An Antigen-targeted Approach to Adoptive Transfer Therapy of Cancer

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ABSTRACT

Previous attempts to treat human malignancies by adoptive transfer of tumor-specific CTLs have been limited by the difficulty of isolating T cells of defined antigen specificity. The recent development of MHC class I/antigenic peptide tetrameric complexes that allow direct identification of antigen-specific T cells has opened new possibilities for the isolation and in vitro expansion of tumor-specific T cells. In the present study, we have derived polyclonal monospecific cell lines from circulating Melan-A-specific CTL precursors of HLA-A*0201 melanoma patients by combining stimulation with recently identified peptide analogues of the immunodominant epitope from the melanoma-associated antigen Melan-A with staining with fluorescent HLA-A*0201/Melan-A peptide tetramers. In vitro expansion of antigen-specific CD8+ T cells was monitored by flow cytometry with the fluorescent tetramers and anti-CD8 monoclonal antibody. This analysis revealed that Melan-A 26–35 peptide analogues were much more efficient than the parental peptides in stimulating a rapid in vitro expansion of antigen-specific CD8+ T cells. These cells were then isolated by tetramer-guided cell sorting and subsequently expanded in vitro by mitogen stimulation. The resulting polyclonal but monospecific CTLs fully cross-recognized the parental peptides and were able to efficiently lyse Melan-A-expressing tumor cells. Altogether, these results pave the way to a molecularly defined approach to antigen-specific adoptive transfer therapy of cancer.

INTRODUCTION

The identification of MHC class I-restricted CTL epitopes derived from melanoma-associated antigens has opened new possibilities for antigen-specific immunotherapy of melanoma (1, 2). Different strategies can be used, including direct immunization with synthetic peptides corresponding to defined melanoma-derived MHC class I-restricted antigens or adoptive transfer of in vitro expanded CTLs. On the basis of studies on animal tumor models, the latter approach might be a more realistic goal in the treatment of large metastatic tumors. Indeed, this form of immunotherapy has been most successful in the eradication of large established tumors, whereas vaccination has only been efficient in preventing outgrowth of subsequently inoculated tumors (3–7).

In humans, adoptive transfer trials have been performed with large numbers of in vitro expanded autologous TILs in metastatic melanoma or other advanced malignancies (8, 9). Despite some encouraging results, a rigorous evaluation of the therapeutic activity of the transferred T lymphocytes has been hampered by the difficulty of isolating and expanding in vitro tumor-reactive T cells of defined antigen specificity. In addition, TILs are available only for a minority of patients, thus greatly limiting the number of patients who could benefit from this therapeutic approach.

We have recently synthesized fluorescent HLA-A*0201/peptide tetramers (10) containing a modified Melan-A 26–35 peptide named A27L. A27L is an analogue of the Melan-A 26–35 epitope carrying a substitution of Ala for Leu at position 2 from the NH2 terminus. We have demonstrated previously that this modified antigenic peptide forms relatively stable complexes with HLA-A2 and is a more potent immunogen than the natural Melan-A peptide (11). In addition, we have shown that tetramers synthesized around Melan-A A27L modified peptide or the natural Melan-A peptide are interchangeable for staining Melan-A-specific T cells.

In the present study, we have used HLA-A*0201/A27L tetramers (A2/Melan-A tetramers thereafter) to monitor the magnitude of the in vitro expansion of Melan-A-specific CTL precursors from PBMCs of melanoma patients upon stimulation with autologous antigen-presenting cells pulsed with parental or modified Melan-A peptides as well as to purify them by cell sorting. Our results show that Melan-A-specific lymphocytes are readily detected by A2/Melan-A tetramer staining in PBMCs from the majority of HLA-A*0201 melanoma patients. By monitoring Melan-A-specific cells with fluorescent tetramers, we observed that Melan-A-specific precursors can be expanded in vitro by restimulation with autologous PBMCs pulsed with parental Melan-A peptide. However, a much more efficient in vitro expansion was obtained by using Melan-A peptide analogues. The frequency of A2/Melan-A tetramer+ T cells detected in bulk cultures directly correlated with peptide-specific cytotoxicity measured by conventional chromium release assay. A2/Melan-A tetramer+ cells were purified by cell sorting and further expanded by mitogen stimulation. The resulting polyclonal monospecific cell lines were fully cross-reactive with the parental peptide and efficiently lysed Melan-A-expressing tumors. Overall, these results illustrate a novel strategy to rapidly generate large numbers of antigen-specific and tumoricidal CTLs from PBMCs of HLA-A*0201 melanoma patients.

