Simultaneous CD8+ T cell responses to multiple tumor antigen epitopes in a multipeptide melanoma vaccine

Danila Valmori1,4✉, Valérie Dutoit1*, Maha Ayyoub1,4*, Donata Rimoldi2, Philippe Guillaume2, Danielle Liénard1,3, Ferdy Lejeune3, Jean-Charles Cerottini2, Pedro Romero1, and Daniel E. Speiser1

1Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, University Hospital (CHUV), 1011 Lausanne, Switzerland
2Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland
3Multidisciplinary Oncology Center, University Hospital (CHUV), 1011 Lausanne, Switzerland
4Ludwig Institute Clinical Trial Center at Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, USA (current address)
*These authors contributed equally to this work

Keywords: human, melanoma, vaccination, immunological monitoring, cytotoxic T cells

Abstract

The recent identification and molecular characterization of tumor-associated antigens recognized by tumor-reactive CD8+ T lymphocytes has led to the development of antigen-specific immunotherapy of cancer. Among other approaches, clinical studies have been initiated to assess the in vivo immunogenicity of tumor antigen-derived peptides in cancer patients. In this study, we have analyzed the CD8+ T cell response of an ocular melanoma patient to a vaccine composed of four different tumor antigen-derived peptides administered simultaneously in incomplete Freund's adjuvant (IFA). Peptide NY-ESO-1157-165 was remarkably immunogenic and induced a CD8+ T cell response detectable ex vivo at an early time point of the vaccination protocol. A CD8+ T cell response to the peptide analog Melan-A26-35 A27L was also detectable ex vivo at a later time point, whereas CD8+ T cells specific for peptide tyrosinase368-376 were detected only after in vitro peptide stimulation. No detectable CD8+ T cell response to peptide gp100457-466 was observed. Vaccine-induced CD8+ T cell responses declined rapidly after the initial response but increased again after further peptide injections. In addition, tumor antigen-specific CD8+ T cells were isolated from a vaccine injection site biopsy sample. Importantly, vaccine-induced CD8+ T cells specifically lysed tumor cells expressing the corresponding antigen. Together, these data demonstrate that simultaneous immunization with multiple tumor antigen-derived peptides can result in the elicitation of multiepitope-directed CD8+ T cell responses that are reactive against antigen-expressing tumors and able to infiltrate antigen-containing peripheral sites.
Introduction

The initial observation that tumor cells can be efficiently and specifically killed in vitro by the host's specific CD8+ cytolytic T cells (CTLs) has encouraged the search for tumor antigens expressed by cancer cells. Many such antigens have been identified, particularly in melanoma, and have been classified in several categories mostly based on their expression pattern. Among them are tissue-specific antigens such as Melan-A, tyrosinase and gp100, which are expressed by the majority of melanoma cells as well as by cells of the melanocytic lineage but not in other normal tissues (1, 2, 3). Another group of antigens that is receiving increasing attention is that of the so-called cancer-testis (CT) antigens. CT antigens are expressed in variable proportions in tumors of different histological types, whereas their expression in normal tissues is limited to germ cells [e.g. MAGE genes (4), NY-ESO-1 (5)]. For antigens in each group, short peptides recognized by tumor-reactive CTLs in association with frequently expressed HLA class-I alleles (e.g. HLA-A*0201 (A2) expressed in about 50% of Caucasians) have been identified (6, 7, 8, 9).

Based on this knowledge, clinical studies have been initiated to assess both the immunogenicity and the therapeutic potential of vaccination with tumor antigen-derived peptides. In most studies performed thus far, single tumor antigen-derived peptides have been administered, sometimes in various formulations (10, 11, 12, 13, 14, 15, 16). Alternatively, several peptides derived from a single antigen have been administered simultaneously (17). However, tumor cells can express multiple tumor antigens and the expression of each of these can vary within the tumor. Therefore, a vaccine containing peptides derived from several of these antigens could be more effective than a vaccine directed against a single antigen. In addition, elicitation of immune responses directed against several antigens can minimize the risk of selecting antigen loss tumor variants (18).

Recent attempts to apply this concept to the generation of cancer vaccines have mostly involved the use of recombinant viruses encoding strings of peptides in murine models (19, 20) or in vitro studies using dendritic cells genetically engineered to express a polyepitope DNA vaccine (21). However, few data are available thus far on the in vivo immunogenicity of cancer vaccines composed of several peptides derived from distinct tumor antigens administered simultaneously to cancer patients (22).

