previously (Nijman *et al.*, 1993). Briefly, peptides were diluted from DMSO stocks to 50, 25 and 12.5 μ g/ml (final DMSO concentration 0.5%) and incubated together with 10^5 T2 cells for 14 hr at 37°C, 5% CO $_2$ in serum-free Iscove's medium in a volume of 100 μ l in the presence of 3 μ g/ml human β_2 -microglobulin (Sigma, St Louis, MO). Stabilization of HLA-A2.1 molecules at the surface of T2 cells was analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Anti-HLA-A2 MAb BB7.2 (culture supernatant) (Parham and Brodsky, 1981) was used as a primary reagent. FITC-

conjugated GAM-IgG-F(ab') $_2$ (Zymed, San Francisco, CA) was used for the second incubation.

RESULTS

TIL 1200 is reactive with more than one peptide derived from gp100

To determine the nominal gp100 epitope being recognized by TIL 1200, a number of gp100-derived peptides were synthesized according to the HLA-A2.1 binding motifs (Falk *et al.*, 1991; Hunt *et al.*, 1992; Ruppert *et al.*, 1993) and tested for their ability to sensitize target cells (Kawakami *et al.*, 1994b). Only one of these peptides, corresponding to amino acids 457-466, was able to induce target-cell lysis by TIL 1200. However, when this peptide was used in chromium-release assays (Fig. 1), we observed only a low percentage of specific release (20-25%). Furthermore, a relatively high concentration of peptide (~ 100 ng/ml) was needed to induce target-cell lysis by TIL 1200. We therefore questioned whether other gp100-derived peptides could be recognized by TIL 1200, a TIL line that was found to be oligoclonal when analyzed at the T-cell receptor (TcR) level (Shilyanski *et al.*, 1994).

A putative second gp100 peptide which was a candidate for recognition by TIL 1200, was peptide 280-288. This naturally processed peptide was identified using tandem mass spectroscopy and found to be recognized with high affinity by 5 melanoma-specific CTL lines (Cox *et al.*, 1994). We tested this peptide for recognition by TIL 1200 but did not observe any reactivity (data not shown).

To address the question whether TIL 1200 might recognize another peptide derived from gp100, we constructed a mutant gp100 cDNA. The resulting construct, pBJ1-gp100DEL454-481neo, encodes a protein in which the 457-466 peptide is absent. We transfected human HLA-A2.1 $^+$ gp100 $^-$ BLM melanoma cells with pBJ1-gp100DEL454-481neo DNA. Subsequently, G418-resistant BLM gp100 DEL454-481 clones were screened for expression of mutated gp100 using MAb NKI-beteb and tested in chromium-release assays using TIL 1200. As shown in Figure 2, elimination of the 457-466 epitope did