MATERIALS AND METHODS

Synthetic Peptides. Peptides were synthesized by standard solid phase chemistry on a multiple peptide synthesizer (Applied Biosystems, Foster City, CA) by using F-moc for transient NH2-terminal protection and were analyzed by mass spectrometry. All peptides were $>$90% pure as indicated by analytical high-performance liquid chromatography. Lyophilized peptides were diluted in DMSO and stored at −20°C.

Cytokines. Human rIL-2 (Glaxo, Geneva, Switzerland) was kindly provided by Dr. M. Nabholz (ISREC, Epalinges, Switzerland), and human IL-7 was donated by Dr. N. Vita of Sanofi Recherche (Lambéz, France). One unit/ml of IL-2 is defined as the concentration that gives 50% maximal proliferation of CTLL-2.

Cells. Tumor cell lines and T2 cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, 0.55 mM Arg, 0.24 mM Asn, and 1.5 mM Gln. Melanoma cell line Me 290 (HLA-A*0201+ and Melan-A+) was established at the Ludwig Institute for Cancer Research, Lausanne Branch, from a surgically excised melanoma metastasis from patient LAU 203. HLA-A2 antigen expression and Melan-A antigen expression were assessed by fluorescence-activated cell sorting analysis and

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The abbreviations used are: TIL, tumor-infiltrating lymphocyte; PBMC, peripheral blood mononuclear cell; IL, interleukin; mAb, monoclonal antibody; CMV, cytomegalovirus.
Western blot analysis with BB7.2 mAb (HLA-A2 specific; Ref. 11) and A103 mAb (12), respectively. T2 cells are HLA-A*0201 human lymphoid cells that are defective in antigen processing but effectively present exogenously supplied peptides (13). peptide-specific CTLs were generated as described previously (11) with minor modification. Briefly, PBMCs from HLA-A*0201+ melanoma patients were isolated by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMCs were plated at 10 × 10^6 cells/well in 2 ml of Iscove’s medium supplemented with 10% human serum, Asp, Arg and Gln (complete medium) in the presence of IL-7 (10 ng/ml) and IL-2 (10 units/ml). Cells were initially stimulated by adding 1 μM of peptide directly into the culture medium. Thereafter, cultures were stimulated weekly with autologous PBMCs (8 × 10^6/well) pulsed during 2 h at 37°C in serum-free medium (X-VIVO 10; BioWhittaker) with the appropriate peptide (1 μM) and human β2 microglobulin (3 μg/ml). peptide-pulsed PBMCs were then extensively washed, irradiated (3000 rad), and adjusted to the appropriate volume before addition to the responder cell population. IL-2 (10 units/ml) and IL-7 (10 ng/ml) were added during the first two stimulation cycles and IL-2 alone (10 ng/ml) thereafter.

Tetramers. Complexes were synthesized as described (10, 14). Briefly, purified HLA heavy chain and β2-microglobulin were synthesized by means of a prokaryotic expression system (pET; R&D Systems, Inc., Minneapolis, MN). The heavy chain was modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a peptide sequence containing the BirA enzymatic biotinylation site. Heavy chain, β2-microglobulin, and peptide were refolded by dilution. The Mf, 45,000 refolded protein was isolated by fast protein liquid chromatography and then biotinylated by recombinant BirA (Avidi, Denver, CO) in the presence of biotin, ATP, and Mg2+ (all from Sigma Chemical, St. Louis, MO). Streptavidin-phycoerythrin conjugate (Sigma) was added in 1:4 molar ratio, and the tetrameric product was concentrated to 1 mg/ml.