To gain insight into the immunogenicity of such vaccines, we analyzed the antigen-specific CD8+ T cell responses of an ocular melanoma patient elicited by a vaccine composed of three melanocyte differentiation antigen-derived peptides and one CT antigen-derived peptide that were simultaneously administered in incomplete Freund's adjuvant. The data obtained demonstrate that simultaneous immunization with multiple tumor antigen-derived peptides can result in the elicitation of CD8+ T cells directed against multiple epitopes that are reactive against antigen-expressing tumors and able to migrate into antigen-containing peripheral sites.

Results

Vaccine-induced circulating CD8+ T cells specific for tumor antigen-derived peptides

Monitoring of vaccine-induced CD8+ T cells was performed using fluorescent multimers incorporating each of the peptides used for the vaccination either ex vivo or after in vitro stimulation with the corresponding peptide during one week. The response of patient LAU 455 to vaccination was first evaluated after the first vaccination cycle (day 39 after the beginning of the treatment), which consisted of three injections given at two-week intervals. In the case of the ex vivo analysis, the phenotype of antigen-specific T cells was simultaneously assessed by staining with anti-CD45RA and anti-CD28 mAbs. As illustrated in Figure 1A, A2/NY-ESO-1 multimer+ CD8+ T
cells were not detectable, either ex vivo or after in vitro peptide stimulation, in the prevaccination sample (day -23). This is consistent with our previous results in both normal donors and in the majority of melanoma patients bearing tumors that do not express NY-ESO-1 (23). However, in the postvaccination sample, A2/NY-ESO-1 multimer+ CD8+ T cells expressing an antigen experienced phenotype (CD45RA- CD28+, Figure 1 and data not shown) were clearly detectable ex vivo and were readily expanded in vitro by peptide stimulation.

Figure 1. Pre- and post-vaccination CD8+ T cell responses to tumor antigen-derived peptides in patient LAU 455. Pre- and post-vaccination (day 39) PBMC samples from patient LAU 455 were analyzed either ex vivo (upper panels) or one week after in vitro stimulation with the corresponding peptide (1 µM) (lower panels). Staining with anti-CD45RA FITC mAb and with the indicated A2/peptide multimers PE on gated CD8+ T cells is shown in the upper panels. Staining with anti-CD8 FITC and with the indicated A2/peptide multimers PE on gated lymphocytes is shown in the lower panels.

We have previously reported that naive A2/Melan-A multimer+ CD8+ T cells are, at variance with other single antigen-specific naive T cells, present in most A2-expressing normal individuals at a frequency (about 10^-3 of circulating CD8+ T cells) that allows their detection ex vivo (24). However, in addition to the A2/Melan-A multimer+ T cell naive population, a variable proportion of A2/Melan-A multimer+ CD8+ T cells expressing an antigen experienced phenotype can also be visualized ex vivo in about 1/3 of melanoma patients and most likely represent spontaneous responses to Melan-A. Patient LAU 455 belonged to this group as both CD45RA+ and CD45RA- CD28+ A2/Melan-A multimer+ CD8+ T cells were detectable in the prevaccination sample (Figure 1B and data not shown). No differences were detectable between pre- and day 39 post-vaccination samples, both in terms of frequency and of phenotype of the A2/Melan-A multimer+ T cells. In line with these data, similar proportions of A2/Melan-A multimer+ T cells were detected after in vitro peptide stimulation in the pre- and post-vaccination samples. Thus, no vaccine-induced CD8+ T cell response to Melan-A peptide was detected at this time point.

No significant proportion of A2/tyrosinase multimer+ CD8+ T cells was detectable either ex vivo or after in vitro peptide stimulation in the pre-vaccination sample (Figure 1C). However, after in vitro stimulation, a low but significant proportion of A2/tyrosinase multimer+ CD8+ T cells became apparent. Finally, in the case the gp100-derived peptide, no significant response was detected either ex vivo or in in vitro stimulated samples (data not shown).
Longitudinal analysis of vaccine-induced circulating CD8+ T cell responses

Molecular monitoring was carried out similarly at later time points during the 7 months following the beginning of treatment. Figure 2 summarizes the longitudinal analysis of vaccine-elicited CD8+ T cells specific for each peptide (except the gp100-derived peptide for which no specific response was detected at any time point) in this time period and in relation to the treatment. Between day 39 and day 84 the patient received one additional vaccine injection, which was followed by two additional injections before the next blood sample was taken at day 105. The patient did not receive any additional injections until the next blood sample was withdrawn on day 181. From this date onwards, the patient received 3 additional injections and blood was taken again on day 221.

