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Efficient Simultaneous Presentation of NY-ESO-1/LAGE-1 Primary and Nonprimary Open Reading Frame-Derived CTL Epitopes in Melanoma

Donata Rimoldi,[1]* Verena Rubio-Godoy,[‡] Valerie Dutoit,[‡] Danielle Lienard,[‡§] Suzanne Salvi,* Philippe Guillaume,[¶] Daniel Speiser,[§] Elisabeth Stockert,[¶] Giulio Spagnoli,[‖] Catherine Servis,[†] Jean-Charles Cerottini,*[‡] Ferdy Lejeune,[§] Pedro Romero,* and Danila Valmori*

Recent studies have shown that CTL epitopes derived from tumor-associated Ags can be encoded by both primary and nonprimary open reading frames (ORF). In this study we have analyzed the HLA-A2-restricted CD8$^+$ T cell response to a recently identified CTL epitope derived from an alternative ORF product of gene LAGE-1 (named CAMEL), and the highly homologous gene NY-ESO-1 in melanoma patients. Using MHC/peptide tetramers we detected CAMEL$_{1-11}$-specific CD8$^+$ T cells in peptide-stimulated PBMC as well as among tumor-infiltrated lymph node cells from several patients. Sorting and expansion of tetramer$^+$ CD8$^+$ T cells allowed the isolation of tetramer^{bright} and tetramer^{dull} populations that specifically recognized the peptide Ag with high and low avidity, respectively. Remarkably, only high avidity CAMEL-specific CTL were able to recognize Ag-expressing tumor cells. A large series of HLA-A2-positive melanoma cell lines was characterized for the expression of LAGE-1 and NY-ESO-1 mRNA and protein and tested for recognition by CAMEL-specific CTL as well as CTL that recognize a peptide (NY-ESO-1$_{157-165}$) encoded by the primary ORF products of the LAGE-1 and NY-ESO-1 genes. This analysis revealed that tumor-associated CD8$^+$ T cell epitopes are simultaneously and efficiently generated from both primary and nonprimary ORF products of LAGE-1 and NY-ESO-1 genes and, importantly, that this occurs in the majority of melanoma tumors. These findings underscore the in vivo immunological relevance of CTL epitopes derived from nonprimary ORF products and support their use as candidate vaccines for inducing tumor specific cell-mediated immunity against cancer. ***The Journal of Immunology*****, 2000, 165: 7253–7261.**

The molecular definition of Ags recognized by CTL in human cancer constitutes an important step toward understanding anti-tumor CTL reactivity. Besides CTL epitopes traditionally encoded by primary open reading frames (ORF1),[2] epitopes encoded by alternative sources such as noncoding regions and nonprimary ORF have been described in both murine models and human cells (reviewed in Ref. 1). To date, the majority of the identified, naturally occurring CTL epitopes encoded by nonprimary ORF are derived from tumors (2–5), and the list is likely to lengthen due to the bias toward increased frameshifting as well as other genetic events observed in neoplastic cells (6). Thus, CTL epitopes derived from nonprimary ORF products in principle represent suitable targets for a tumor-associated Ag-targeted CTL therapy of cancer. However, one potential pitfall is represented by the relatively low abundance with which most nonprimary ORF-encoded products are generated compared with primary ORF-derived proteins (1).

NY-ESO-1 and LAGE-1 are two highly homologous genes encoding tumor-specific Ags expressed in a significant proportion of tumors of different histological types (7, 8). These genes are not expressed in normal tissues, except testis and, to a much lower extent, placenta, ovary, and uterus and thus belong to the so-called cancer-testis gene family. NY-ESO-1 and LAGE-1 mRNAs and their predicted protein products are summarized in Fig. 5. Two forms of LAGE-1 transcripts, LAGE-1$_S$ and LAGE-1$_L$, have been identified, the latter deriving from retention of intron 2 (8). NY-ESO-1 and LAGE-1$_S$ primary ORF code for homologous proteins of 180 aa. The partially spliced LAGE-1$_L$ mRNA contains an ORF encompassing most of intron 2 and encodes a putative protein of 210 aa. In addition, LAGE-1 and NY-ESO-1 ORF2 code for two putative products of 109 and 58 aa, respectively. Several NY-ESO-1- and LAGE-1-derived CTL epitopes have been identified (3, 4, 9). In particular, an HLA-A2-restricted CTL epitope derived from the primary ORF of NY-ESO-1 spanning residues 157–165 has been recently described (9, 10). An identical sequence is present in the LAGE-1$_S$ protein as predicted by the primary ORF. In addition, a second HLA-A2-restricted CTL epitope derived from a LAGE-1 gene product, named CAMEL, and encoded by ORF2 of LAGE-1$_S$ and LAGE-1$_L$, has also been identified (4). The antigenic peptide has been mapped to residues 1–11 of this putative protein. An identical sequence is found in the putative product of NY-ESO-1 ORF2, and indeed, CTL specific for CAMEL have been shown to recognize NY-ESO-1-transfected COS-7 cells (4).