Abs and Flow Cytometry Immunofluorescence Analysis. Anti-CD8 FITC was purchased from Becton Dickinson. Cells were stained with tetramers in 20 μl of PBS 2% FCS during 15 min at 4°C, then 20 μl of anti-CD8 FITC mAbs were added and incubated for an additional 30 min at 4°C. Cells were washed once in the same buffer and analyzed by flow cytometry. Data analysis was performed using Cell Quest ™ software. When indicated, CD8+ lymphocytes were enriched from PBMCs by positive selection by magnetic cell sorting using a miniMACS device (Miltenyi Biotec GmbH, Sunnyvale, CA). The resulting cells, which were >99% CD3+ CD8+, were stained for flow cytometry analysis as described above.

Chromium Release Assay. Antigen recognition was assessed using target cells (T2 or melanoma) labeled with 51Cr for 1 h at 37°C and washed twice. Labeled target cells (1000 cells in 50 μl) were then added to varying numbers of effector cells (50 μl) in V-bottomed microc Wells in the presence or absence of 1 μg/ml of the antigenic peptide (50 μl). The effector cells were preincubated for at least 20 min at 37°C in the presence of unlabeled K562 cells (50,000/well) to eliminate nonspecific lysis due to natural killer-like effectors present in stimulated T-cell populations. In the peptide titration experiments, target cells (1000 cells in 50 μl) were incubated in the presence of various concentrations of peptide (50 μl) for 15 min at room temperature before the addition of effector cells. Chromium release was measured after incubation for 4 h at 37°C. The percentage of specific lysis was calculated as:

% specific lysis = \[ \frac{\text{Experimental} - \text{spontaneous release}}{\text{Total} - \text{spontaneous release}} \times 100 \]

The concentration of each peptide required to achieve 50% maximal lysis of target cells was then determined and indicated as [μM] 50%. To facilitate comparison, the relative activity of each peptide was calculated as the [μM] 50% of the parental Melan-A nonapeptide AAGIGILTV divided by the [μM] 50% of the tested peptide. The correlation between percentage of specific cytotoxicity and frequency of CD8+ A2/Melan-A tetramer+ lymphocytes was assessed by the linear regression test.

RESULTS

Direct Assessment of in Vitro Expansion of Melan-A-specific Precursors from PBMCs of Melanoma Patients. The frequency of A2/Melan-A tetramer+ T lymphocytes was measured by two-color flow cytometry analysis of CD8+–enriched peripheral blood lymphocytes from seven melanoma patients. For each patient tested, A2/Melan-A tetramer+ T lymphocytes were detected selectively within the CD8+ bright population. The frequency of positive cells varied between 0.03 and 0.09% of CD8+ cells (LAU 50, LAU 56, and LAU 203, see Fig. 1A; LAU 240, LAU 267, LAU 132, and LAU 97: 0.04%, 0.03%, 0.07%, and 0.09%, respectively; data not shown). We have recently documented the functional specificity of A2/Melan-A tetramer+ cells found at relatively high proportions in tumor-infiltrated lymph nodes (10). Because the frequencies of positive cells found in peripheral blood were close to the detection limit of the flow cytometer, it was essential to further document the specificity and the proliferation potential of A2/Melan-A tetramer+ cells.