Figure 2. Longitudinal analysis of vaccine-induced circulating CD8+ T cell responses. An analysis similar to that illustrated in Figure 1 was performed for samples obtained at different time points during the treatment. Results of the longitudinal analysis are shown separately for each tumor antigen-derived peptide as bar graphs. Numbers on the y-axis correspond to the percentage of CD8+ multimer+ T cells detected \textit{ex vivo} using multimers incorporating the corresponding peptide at the time points indicated. Numbers on bars correspond to the percentage of CD8+ multimer+ T cells detected in the corresponding peptide-stimulated cultures. Time points analyzed and vaccination injections (arrows) are shown in the x-axis.

After the initial expansion detected at day 39 post-vaccination, the proportion of A2/NY-ESO-1 multimer+ CD8+ T cells decreased moderately at day 84 and then increased at day 105 to reach a level similar to that at day 39. In contrast, at day 181 A2/NY-ESO-1 multimer+ CD8+ T cells were not detectable either \textit{ex vivo} or after \textit{in vitro} peptide stimulation. However, at day 221, after three boost vaccine injections, A2/NY-ESO-1 multimer+ CD8+ T cells were detected again in proportions similar to those observed after the first injection cycle. Results were qualitatively similar in the case of Melan-A and tyrosinase, albeit in the latter case the proportion of CD8+ multimer+ T cells remained close to the \textit{ex vivo} multimer detection limit. It is of note that the increase in the proportion of A2/Melan-A multimer+ T cells was mostly confined to the CD45RA- compartment whereas the proportion of CD45RA+ A2/Melan-A multimer+ T cells remained stable. Further phenotypic analysis was performed for both A2/Melan-A and A2/NY-ESO-1 multimer+ CD8+ T cells detected \textit{ex vivo} at day 105. Interestingly, among A2/Melan-A multimer+ CD8+ T cells, the fraction expressing CD45RA was CCR7+ whereas...
in the CD45RA- fraction similar proportions of CCR7+ and CCR7- T cells were found. In contrast, A2/NY-ESO-1 multimer+ T cells were mostly CCR7- (data not shown). Together, the results of the longitudinal monitoring show that, for all peptides, the vaccine-induced CD8+ T cells were not persistent in the circulation and dropped to levels found prior to initiation of immunotherapy when peptide administration was not repeated. However, boost injections resulted in the reappearance of specific populations in proportions similar to or higher than those observed (i.e. for Melan-A) when these responses were initially detected.

Figure 3. Functional characterization of vaccine-induced circulating CD8+ T cells isolated by multimer-guided cell sorting. The functional avidity of antigen recognition and tumor reactivity of each population was assessed in CTL assays. For each tumor antigen-derived peptide, specific tumor-reactive clones LAU 156/5 (NY-ESO-1), LAU 156/34 (tyrosinase) and LAU 203/17 (Melan-A) were used as internal controls. (A) Functional avidity of antigen recognition was determined by assessing peptide recognition on T2 target cells in the presence of increasing concentrations of the peptides NY-ESO-1157-165 (open circles), Melan-A27-35 (closed diamonds), Melan-A26-35 (closed squares), Melan-A26-35 A27L (open squares), tyrosinase368-376 (closed circles), or influenza matrix58-66 (open triangles). (B) and (C) Tumor recognition was assessed on Me 275 (A2+, NY-ESO-1+, Melan-A+, tyrosinase+ and gp100+) and NA8-MEL (A2+, NY-ESO-1-, Melan-A-, tyrosinase- and gp100-) melanoma cell lines in the absence (closed symbols) or presence (open symbols) of the relevant peptide added exogenously.
Isolation and functional characterization of vaccine-induced circulating CD8+ T cells

To functionally characterize the vaccine-induced circulating CD8+ T cells, we isolated A2/Melan-A, A2/NY-ESO-1 and A2/tyrosinase multimer+ CD8+ T cells from peptide-stimulated cultures of samples at days 84 and 105 by multimer guided cell sorting as previously described (25). Isolated CD8+ multimer+ cells were expanded in vitro by stimulation with PHA and irradiated feeder cells in order to obtain polyclonal monospecific T cell populations. The T cells making up these populations exhibited a large repertoire of TCR beta chain (data not shown). Functional avidity of antigen recognition and tumor reactivity of each population was assessed in CTL assays (Figure 3). For each peptide, specific tumor-reactive T cell clones that have previously been described were used as internal controls. As illustrated in Figure 3A, all populations displayed a functional avidity of antigen recognition roughly similar to that of the corresponding control clone. In addition, each population specifically lysed A2+ tumor targets expressing the relevant antigen both in the presence and in the absence of exogenously added peptide (Figure 3B). In contrast, A2+ but antigen negative tumor targets were lysed in the presence but not in the absence of peptide (Figure 3C).