In the present study we have derived CAMEL$_{1-11}$-specific CD8$^+$ T cell populations from HLA-A2$^+$ melanoma patients and analyzed their ability to recognize and lyse NY-ESO-1- and/or

*Ludwig Institute for Cancer Research, Lausanne Branch, [†]Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland; [‡]Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne Branch, and [§]Multidisciplinary Oncology Center, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; [¶]Ludwig Institute for Cancer Research, New York Branch, New York, NY 10021; and [‖]Department of Surgery, University of Basel, Basel, Switzerland

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[1] Address correspondence and reprint requests to Dr. Donata Rimoldi, Ludwig Institute for Cancer Research, 155 Chemin des Boveresses, 1066 Epalinges, Switzerland. E-mail address: donata.rimoldi@isrec.unil.ch

[2] Abbreviations used in this paper: ORF, open reading frame; TILN, tumor-infiltrated lymph node cells.

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LAGE-1-expressing melanoma cell lines. We observed that only high avidity CAMEL-specific T cells are able to specifically recognize Ag-expressing tumor cells. Analysis of CTL recognition of a panel of Ag-expressing melanoma lines by both high avidity $CAMEL_{1–11}$- and NY-ESO-$1_{157–165}$-specific CTL revealed that tumor-associated $CD8^+$ T cell epitopes can be simultaneously generated from both primary and nonprimary ORF with similar efficiency.

Materials and Methods

Patients, tumors, and melanoma cell lines

Frozen tumor samples from 62 stage II–IV melanoma patients were selected for analysis of NY-ESO-1 and LAGE-1 expression. Melanoma cell lines were established in our laboratory from surgically excised melanoma metastases and were cultured in RPMI 1640/10% FCS medium. The cells were characterized for surface expression of total HLA class I or HLA-A2 molecules by FACS analysis using W6-32 and BB7.2 Abs, respectively. All lines expressed the melanoma marker HMW-MAA and the adhesion molecule ICAM-1/CD54. The melanoma cell lines NA8-MEL, SK-Mel-37, 518/IL2.14, and FM6 were provided by Drs. F. Jotereau (U211, Institut National de la Santé et de la Recherche Médicale, Nantes, France), Y. T. Chen (Ludwig Institute for Cancer Research, New York Branch, New York, NY), P. Schrier (Leiden University Medical Center, Leiden, The Netherlands), and J. Zeuthen (Danish Cancer Society, Copenhagen, Denmark), respectively.

PCR analyses

RNA extraction from frozen tissue samples and cell lines was performed by the guanidinium thiocyanate/CsCl gradient method (11) and with TRIzol reagent (Life Technologies, Basel, Switzerland), respectively. cDNA synthesis was performed as previously described (11), and aliquots (equivalent to 100 ng of RNA) were used for different PCR using Qiagen DNA Taq polymerase (Basel, Switzerland). Amplification of NY-ESO-1 was performed for 35 cycles with primers ESO-1A (5′-ATGGATGCTG CAGATGCGG-3′) and ESO-1B (5′-GGCTTAGCGCCTCTGCCCTG-3′) with an annealing temperature of 60°C. Specific amplification of LAGE-1 sequences was performed as previously described (8). Both $LAGE\text{-}1_S$ and $LAGE\text{-}1_L$ are amplified by this PCR assay, yielding products of 399 and 628 bp, respectively. Amplification of actin for 21 cycles was performed on each cDNA sample to assess the quality and quantity of input RNA (11). Dilutions of input RNA from a reference cell line expressing both NY-ESO-1 and LAGE-1 (SK-Mel-37) during each cDNA synthesis allowed a semiquantitative analysis of NY-ESO-1 and LAGE-1 expression.