To this end, PBMCs from three selected melanoma patients were stimulated three times at weekly intervals with the parental Melan-A 26–35 decapeptide or the A27L analogue using autologous PBMCs as a source of antigen-presenting cells. On day 7 after each stimulation cycle, cultures were monitored for the presence of CD8+ A2/Melan-A tetramer+ cells. The number of CD8+ A2/Melan-A tetramer+ lymphocytes relative to the total number of CD8+ T cells detected in each culture and the percentage of CD8+ T cells at the end of each restimulation cycle are reported in Fig. 1B. During the first week of stimulation, the total cell number consistently declined, then moderately increased after the second stimulation cycle, and significantly increased after the third stimulation cycle (data not shown). Stimulation with the Melan-A peptide analogue A27L resulted in a more vigorous expansion of CD8+ A2/Melan-A tetramer+ cells as compared with the parental peptide. The difference between the stimulatory capacity of parental and modified peptides was already apparent after the first stimulation cycle and became very marked after the third cycle. For example, in patient LAU 203, as many as 44% of CD8+ T cells resulting from stimulation with the peptide analogue were also A2/Melan-A tetramer+ in contrast to only 0.78% in cultures stimulated with the parental Melan-A peptide. Dramatic A2/Melan-A tetramer+ lymphocyte expansion occurred during the third stimulation cycle with the peptide analogue in parallel with the increase in total cell growth. An exception was patient LAU 50, for whom no significant increase of A2/Melan-A tetramer+ lymphocytes was apparent at the third stimulation cycle in cultures stimulated with the peptide analogue A27L, despite a significant increase in total cell number. T-cell unresponsiveness in vitro can be due in certain cases to activation in vivo (15). However, this is unlikely the case for patient LAU 50, because his A2/Melan-A tetramer+ precursors exhibited a naive CD45RAhigh CD28bright phenotype (data not shown).

Cultures stimulated with either the parental peptide or the peptide analogue were tested on day 7 after the third stimulation cycle for their capacity to lyse T2 cells in the absence or presence of each of the two peptides (Fig. 2). The frequency of A2/Melan-A tetramer+ T cells detected in the cultures directly correlated with peptide-specific cytotoxicity measured by chromium release (P < 0.01). In addition, CTLs generated upon stimulation with the peptide analogue A27L cross-recognized the parental decapeptide.

A2/Melan-A Tetramer-guided Isolation and Functional Characterization of in Vitro-stimulated Melan-A-specific T Cells. To further document the antigen specificity of CD8+ A2/Melan-A tetramer+ lymphocytes generated upon stimulation with Melan-A peptides, PBMCs from melanoma patient LAU 203 were stimulated as described above with autologous PBMCs pulsed with either an irrelevant peptide (corresponding to the immunodominant HLA-A2 restricted epitope from influenza matrix protein), the parental peptide Melan-A 27–35, the parental peptide Melan-A 26–35, or various Melan-A 26–35 peptide analogues. In addition to the peptide analogue A27L, the latter included two recently identified analogues.
Peptide E26A is an analogue of Melan-A 26–35 epitope carrying a substitution of Ala for Glu at position 1, whereas peptide E26A/A27L contains both substitutions. These new peptide variants share characteristics similar to those of peptide A27L including improved HLA-A2 binding, improved stability of HLA-A2/peptide complexes, and increased efficiency of recognition by a large fraction of Melan-A-specific T cells (Refs. 11 and 16 and data not shown). As described above, cultures were monitored at day 7 of each stimulation cycle for the presence of CD8$^+$ A2/Melan-A tetramer$^+$ cells (Fig. 3).

The small number of A2/Melan-A tetramer$^+$ cells detected at the end of the first stimulation cycle in PBMC cultures stimulated with the irrelevant influenza peptide was equivalent to the number of A2/Melan-A tetramer$^+$ cells detected in uncultured PBMCs and declined after an additional round of stimulation with the influenza peptide. Expansion of CD8$^+$ A2/Melan-A tetramer$^+$ lymphocytes did occur upon stimulation with both parental peptides. This demonstrates directly the ability of both parental peptides to trigger the expansion in vitro of Melan-A-specific T cells present in peripheral blood. Moreover, their stimulatory capacity appears to be similar. Nonetheless, the increase in the proportion of CD8$^+$ A2/Melan-A tetramer$^+$ lymphocytes was much more pronounced in the cultures stimulated with the decapeptide analogues. The largest expansion occurred upon stimulation with the peptide E26A/A27L. Antigen-specific cytotoxicity was assayed on day 7 after the third stimulation cycle (Fig. 4). The frequency of A2/Melan-A tetramer$^+$ cells directly correlated with the level of specific cytotoxicity ($P < 0.01$), and importantly, the CTLs resulting from decapeptide analogue-driven expansion efficiently lysed target cells sensitized with the parental peptide.