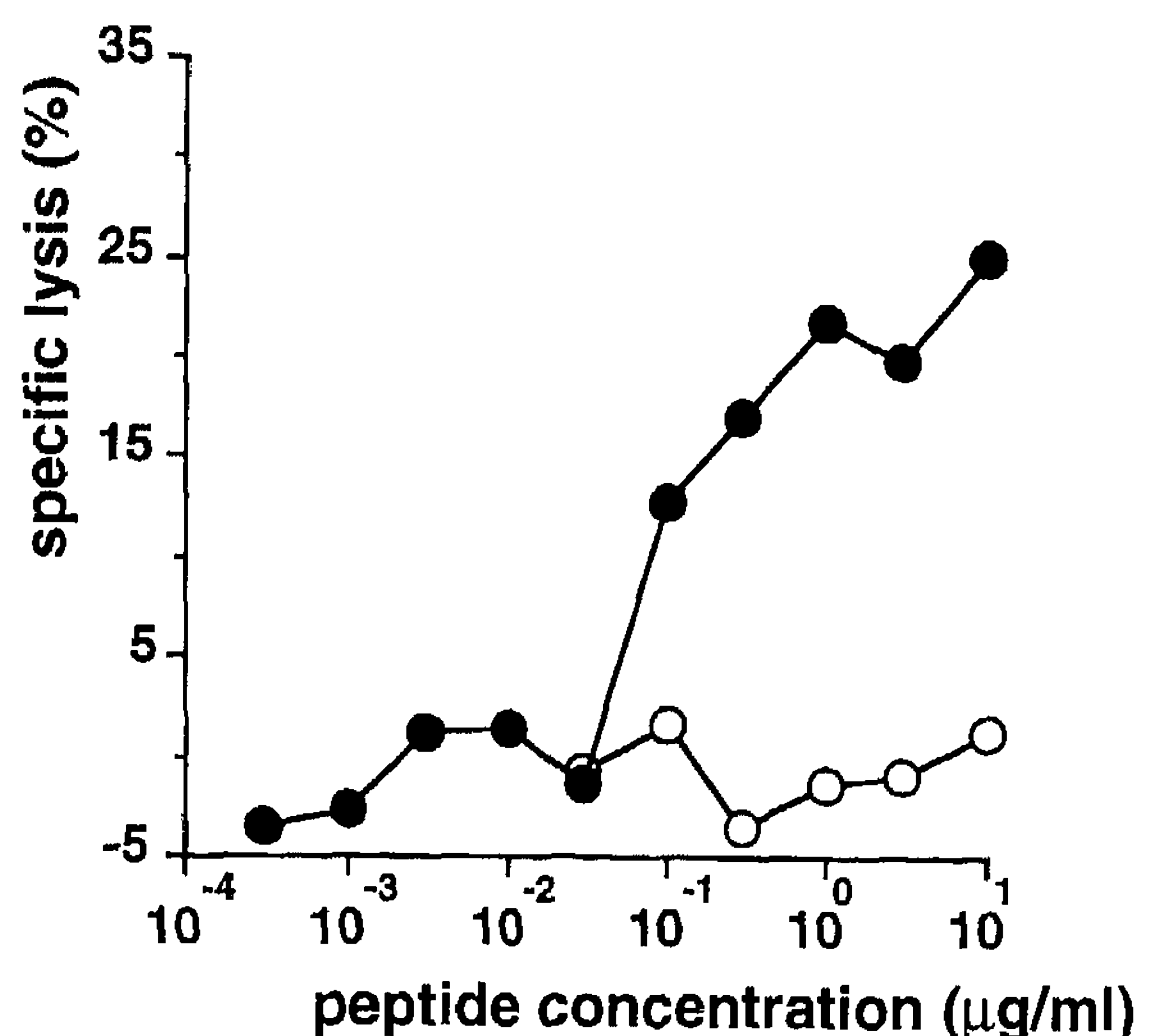


FIGURE 1 - TIL 1200 recognizes the gp100 457-466 peptide. Chromium-labelled T2 target cells were pre-incubated for 90 min with various amounts of peptide. TIL 1200 lymphocytes were added at an effector to target ratio of 30:1. After 5 hr of incubation, chromium release was measured. Peptide 96-105 is an irrelevant gp100-derived peptide that binds to HLA-A2.1. Peptides: ○—○, gp100 96-105; ●—●, gp100 457-466.