Tetramers, mAbs, and flow cytometric immunofluorescence analysis

HLA-A2/peptide tetramers were synthesized as previously described (12, 13). The $CAMEL_{1–11}$ sequence (MLMAQEALAFL) was used as the antigenic peptide. Cells were stained with tetramers (20 μl of 10 μg/ml in PBS, 2% BSA, and 0.2% NaN_3) for 1 h at room temperature, then further incubated with anti-CD8-FITC (20 μl; Becton Dickinson, Mountain View, CA) at 4°C for 30 min. Cells were washed once with the same buffer and analyzed by flow cytometry. Data analysis was performed using CellQuest software.

MLC, isolation of CAMEL-specific $CD8^+$ T cells, and cloning

For peptide stimulation experiments, $CD8^+$ lymphocytes were positively selected by magnetic cell sorting from PBMC of HLA-A*0201 melanoma patients using a miniMACS device (Miltenyi Biotec, Sunnyvale, CA). Cells from the $CD8^-$ fraction were irradiated (3000 rad) and used as APC. $CD8^+$ highly enriched lymphocytes (1×10^6/well) were stimulated with peptide (1 μM) and irradiated autologous APC in 2 ml of CTL medium (14) containing human rIL-2 (100 U/ml; Glaxo Welcome, Geneva, Switzerland; provided by Dr. M. Nabholz, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) and human rIL-7 (10 ng/ml; R&D Systems Europe, Oxon, U.K.). Cells were cultured for 2 wk before A2/CAMEL peptide tetramer analysis. A2/CAMEL $tetramer^+$ cells were isolated by FACS as previously described. CAMEL-specific CTL clones were derived from A2/CAMEL $tetramer^+$-sorted cells by limiting dilution cultures in the presence of irradiated allogeneic PBMC, PHA, and rIL-2 as described previously (15).

Chromium release assay

Ag recognition was assessed using chromium release assay. Target cells were labeled with ^{51}Cr for 1 h at 37°C and washed twice. Labeled 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 (50 μl). Chromium release was measured in the supernatant (100 μl) harvested after 4-h incubation at 37°C. The percent specific lysis was calculated as: $100 \times$ [(experimental − spontaneous release)/(total − spontaneous release)].

Transient transfections and TNF release assay

Plasmids containing full-length NY-ESO-1, $LAGE\text{-}1_S$, and $LAGE\text{-}1_L$ cDNAs were gifts from B. Lethé (Ludwig Institute for Cancer Research, Brussels, Belgium). Minigene-containing plasmids were based on the previously described pGFP/Ub plasmid (16). Sites *Sac*II and *Ava*I were used to insert minigenes at the 3′ end of the ubiquitin sequence. Minigenes encoding the NY-ESO-$1_{157–165}$- and $CAMEL_{1–11}$-derived sequences were obtained by annealing of complementary synthetic oligonucleotides (Microsynth, Balgach, Switzerland) designed so as to reconstitute the *Sac*II and *Ava*I sites and including a stop codon. NA8-MEL and COS-7 cells were transiently transfected using Lipofectamine Plus reagent according to the manufacturer's instructions (Life Technologies) and as previously described (16). COS-7 cells were cotransfected with a plasmid encoding HLA-A2. Transfected cells were then tested for their ability to stimulate the release of TNF by the NY-ESO-$1_{157–165}$- and $CAMEL_{1–11}$-specific CTL. In brief, CTL were added at the appropriate effector to stimulator cell ratio in 200 μl of IMDM supplemented with 10% human serum and 20 U/ml rIL2 (Glaxo Welcome). Where indicated, synthetic peptides (1 μM) were added. After a 24-h incubation at 37°C supernatants were collected, and the TNF content was determined in a functional assay using WEHI-164 clone 13 cells (17) as previously described (18).

Western blot analysis

Cells lysates were prepared with a Nonidet P-40 buffer and subjected to SDS-PAGE (15% gel) under reducing conditions. Western blotting was performed as previously described (19). The anti-NY-ESO-1 mouse mAbs used were ES121 (20) (A. Jungbluth et al., manuscript in preparation), a mouse mAb recognizing specifically the NY-ESO-1 protein, and B9.8, recognizing both NY-ESO-1 and $LAGE\text{-}1_S$ (21). Peroxidase-conjugated anti-mouse secondary Ab and ECL detection system were obtained from Amersham Pharmacia Biotech Europe (Duebendorf, Switzerland). The primary Ab specificities were confirmed by Western blot analysis of lysates of COS-7 cells transfected with NY-ESO-1, $LAGE\text{-}1_S$, and $LAGE\text{-}1_L$ cDNAs.