Mitogen-driven Expansion of Sorted A2/Melan-A Tetramer$^+$ Lymphocytes. The ability to directly visualize antigen-specific T lymphocytes with fluorescent tetramers opens the possibility to separately them from the rest of the bulk cultures at the early stages of in vitro stimulation with peptide and to continue their expansion in an antigen-independent fashion. To test the feasibility of this approach, CD8$^+$ A2/Melan-A tetramer$^+$ lymphocytes were isolated from each culture, by flow cytometry sorting, on day 7 after the second cycle of in vitro stimulation. Sorted cells were then expanded in vitro by mitogen stimulation by phytohemagglutinin and tested for their lytic activity on T2 cells in the absence or presence of either the peptide used during in vitro stimulation or the parental peptide Melan-A 26–35. In addition, the tumoricidal activity of each cell population was measured by assessing their capacity to lyse the autologous Melan-A$^+$ tumor cell line Me 290. As illustrated in Fig. 5, all of the cell populations tested exhibited a high level of specific lysis against
target cells pulsed with the corresponding stimulating peptide as well as with the parental Melan-A 26–35 peptide. Furthermore, they exhibited a high tumoricidal activity against the autologous melanoma line Me 290 (Fig. 5) and were able to lyse other Melan-A-expressing tumor cell lines but did not lyse Melan-A-negative cell lines (data not shown). Half maximal tumoricidal activity was obtained at an E:T ratio of 3:1 for the line Melan-A 27–35 (named according to the peptide used during the initial in vitro expansion of Melan-A-specific precursors), 7:1 for the line Melan-A 26–35, 4:1 for the line E26A, 5:1 for the line A27L, and 15:1 for the line E26A/A27L. Although this study was not intended to optimize protocols of expansion of specific CTLs, we could obtain as much as 10^7 polyclonal Melan-A-specific CTLs from an initial number of about 1000 A2/Melan-A tetramer^+^ CD8^+^ T lymphocytes (corresponding to ~10,000-fold expansion) in a 3-week period including 1 week of stimulation with peptide analogue and 2 weeks of mitogen stimulation of the A2/Melan-A tetramer^+^ sorted populations.

The relative avidity of peptide antigen recognition of the different cell populations obtained was assessed in a standard CTL assay. To this purpose, titration curves over a wide range of peptide concentrations were generated for each CTL line and for each parental peptide and analogue. Data obtained from this set of experiments are summarized in Table 1. Numbers represent the peptide concentration required for 50% maximal activity. The relative avidity of the different lines for the parental sequences was remarkably similar, with the exception of the line obtained after in vitro stimulation with the analogue E26A/A27L, which recognized peptide Melan-A 27–35 about 3-fold less efficiently and Melan-A 26–35 5–7-fold less efficiently as compared with the other cell lines. This decreased avidity could provide an explanation for the slightly decreased tumoricidal activity exhibited by this line. Results were also expressed as relative antigenic activity. It is of note that, irrespective of the peptide used for in vitro expansion, all of the lines recognized peptide Melan-A 26–35 more efficiently than peptide Melan-A 27–35. Peptide analogues were recognized more efficiently than both parental sequences by all of the lines, even if some differences in the relative antigenicity of peptide analogues could be detected for different lines. However, the preference of a line for a certain analogue did not correlate with the analogue used to generate the line. In all cases, the peptide analogue E26A/A27L was the peptide more efficiently recognized.