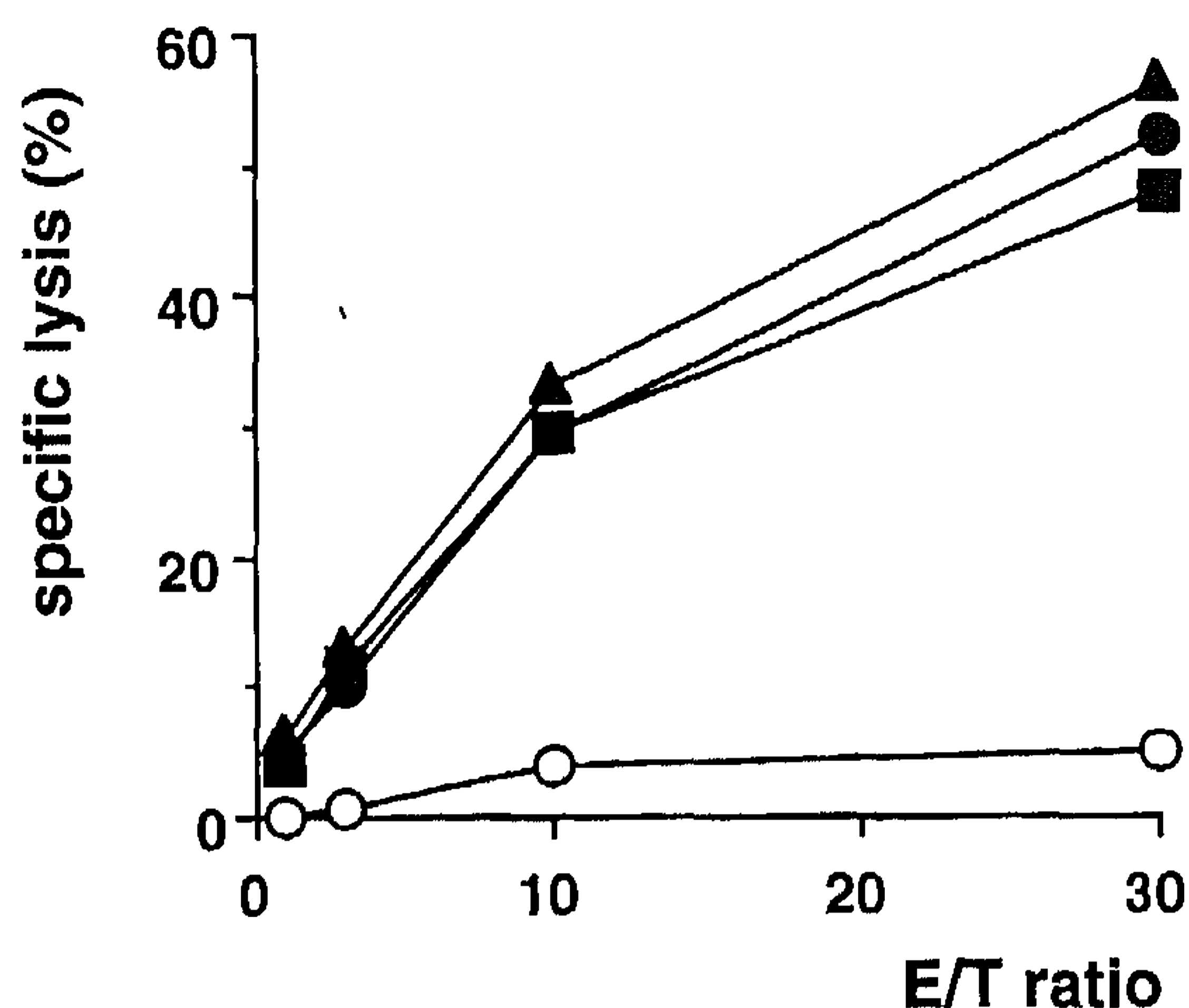


FIGURE 2 – TIL 1200 recognizes more than one epitope derived from gp100. HLA-A2.1⁺ BLM melanoma cells were used as target cells. BLM gp100 transfectants that do (H2.3) or do not express the gp100 457–466 epitope (DEL 454–481 IIA1/ID5) were recognized by TIL 1200. Targets: ○—○, BLM; ●—●, BLM gp100 H2.3; ▲—▲, BLM gp100 DEL454–481 IIA1; ■—■, BLM gp100 DEL454–481 ID5.

not abolish TIL 1200 reactivity; BLM gp100 DEL454–481 clones were efficiently lysed. We therefore conclude that the TIL 1200 line recognizes more than one epitope derived from gp100.

Gp100 peptide 154–162 is the second gp100 epitope recognized by TIL 1200

Subsequently, to define the region of the gp100 protein that contains the unknown second epitope, a number of different gp100 cDNA deletion mutants were generated that all lacked the sequence encoding the 457–466 epitope (Fig. 3). These mutant gp100 cDNAs, cloned into the expression vectors pBJ1-neo or pSVL, were transfected into COS-7 cells together with an expression vector carrying the HLA-A2.1 cDNA. These COS-7 transfectants were then tested for their ability to induce IFN γ secretion by TIL 1200 upon TcR engagement.

As shown in Figure 3, TIL 1200 specifically secreted IFN γ when stimulated with COS-7 cells transfected with HLA-A2.1 and the full-length gp100 cDNA. In accordance with the cytotoxicity data (Fig. 2), we again observed TIL 1200 reactivity against the gp100 DEL454–481 mutant that does not encode the 457–466 epitope. From the other gp100 deletion mutants, only the DEL100–661 and DEL149–661 constructs were not recognized, thereby excluding the possibility that TIL 1200 was reactive with a peptide located N-terminal from amino-acid position 148 in the gp100 protein. We could also exclude the C-terminal region of the gp100 protein, because we observed TIL 1200 reactivity using a mutant construct, DEL454–661, encoding the first 453 amino acids of gp100. The observation that a construct encoding the N-terminal region up to amino acid 166 was able to stimulate TIL 1200 (DEL167–508), demonstrated that a presumptive second epitope was localized between amino acids 148–166 of the gp100 protein.

Several motifs have been described for 9-mer to 10-mer peptides binding to HLA-A2.1, that were deduced from naturally processed and synthetic HLA-A2.1 binding peptides (Falk *et al.*, 1991; Hunt *et al.*, 1992; Ruppert *et al.*, 1993). When these motifs were applied to the 148–166 region of the gp100 protein, no putative HLA-A2.1-binding peptides were found.