Retrieval of tumor antigen-specific T cells at the injection site

Injection of the vaccine emulsion led to local indurations at the injection sites, which in some cases persisted for several months. During the first week following some of the injections, previous injection sites showed signs of inflammation. One such event occurred at a site where the fourth vaccine injection had been given at day 70 following the sixth injection on day 97. A fine needle biopsy of the subcutaneous tissue at this site was taken and, after mechanical dissection, placed in culture in the presence of cytokines. At day 8, T lymphocytes (mostly CD8+, Figure 4) had outgrown the culture. Among CD8+ T cells, 4.6% of the CD8+ T cells in the culture were specifically stained by A2/Melan-A multimers whereas a lower, but clearly detectable population of CD8+ A2/NY-ESO-1 multimer+ T cells was also found (0.11%, Figure 4). In contrast, neither A2/tyrosinase nor A2/gp100 multimer+ T cells were detected (data not shown). It is of note that retrieval of tumor antigen-specific T cells at the site of vaccination did not simply reflect the presence of specific T cells in the circulation. Indeed, CD8+ multimer+ T cells specific for two virally derived peptides (influenza matrix58-66 and EBV pp65495-503) were not detectable in the cultured lymphocytes derived from the biopsy, despite their presence at relatively high proportions among the circulating CD8+ T cells of patient LAU 455 (0.05% and 0.11% respectively) (data not shown).

![Figure 4. Detection of tumor antigen-specific T cells at the site of vaccination.](http://www.cancerimmunity.org/v3p15/030915.htm)
Discussion

The most remarkable finding of this study is the high immunogenicity of peptide NY-ESO-1_{157-165} in IFA. Consistent with our previous findings in which no detectable CD8+ T cell responses were seen in most normal donors and in patients with tumors that do not express NY-ESO-1, no A2/NY-ESO-1 multimer+ T cells were detectable among the CD8+ T lymphocytes of patient LAU 455 prior to vaccination. However, after a single injection cycle (three injections) about 1 in 2000 circulating CD8+ T cells were specifically stained by A2/NY-ESO-1 multimers. These cells exhibited an antigen-experienced phenotype (CD45RA-, CCR7-, CD28+). Although vigorous vaccine-induced CD8+ T cell responses to peptides derived from melanocyte differentiation antigens have been described (16, 26), no vaccine-induced CD8+ T cell responses to a CT antigen-derived peptide, of the magnitude reported here, have been found previous to this study.

Three peptides, corresponding to overlapping sequences in the 155-167 region of the NY-ESO-1 protein (155-163 QLSLLMWIT, 157-165 SLLMWITQC and 157-167 SLLMWITQCFL), were initially shown to be recognized by tumor-reactive NY-ESO-1-specific CTLs (27). These peptides were administered together intradermally to cancer patients, either alone or in combination with GM-CSF as a systemic adjuvant (28). No reactivity to peptide 155-163 was detected in any of the vaccinated patients and this peptide was therefore not included in subsequent vaccination protocols. In contrast, CD8+ T cell responses to peptide 157-167 were detected soon after the beginning of the treatment, whereas reactivity against peptide 157-165 appeared later and was to a lesser extent. In a cancer patient vaccination trial that is currently ongoing at the Ludwig Institute Clinical Trial Center, New York Branch, patients bearing NY-ESO-1-expressing tumors are immunized with peptides 157-165 SLLMWITQC and 157-167 SLLMWITQCFL administered intradermally together with GM-CSF. In agreement with previous studies, we detected a significant CD8+ T cell response to the vaccine in these patients. By analyzing vaccine-elicited CD8+ T cell responses in more detail, we found that the majority of vaccine-elicited CD8+ T cells were directed against multiple distinct epitopes in the 157-167 region and were not tumor-reactive (29). In addition, only a minor fraction of elicited CD8+ T cells directed against peptide 157-165 and of sufficiently high functional avidity recognized the naturally processed target on NY-ESO-1+ tumor cells.