**DISCUSSION**

Previous attempts to determine the impact of adoptive transfer in melanoma have been limited by the difficulty of isolating T cells of known antigen specificity. In an extensive study, adoptive transfer of autologous TILs in combination with high-dose IL-2 resulted in an objective response rate of about 30% of patients (17). A parallel study using indium-111-labeled TILs showed tumor localization of the infused cells (18). The limited antitumor effect recorded in these trials might be explained, at least in part, by the fact that the TILs were heterogeneous populations of lymphocytes, only some of which may have had antitumor activity. The recent development of fluorescent tetrameric complexes of HLA class I/antigenic peptide has allowed the direct identification and isolation of antigen-specific T cells by cell sorting. This, together with the recent identification of numerous melanoma-associated CTL epitopes, has enabled us to revisit the approach to adoptive transfer therapy.

The novel strategy to targeted adoptive T-cell transfer outlined in this report consists of a combination of short-term in vitro stimulation of circulating specific CTL precursors with antigenic peptide analogues and tetramer-guided sorting of the specific lymphocyte fraction. Further expansion in vitro to obtain the large numbers of lymphocytes required for infusion to the patient is achieved by potent mitogen in vitro stimulation. An important advantage of this approach is its applicability to a high proportion of patients because it does not depend on the availability of TILs. Instead, the source of specific lymphocytes are the circulating CTL precursors present in the peripheral blood compartment.

However, to implement this novel approach, a number of constraints should be identified and overcome. The first relates to the generation of the fluorescent class I MHC molecule/antigenic peptide tetramers. We have shown previously that both Melan-A nona- and decapeptides bind poorly to HLA-A2 molecules and form unstable MHC/peptide complexes. This holds also true for at least part of the synthetic peptides corresponding to defined melanoma-associated immunodominant epitopes identified thus far that bind to HLA class I molecules with low to intermediate affinities (19). This may lead to difficulties in generating stable tetramer preparations. Thus, for this category of peptides, it may be necessary to design peptide variants containing amino acid substitutions that enhance peptide binding to MHC molecules without negatively affecting T-cell recognition. This was the case for the Melan-A antigen, for which a much more efficient tetramer formation as well as staining of specific T lymphocytes was obtained with HLA-A2 decapeptide analogue Melan-A 26–37 A27L tetramers, as compared with the natural decapeptide (10).

We have recently used fluorescent tetramers synthesized around peptide A27 to detect specific T cells ex vivo in lymphocyte suspensions from metastatic lymph nodes from HLA-A^*0201 melanoma...
patients. It was clear that antigen-experienced Melan-A-specific T lymphocytes can be found at relatively high frequencies (up to 3%) in tumor-invaded lymph nodes. In contrast, frequencies <0.1% are usually detected among circulating CD8+ lymphocytes of melanoma patients.

Thus, a second limitation of our approach relates to the low frequency of tumor-reactive circulating T cells. In this study, A2/Melan-A tetramer+ cells were detected in PBMCs of HLA-A*0201+ melanoma patients at an average frequency of about 1/2000. These frequencies may be lower for other epitopes for which in vitro-specific activities have been less frequently obtained (20). In these cases, although tetramer staining has shown thus far to be highly specific, it is crucial to rigorously assess the antigen specificity and the proliferation potential of rare tetramer+ cells through repeated in

Fig. 3. Differential stimulatory capacity of parental and analogue Melan-A peptides monitored by flow cytometry with A2/Melan-A tetramers and anti-CD8 FC. Total PBMCs were initially stimulated by adding 1 μM of the indicated peptide directly into the culture medium. Thereafter, the cultures were stimulated weekly with irradiated autologous PBMCs pulsed with the indicated peptide. Cultures were stained 7 days after each stimulation with A2/Melan-A tetramers together with anti-CD8 PE mAb. Histograms are shown for gated CD8+ cells. Numbers in the upper right quadrant represent the percentage of A2/Melan-A tetramers and anti-CD8 double+ cells. *, the percentage of CD8dimw cells in the culture.