We therefore screened the 148–166 region of the gp100 protein for peptides that fitted into a less strict motif, including threonine residues at position 2. Using such a motif, a number of potential HLA-A2.1 binding peptides from the gp100 148–166 region were synthesized. These peptides were loaded onto HLA-A2.1⁺ T2 cells and tested for their ability to induce TIL-1200-mediated target-cell lysis (Fig. 4). The 5 peptides tested were all able to sensitize T2 cells for lysis by TIL 1200 when applied at a concentration of 10 μ g/ml. All these peptides contain the 8-mer peptide TWGQYWQV, corresponding to gp100 amino acids 155–162. Next, all peptides were titrated to evaluate their relative ability to sensitize T2 target cells for lysis by TIL 1200. Figure 4 shows that the 9-mer peptide KTWGQYWQV elicited 50% of maximal specific lysis by TIL 1200 when applied at a concentration of 3 ng/ml, whereas the other peptides had to be applied at higher concentrations. Therefore, the 9-mer 154–162 peptide is most likely the naturally processed second gp100 epitope that is recognized by TIL 1200.

Binding of gp100 epitopes 154–162, 280–288 and 457–466 to HLA-A2.1

We compared the gp100 155–162 and 457–466 epitopes, that are recognized by TIL 1200, to the above-mentioned gp100 280–288 peptide and 2 known viral epitopes presented in HLA-A2.1: the influenza matrix 58–66 peptide (Gotch *et al.*, 1987) and the HIV polymerase 510–518 peptide (Tsomides *et al.*, 1991). We analyzed the HLA-A2.1 binding capacity of the different gp100 epitopes by means of an indirect binding assay using the processing-defective cell line T2 (Nijman *et al.*, 1993). With this assay, we observed similar HLA-A2.1 stabilization with the gp100 280–288 epitope and the tested viral epitopes (Fig. 5). Both the 154–162 and 457–466 gp100 epitopes bind to HLA-A2.1 with a low affinity compared to the gp100 280–288 epitope and the viral epitopes. From these data we conclude that the gp100 epitopes bind to HLA-A2.1 with distinct affinities.

DISCUSSION

In the present report, we describe the identification of a novel gp100 epitope, corresponding to gp100 amino acids 154–162, that is presented to anti-melanoma CTL in the context of HLA-A2.1. We show that the gp100-reactive TIL 1200 line, consisting of several TcR specificities (Shilyanski *et al.*, 1994), recognizes not only the gp100 457–466 epitope, but also the gp100 154–162 epitope. HLA-A2.1 binding experiments revealed that the gp100 154–162 epitope and the gp100 457–466 epitope bind with relatively low affinity to HLA-A2.1, compared to the recently reported gp100 280–288 epitope (Cox *et al.*, 1994) and HLA-A2.1 restricted viral epitopes. This may be partly explained by the amino-acid composition of the gp100 epitopes recognized by TIL 1200. The lower affinity for HLA-A2.1 of peptide 457–466 may be due to the charged residues at positions 3 and 9 (Ruppert *et al.*, 1993). Inefficient binding of the gp100 154–162 epitope to HLA-A2.1 may be caused by the presence of a threonine residue at the N-terminal anchor position since, in most HLA-A2.1 binding peptides, leucine or methionine residues are present at this site. While both peptides bind to HLA-A2.1 with low affinity, a 100-fold lower concentration of gp100 peptide 154–162 was required for the sensitization of T2 target cells for lysis by TIL 1200, when compared to gp100 peptide 457–466. We therefore consider the 154–162 peptide to be the dominant gp100 epitope recognized by TIL 1200. Attempts to further investigate the recognition of the different gp100 epitopes using