These data are in agreement with our previous finding that, among the NY-ESO-1-derived natural peptides initially described (27), peptide 157-165 is the one that is the most efficiently recognized by tumor-reactive CTLs and therefore most likely corresponds to the natural target peptide on NY-ESO-1-expressing tumor cells. Further support for this view comes from the results of this study that shows that vaccination with peptide 157-165 in IFA resulted in the rapid induction of high levels of specific, high affinity, tumor-reactive CD8+ T cells. It is of note that upon in vitro stimulation of PBMCs from patient LAU 455 with peptide 157-167, no response against any epitope in the 157-167 region other than peptide 157-165 was detected (data not shown). Interestingly, we have observed that peptide 157-165 is particularly resistant to degradation by serum proteases (29), a characteristic that may explain its natural high immunogenicity. Together, these data clearly indicate that peptide 157-165 should be specifically selected for inclusion in upcoming vaccination protocols.

CD8+ T cell responses specific for the two other peptides, Melan-A analog_{26-35 A27L} and tyrosinase_{368-376}, contained in the vaccine administered to patient LAU 455 were also detected. An antigen experienced (CD45RA-) A2/Melan-A multimer+ CD8+ T cell population was already detectable ex vivo prior to vaccination and most likely represented a spontaneous tumor-specific response as previously reported (24). However, a significant increase in the size of this population was detected ex vivo following vaccination although at a later time point as compared to peptide NY-ESO-1. Importantly, A2/Melan-A multimer+ CD8+ T cells isolated at two different time points along the vaccination protocol specifically recognized Melan-A parental peptides as well as Melan-A-expressing tumor cells. These results are in agreement with those that we recently obtained in a study where Melan-A analog_{26-35 A27L} was administered to patients with malignant melanoma and elicited responses that were detectable in the lymph nodes draining the injection site (30). Taken together the data obtained show that
peptide Melan-A$_{26-35}$ A$_{27L}$ is consistently immunogenic in cancer patients and able of eliciting tumor-reactive CD8$^+$ T cells. It is of note that experimental evidence ($^{16, 31}$) suggests that the natural Melan-A-derived peptide most abundantly expressed by melanoma tumor cells is the nonapeptide 27-35 (AAGIGILTV). The latter, however, displays poor binding to the HLA-A$^*$0201 molecule and is relatively poorly immunogenic in vitro ($^{25}$). We observed that the decapeptide 26-35 (EAAGIGILTV) is recognized more efficiently than peptide 27-35 by Melan-A-specific tumor-reactive CTLs and binds to HLA-A$^*$0201 more efficiently. However, both nona- and decapeptides lack a canonical "anchor" for binding to HLA-A$^*$0201. This prompted us to design the peptide analog Melan-A$_{26-35}$ A$_{27L}$ bearing a single A to L substitution at position 2 of the decapeptide. As expected, the Melan-A$_{26-35}$ A$_{27L}$ peptide exhibited increased in vitro and in vivo immunogenicity in preclinical models ($^{25, 32, 33}$) and was therefore selected as immunogen for ongoing trials.

An inherent risk of using amino acid substituted peptide analogs of enhanced immunogenicity, even when these have been designed such that the substitution affects peptide recognition minimally, is the recruitment of cells of increasingly high avidity towards the immunogen and lower reactivity towards the natural peptide and hence the tumor ($^{16}$). This risk could become increasingly higher with repeated vaccinations. Therefore, whereas peptide Melan-A$_{26-35}$ A$_{27L}$ presently appears as a good compromise between peptide immunogenicity and tumor reactivity of the elicited CD8$^+$ T cell populations, efforts to increase the immunogenicity of the nonapeptide by administering it in highly immunogenic formulations should be pursued. Alternatively, vaccination protocols could be designed such that peptide Melan-A$_{26-35}$ A$_{27L}$ is used for priming followed by peptide Melan-A$_{27-35}$ for boost injections after a primary response has appeared.