Fig. 4. Differential stimulatory capacity of parental and analogue Melan-A peptides measured by 51Cr release assay. Viable cells recovered on day 7 after the third restimulation with peptides in the experiment described in Fig. 3 were titrated as effectors against chromium-labeled T2 target cells in a 4-h 51Cr release assay in the absence (○) or presence of exogenously added (1 μM) parental Melan-A 26–35 peptide (●).
therapy with TILs also requires concurrent administration of IL-2 to in vitro PBMCs pulsed with the indicated peptide. Seven days after the second cycle of in vitro stimulation, Sorted cells were expanded in vitro on day 7 after the second in vitro stimulation. Activated cell sorting from the bulk cultures of Fig. 3, on day 7 after the second in vitro stimulation, was then quantitated in a standard CTL assay.

In vitro stimulation with the corresponding synthetic peptides. However, as it is illustrated by the Melan-A parental peptides, peptides suboptimally binding to MHCs are generally relatively poor stimulators of specific T cells (Figs. 1 and 3; Refs. 11, 21, and 22). Again, the use of peptide analogues may significantly improve the efficiency of this important in vitro restimulation step, provided that, as illustrated in this study, the T-cell populations derived from peptide analogue-driven isolation and expansion are able to fully cross-react with the parental peptides and to efficiently recognize antigen-expressing tumor cells (Figs. 2 and 5).

In animal models of tumor immunotherapy, efficient tumor eradication by CD8+ T lymphocytes requires CD4+ helper T cells or IL-2 (23, 24). In a clinical trial of adoptive transfer of autologous CMV-specific CTLs for prophylaxis from CMV disease in patients undergoing bone marrow transplantation, long term in vivo persistence of transferred CTLs was dependent on the development of an endogenous CMV-specific CD4+ T helper response (25). Human tumor therapy with TILs also requires concurrent administration of IL-2 to provide helper function. However, the limitations imposed by the toxicity of systemic IL-2 administration warrant alternative strategies including, for example, the coinfusion of CD4+ helper T cells specific for defined tumor-associated antigens. The feasibility of this increases as mounting evidence indicates that CD4+ T-cell responses to defined tumor-associated antigens can be frequently found in metastatic melanoma patients (26–29). Moreover, as with antigen-specific CD8+ T cells, it has been shown recently that class II MHC/antigen peptide fluorescent tetramers can specifically stain CD4+ T lymphocytes (30, 31). Thus, future adoptive transfer therapy could also benefit from tetramer-guided isolation of specific CD4+ T lymphocyte populations of genes that can potentially render them independent of a requirement for either CD4+ helper T cells or of exogenous IL-2 (32, 33).

In conclusion, in the present study we have illustrated a novel strategy that combines the use of potent antigen peptide analogues and

Table 1

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<th>Culture stimulated with*</th>
<th>Melan-A 27–35</th>
<th>Melan-A 26–35</th>
<th>E26A</th>
<th>A27L</th>
<th>E26A/A27L</th>
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* Total PBMCs were initially stimulated by adding 1 μg of the indicated peptide directly into the culture medium. Thereafter, cultures were stimulated weekly with autologous PBMCs pulsed with the indicated peptide. Seven days after the second cycle of in vitro stimulation, CD8+ A2/Melan-A tetramer+ cells were purified from each culture by flow cytometry sorting and expanded in vitro by mitogen stimulation. The avidity of antigen recognition as well as the relative antigenic activity of the different cell populations obtained was then quantitated in a standard CTL assay.

† T2 cells were incubated with various concentrations of the different peptide variants. Lysis was measured in a 4-h 51Cr release assay at a lymphocyte to target cell ratio of 10:1. The peptide nanomolar concentration giving 50% of maximal activity, [nM] 50%, was then determined from the titration curve for each peptide.

The relative antigenic activity of each peptide, normalized to that of the parental peptide Melan-A 27–35, was calculated as described in “Materials and Methods.”
fluorescent HLA-class I/peptide tetramers and allows the rapid preparation of large numbers of polyclonal monospecific tumor-reactive CTL populations. This study provides the basis for the evaluation of the tumoricidal potential of CTLs specific for defined tumor-associated epitopes, within appropriate experimental settings, thus opening new perspectives for adoptive transfer therapy of cancer.

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REFERENCES

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