gp100 cDNA constructs

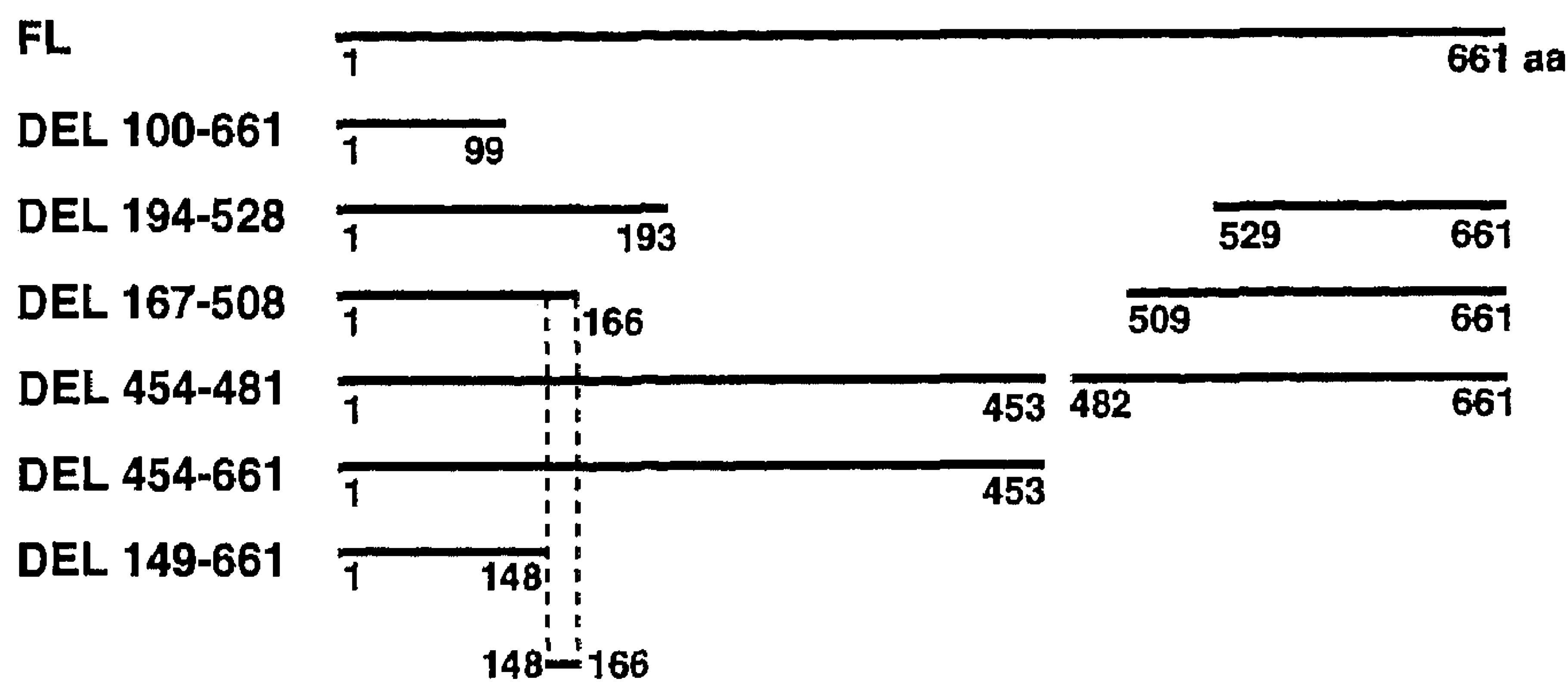