Tumor reactivity was also clear in the case of vaccine elicited tyrosinase$_{368-376}$-specific CD8$^+$ T cells, albeit the frequency of these was close to the limit of multimer staining detection ex vivo and could only be clearly assessed upon in vitro peptide stimulation. It is of note that the tyrosinase$_{368-376}$ variant YMDGTMSQV bearing a D at position 3 as the result of a post-translational modification corresponds to the natural peptide isolated from tyrosinase-expressing tumor cells ($^8$). Our results, together with similar data reported previously ($^{34}$), suggest that peptide tyrosinase$_{368-376}$ exhibits limited immunogenicity in IFA. This is in apparent contradiction with the relatively good binding capacity of this peptide to HLA-A$^*$0201, the relatively high frequency of specific precursors ($^{35}$) and the spontaneous immunogenicity observed in some melanoma patients ($^{35, 36, 37}$).

No significant CD8$^+$ T cell response was detected, either ex vivo or in in vitro stimulated samples, in the case of the gp100$_{457-466}$ peptide at any time point of the vaccination protocol. This is in agreement with the results of a previous study where the peptide was injected intradermally alone for 3 cycles of 4 weekly injections and where GM-CSF was used as a systemic adjuvant during the fourth cycle of vaccination ($^{38}$). It is of note that both for tyrosinase and gp100 several additional peptides corresponding to HLA-A2-restricted epitopes recognized by tumor-reactive CTLs have been described ($^{39, 40}$). Although the results from several studies indicate that some of these peptides could be more immunogenic than the ones selected in this study ($^{10, 13, 38}$), unfortunately, each of these peptides has been administered to patients in independent trials and therefore under very different conditions (different doses, adjuvants, routes and schedules of administration). Therefore, in future trials, the immunogenicity of these different peptides should be directly compared in order to select the most immunogenic.

A common feature of the reported peptide-specific CD8$^+$ T cell responses to vaccination was that they dropped to undetectable levels rapidly after the initial response (compare pre-vaccination, day 105 and day 181) but were clearly detectable again after a boost injection cycle (day 221). Induction of short-lived CD8$^+$ T cell responses could be due to the lack of CD4$^+$ T cell help in the vaccine. CD4$^+$ helper T cells play a critical role in the elicitation and maintenance of CD8$^+$ CTL responses, including those against cancer ($^{41}$). Indeed, the simultaneous elicitation of CD4$^+$ T cells, particularly if specific for the same antigen, can considerably enhance the immunogenicity of MHC class-I peptides, both in terms of the magnitude and of the life span of the vaccine-induced CD8$^+$ T cells ($^{42}$). A situation similar to that found in this study has been observed in a clinical trial of...
vaccination of breast cancer patients with a HER-2/neu peptide vaccine consisting of a single peptide corresponding to a CD8+ T cell epitope (43). In contrast, vaccination with HER-2/neu-derived synthetic peptides encompassing both MHC-class-I and class-II-restricted T cell epitopes resulted in the induction of sustained CD8+ T cell responses (17). In addition to providing help to tumor-reactive CD8+ T cells, tumor antigen-specific CD4+ T cells can help B cells to produce tumor antigen-specific antibodies that, in turn, may play an important role in cancer immunity by promoting cross-presentation of MHC-class-I restricted epitopes by dendritic cells (44). The identification of tumor antigen-derived peptides recognized by tumor-reactive CD4+ T cells to be used for generic vaccination of cancer patients has been hampered by the high polymorphism of the MHC-class-II molecules. Recently, however, two tumor antigen-derived T cell epitopes recognized by CD4+ T cells in association with HLA-DP4 (an MHC class-II allele commonly expressed in Caucasians) have been described (45). One of them, derived from NY-ESO-1, corresponds to a 14 amino acid long peptide that overlaps the relevant CD8+ T cell epitope (157-165) and therefore represents an attractive candidate for the simultaneous elicitation of specific CD8+ and CD4+ T cells. Nonetheless, the results of our recent monitoring of CD8+ T cell responses in patients immunized with peptide 157-167 indicate that immunization with peptides longer than the optimal CD8+ T cell epitope recognized by tumor-reactive CTLs can result in imprecise tumor targeting (29). These results also underline the importance of a thorough monitoring of the complete repertoire of specific T cell responses elicited by such peptides. An alternative approach for the simultaneous elicitation of relevant tumor antigen-specific CD8+, CD4+ T cell and antibody responses could consist in the administration of peptides corresponding to precisely defined CD8+ T cell epitopes in combination with recombinant proteins.