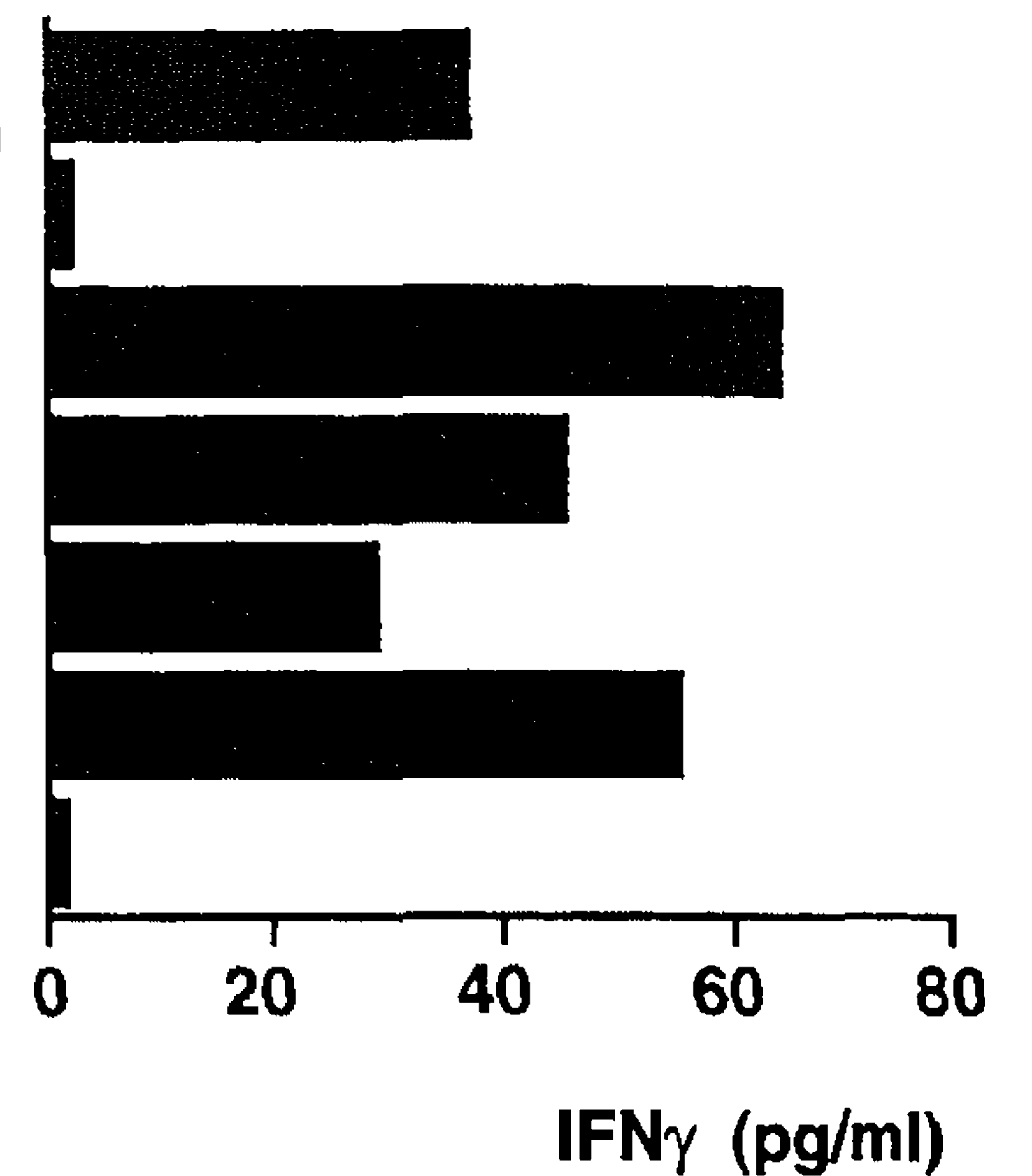
IFN γ released by TIL 1200

FIGURE 3 – TIL 1200 recognizes a second gp100-derived epitope located between amino acids 149 and 166. Gp100 cDNA deletion mutants encoding parts of the gp100 protein were cloned into expression vectors and transfected into COS-7 along with the HLA-A2.1 cDNA (numbers indicate amino acids in the gp100 protein, amino acid 1 refers to the methionine in the signal peptide). TIL 1200 lymphocytes and transfected COS-7 cells were co-incubated for 24 hr and the amount of IFN γ in the supernatant was measured using an immunoradiometric assay.

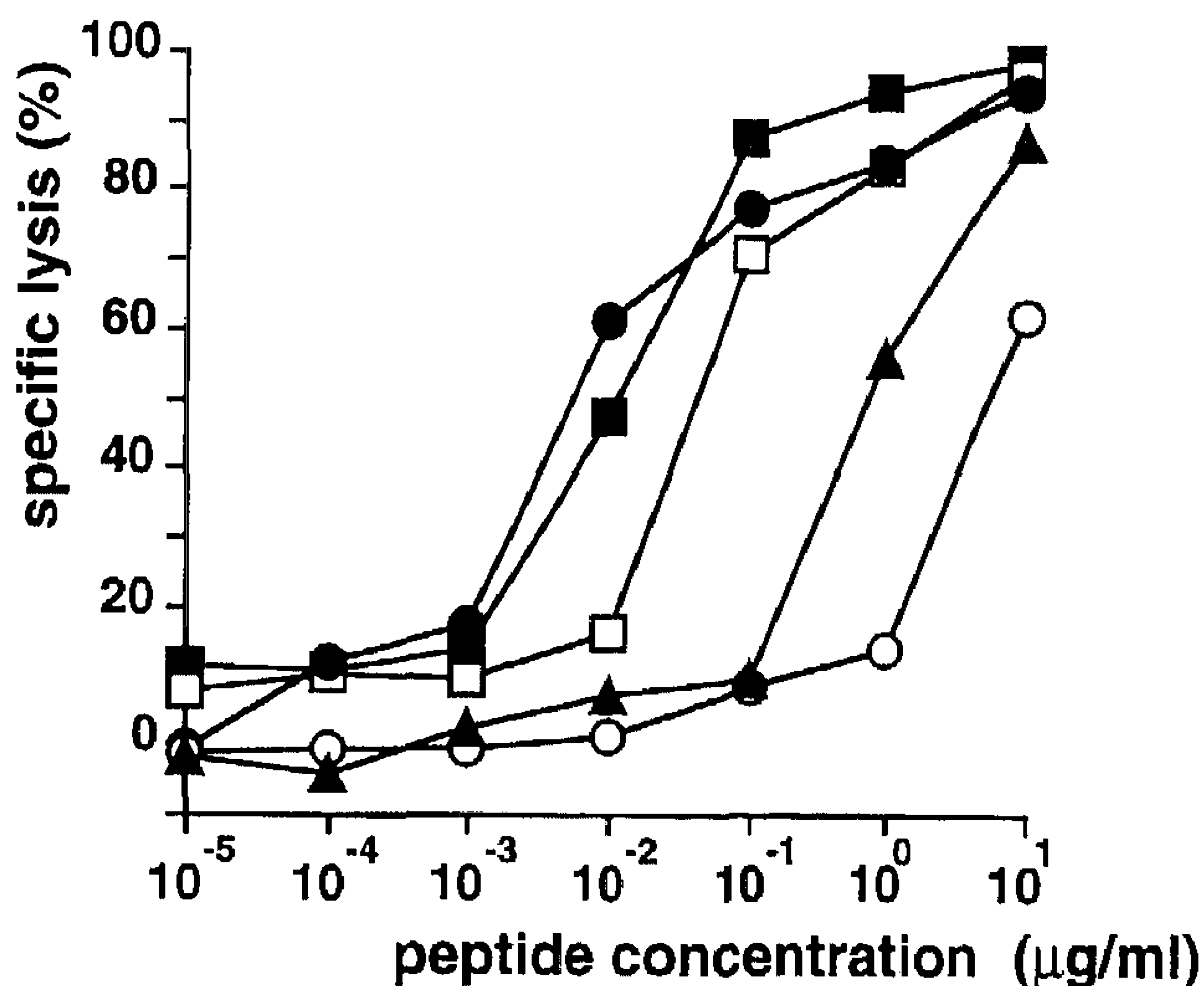


FIGURE 4 – TIL 1200 predominantly recognizes the gp100 154–162 epitope. Chromium release experiments were performed as in Figure 1. Five peptides derived from the gp100 148–166 region, varying from an 8-mer to an 11-mer, were tested for recognition by TIL 1200. T2 target cells were pre-incubated with various concentrations of peptide and specific lysis was determined at an effector to target ratio of 30:1. Peptides: VWKTWGQYWQV, ■—■; KTWGQYWQVL, □—□; KTWGQYWQV, ●—●; TWGQYWQVL, ○—○; TWGQYWQV, ▲—▲.

cloned TIL have not been successful. Since other anti-gp100 CTL-lines (Cox *et al.*, 1994) recognize the gp100 280–288 epitope, it remains to be established which epitope is immunodominant in gp100.

It is surprising that, in a single patient, anti-melanoma CTL have emerged that are reactive with 2 distinct epitopes derived from gp100, a self antigen. The gp100 epitopes are recognized by at least 2 T-cell specificities, co-existing within the TIL 1200 line, that display differences in TcR affinity. T lymphocytes