Another interesting finding of this study was the retrieval of tumor antigen-specific T cells at a site of injection that showed signs of inflammation upon subsequent peptide injection at a distant site. It is generally acknowledged that after injection of the peptides emulsified in IFA, small quantities of antigen are continuously released from the depot, taken up by APCs and transported into lymphoid organs (i.e. lymph nodes) where immune responses are initiated. Primed T cells will then migrate into the circulation and eventually from peripheral vessels into tissues to exert their functions. Retrieval of tumor antigen-specific T cells at a site of injection where antigen persists for a long time trapped into the water/oil emulsion, suggests the potential ability of vaccine-induced CD8+ T cells to migrate from the circulation into peripheral tissues, driven by the presence of antigen, and eventually to destroy metastatic lesions.

The patient in this study had an ocular melanoma which expressed melanocyte differentiation antigens but not NY-ESO-1. Thus, the antigenic profile was similar to the majority of cutaneous melanomas. Nevertheless, further studies are necessary to compare immune responses in ocular versus cutaneous melanoma patients. Since NY-ESO-1 is generally not expressed in ocular melanomas (46), it is likely that none of the tumor cells of this patient expressed this antigen. Thus, the responding NY-ESO-1-specific T cells must have been triggered by the vaccine, demonstrating its strong immunogenicity.

In conclusion, although this study is limited to a single patient, the data clearly show that simultaneous high avidity and potentially tumor-reactive CD8+ T cell responses to multiple tumor antigen-derived peptides can be induced by vaccination with mixtures of synthetic peptides in adjuvant. Responses to individual peptides in the mixture were observed to variable extents, most likely in relation with the individual immunogenicity of each of the peptides. Thus, for each peptide, appropriate peptide variants, doses and formulations that elicit vigorous responses need to be determined. In addition, as the responses elicited seem to be short lived, maintenance of sustained or at least repetitive responses may require frequent boost injections or the concomitant elicitation of helper T cells. Overall these results encourage the development of polyepitope vaccines consisting of a mixture of synthetic peptides in adjuvant. Due to the generally low toxicity and relative ease of administration of peptide vaccines, clinical trials based on vaccination of cancer patients with polyepitope synthetic peptide vaccines provide a platform for the development of cancer immunotherapy approaches with increasing efficacy.
Abbreviations

IFA, incomplete Freund’s adjuvant

Acknowledgements

This study was supported by the Ludwig Institute for Cancer Research. D.V., M.A. and D.S. are recipients of grants from the Cancer Research Institute. M.A. was also the recipient of a postdoctoral fellowship from the NCCR in Molecular Oncology (Swiss National Science Foundation).

We would like to thank Nicole Montandon, Andrée Porret, Christine Geldhof and Danielle Minaïdis for technical assistance. We would like to dedicate this work to Dr. Pascal Batard who performed the flow cytometry cell sorting experiments presented and who disappeared tragically very recently, leaving us all in deep sorrow.

References


Materials and methods

Patient, vaccination protocol and clinical course

Patient LAU 455 was diagnosed with a primary ocular (uveal) melanoma of the left eye in 1980. After local treatment by photocoagulation and proton therapy, the patient had local recurrence and underwent surgical removal of the eye in 1991. Despite systemic chemotherapy, the patient further recurred. A first metastatic lesion was detected in the left lung in 1992 and a rapidly progressing second lesion detected in 1995 required lobectomy. In 2000 the patient presented eight nodular lung metastases and right cervical adenopathy. Two tumor-infiltrated lymph nodes were removed surgically and analyzed for tumor antigen expression by RT-PCR. Expression of melanocyte differentiation antigens Melan-A, tyrosinase and gp100 was detectable in both lymph nodes, whereas no expression of CT antigens MAGE-A1, -A3, -A4, -A10, NY-ESO-1 and Lage-1 was detected (data not shown). Immunotherapy was initiated because additional surgery and chemotherapy were considered unlikely to be beneficial. The patient was treated at the Multidisciplinary Oncology Center, Lausanne University Hospital, after giving written informed consent and after approval by the institutional review boards. Treatment toxicity was evaluated according to the National Cancer Institute Common Toxicity Criteria (CTC) scale. Tumor staging was carried out by physical examination and computer-aided tomography scan analysis. Vaccination was given in 4 cycles, each consisting in 3 subcutaneous injections given at two-week intervals. Each vaccine contained four peptides (synthesized by Multiple Peptide Systems, San Diego, USA): NY-ESO-1_{157-165} (SLLMWITQC), Melan-A_{26-35} A27L (ELAGIGILTV), tyrosinase_{368-376} (YMDGTMSQV) and gp100_{457-466} (LLDGTATLRL). Peptides were conditioned according to Good Manufacturing Practice quality criteria at the LICR Biological Production Facility, Melbourne, Australia. Tyrosinase_{368-376} and gp100_{457-466} were in saline containing 10% DMSO, NY-ESO-1_{157-165} in 100% DMSO, Melan-A_{26-35} A27L in saline containing 30% DMSO. Peptides were administered subcutaneously (50 µg each in 150 µl) emulsified in 600 µl of IFA (Montanide ISA-51, provided by Seppic, France). For the first 3 injections, Melan-A_{26-35} A27L and gp100_{457-466} were administered together at one site and NY-ESO-1_{157-165} and tyrosinase_{368-376} together at a separate site. For subsequent injections, the four peptides were injected at a single site. The treatment was associated with disease stabilization. During the treatment, two lung metastases in the upper left pulmonary lobe showed radiological signs of necrosis. Concomitantly, the patient experienced an episode of thoracic pain and flu-like symptoms. In addition, about one week after some of the injections, local inflammation was observed at previous injection sites.