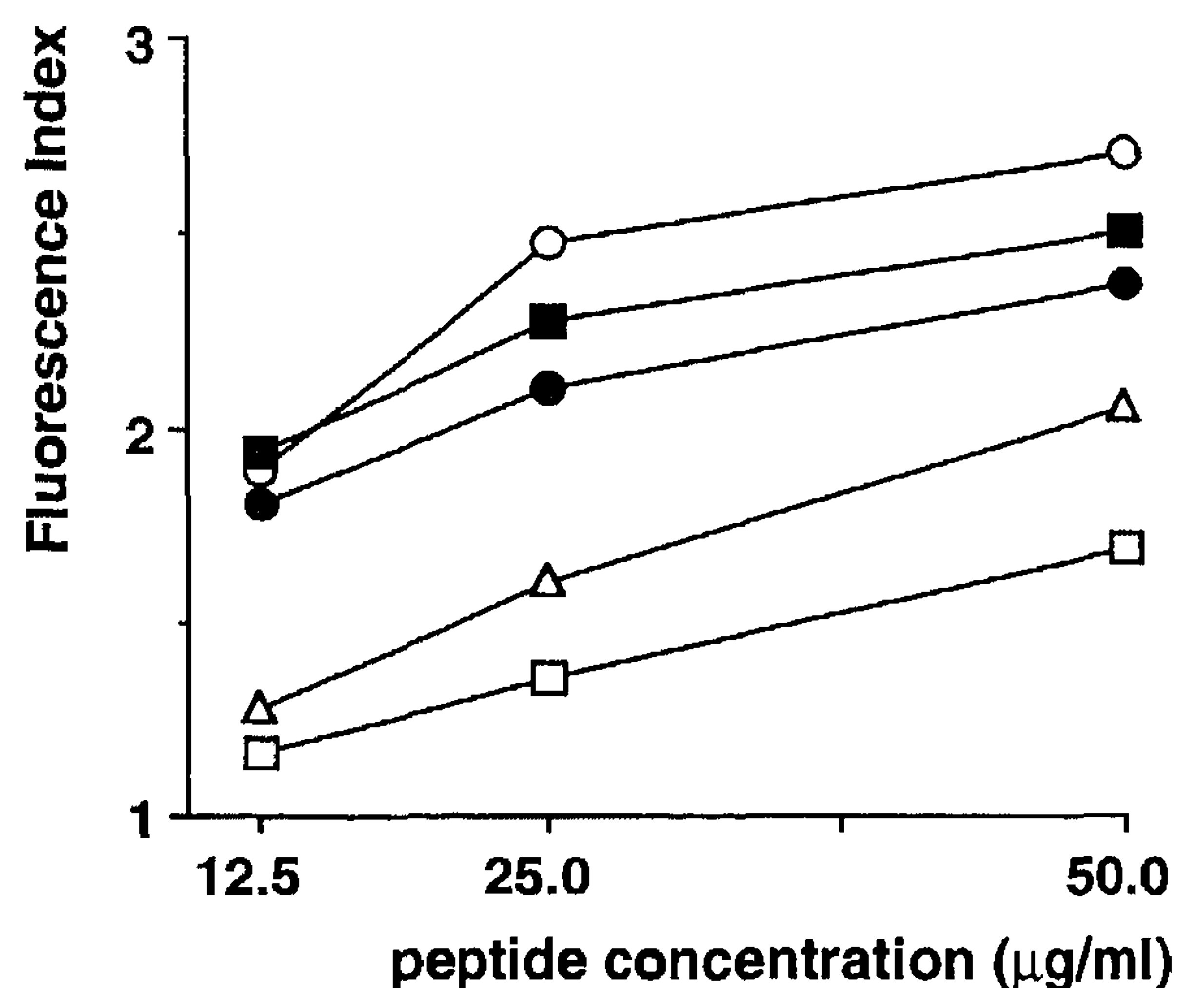


FIGURE 5 – Binding of gp100 epitopes to HLA-A2.1. T2 cells were incubated with various concentrations of the gp100 154–162, 280–288 and 457–466 epitopes or with 2 HLA-A2.1 presentable viral epitopes. HLA-A2.1 stabilization at the cell surface was determined by flow cytometry using MAb BB7.2. The fluorescence index is expressed as the experimental mean fluorescence divided by the mean fluorescence that is obtained when T2 cells are incubated with an HLA-A2.1 non-binding peptide, WMAF-KERKV, at a similar concentration. Peptides: ○—○, INF ma 58–66; ●—●, HIV pol 510–518; □—□, gp100 154–162; ■—■, gp100 280–288; △—△, gp100 457–466.

bearing potential self-reactive TcR can emerge in the periphery. They may have escaped from tolerance induction because they bear low-affinity TcR, whereas potential autoreactive CTL bearing high-affinity TcR are probably deleted in the thymus (Schwartz, 1989) or may be tolerized in the periphery. The anti-tyrosinase CTL clones described by Wölfel and colleagues and the gp100 457–466-reactive TIL 1200 lymphocytes, exhibiting low peptide/MHC affinity, may have emerged from precursor T lymphocytes of this type. High-affinity

autoreactive CTL may have escaped tolerance induction, because they have not encountered their antigenic peptide in the thymus or in the periphery. It is not clear how high- or intermediate-affinity CTL, like gp100 280–288-reactive T lymphocytes (Cox *et al.*, 1994) or gp100 154–162-reactive TIL 1200 lymphocytes, can exist in the periphery as ignorant T lymphocytes without causing apparent autoimmune disease (Ohashi, 1994). A possible explanation is that HLA-A2.1-gp100 154–162/280–288 complexes on normal melanocytes may be recognized by activated CTL, while being incapable of initiating primary immune responses *in vivo*. Apparently, in the case of melanoma, gp100-reactive precursor T lymphocytes can be triggered *in vivo*. This may be the result of a higher expression level of the gp100 antigen in melanoma cells. Alternatively, necrosis in melanoma tumors may lead to uptake and presentation of gp100-derived peptides to T lymphocytes by professional antigen-presenting cells.

Clinical data, indicating that vitiligo (local depigmentation of the skin) is associated with improved survival of melanoma patients (Bystryn *et al.*, 1987), suggest that these patients

benefit from an autoimmune response to melanocytes. In addition, re-infusion of TIL 1200 plus IL-2 into the autologous patient was accompanied by regression of metastatic tumor (Kawakami *et al.*, 1994b). This suggests that the generation of an immune response against the gp100 melanocyte differentiation antigen may have anti-tumor effects *in vivo*. Therefore, the gp100 antigen, of which 3 distinct HLA-A2.1 presentable epitopes have already been identified, is an attractive target antigen in the development of immuno-therapeutic protocols against melanoma.

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