Antibodies, A2/peptide multimers and flow cytometry immunofluorescence analysis

Monoclonal antibodies were obtained from Becton-Dickinson (San José, CA, USA). PE-conjugated multimeric A2/peptide complexes containing peptides NY-ESO-1_{157-165}, Melan-A_{26-35} A27L, tyrosinase_{368-376} and gp100_{457-466} were synthesized as described (47, 48). Uncultured (ex vivo) or peptide-stimulated samples were stained with A2/peptide multimers (4.5 µg/ml) in PBS containing 0.2% BSA and 0.02% sodium azide for 1 hour at room
temperature, washed once in the same buffer, stained with anti-CD8 mAb and/or anti-CD45RA, anti-CD28 and anti-CCR7 mAbs (Becton Dickinson, San Jose, CA, USA, except for the anti-CCR7 mAb which was a gift from Dr. M. Lipp, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) for 20 min at 4°C, washed again and analyzed by flow cytometry (FACScan device, Becton Dickinson, San José, CA, USA). Data analysis was performed using Cell Quest™ software.

**Cells**

Highly enriched CD8+ cells from the patient's PBMCs were cultured in vitro during one week in CTL medium (32) containing hrIL-2 (10 IU/ml, Roche, Basel, Switzerland) and hrIL-7 (10 ng/ml, R&D System, Oxon, UK) in the presence of each of the peptides used (1 µM). A2/peptide multimer+ CD8+ T cells were purified from PBMCs after in vitro peptide stimulation by flow cytometry cell sorting and stimulated in vitro with PHA (Sigma, Basel, Switzerland), allogeneic irradiated PBMCs and cytokines as described (32).

**Assessment of the efficiency of antigen recognition and tumor reactivity**

Antigen recognition was assessed by chromium release assay (CTL assay). The A2+ human mutant cell line CEMx721.T2 (T2, A2+ NY-ESO-1-) (49) or the melanoma cell lines Me 275 (A2+, NY-ESO-1+, Melan-A+, tyrosinase+ and gp100+) and NA8-MEL (A2+, NY-ESO-1-, Melan-A-, tyrosinase- and gp100-) were used as targets. Briefly, after labeling with 51Cr for 1 hr at 37°C followed by extensive washing, target cells (1000/well) were incubated with effector cells at the indicated effector to target cell (E:T) ratio for 4 hr at 37°C in the absence or in the presence of the indicated synthetic peptide (1 µM). In peptide titration experiments, target cells were incubated with effector cells at an E:T ratio of 10:1 in the presence of serial dilutions of the peptide indicated. Antigen recognition experiments were performed under reducing conditions using TCEP as detailed previously (29). Chromium release was measured in the supernatant of the cultures using a gamma counter. The percentage specific lysis was calculated as: 100 x [(experimental-spontaneous release) / (total-spontaneous release)].

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**Contact**

**Address correspondence to:**

Danila Valmori  
Ludwig Institute Clinical Trial Center at Division of Medical Oncology  
Department of Medicine  
Columbia University College of Physicians and Surgeons  
650 W 168th Street, Black Building 20-22  
New York, NY 10032  
USA  
Tel.: + 1 212 305-3923  
Fax: + 1 212 305-7348  
E-mail: valmori@cancercenter.columbia.edu