Results

*Assessment of CAMEL-specific $CD8^+$ T cell responses in HLA-A*0201 melanoma patients and isolation of polyclonal and monoclonal specific T cell populations*

To determine the proportion of HLA-A*0201 melanoma patients responsive to in vitro stimulation with the $CAMEL_{1–11}$ peptide MLMAQEALAFL, highly enriched $CD8^+$ T cells prepared from blood samples of 33 patients were cultured for 2 wk in the presence of peptide (1 μM), autologous APCs, and cytokines as previously described (14). To directly enumerate $CAMEL_{1–11}$-specific $CD8^+$ T cells in these cultures, we prepared fluorescent A2/$CAMEL_{1–11}$ peptide tetramers (12, 13). $Tetramer^+$ $CD8^+$ populations were clearly detected in three melanoma patients, LAU 50, 143, and 342 (Fig. 1*A*). Interestingly, the tumor from patient LAU 50 expressed both NY-ESO-1 and LAGE-1 (see Table I). Gene expression could not be assessed in the lesions from patients LAU 143 and 342 due to lack of tumor material. A2/$CAMEL_{1–11}$ $tetramer^+$ cells were then isolated by cell sorting followed by PHA-driven expansion, either in bulk or as clones by plating at limiting dilution (14, 15). From the culture derived from patient LAU 50 we isolated a polyclonal cell line highly enriched in $tetramer^+$ $CD8^+$ T cells as well as several $tetramer^+$ $CD8^+$ T cell clones (Fig. 1*B*). In addition, a polyclonal monospecific line, but no clones, was isolated from the culture derived from patient LAU 342. Remarkably, whereas the $tetramer^+$ $CD8^+$ cells present

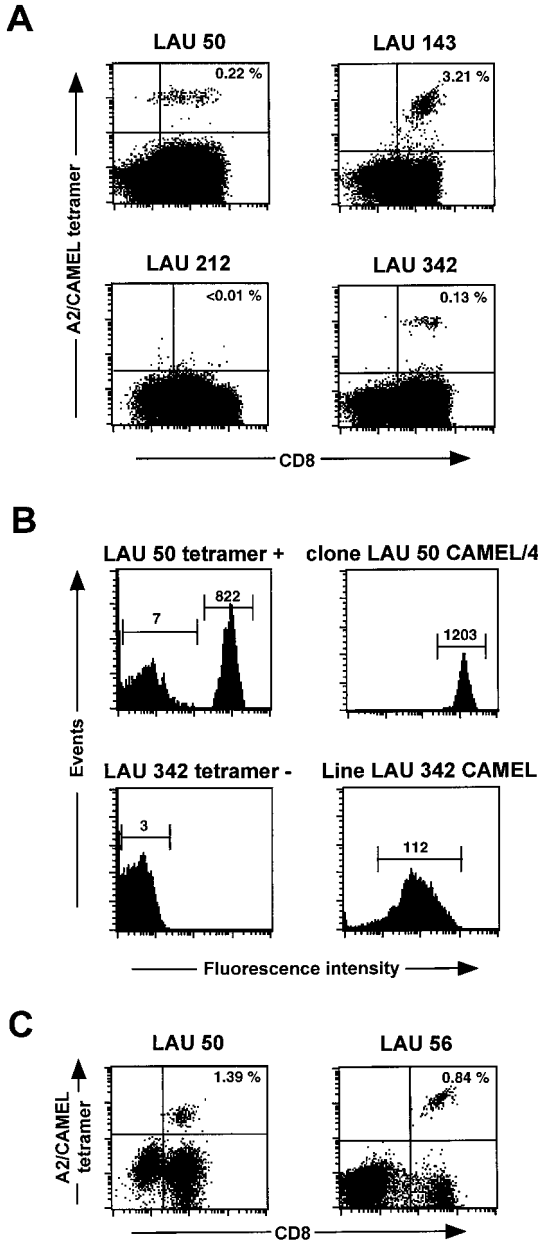

FIGURE 1. Tetramer-guided analysis of CAMEL $_{1–11}$/ORF2-specific CD8$^+$ T cell responses in A2$^+$ melanoma patients. *A*, CD8$^+$-enriched PBMC from melanoma patients were stained with PE-conjugated A2/CAMEL$_{1–11}$ tetramers together with fluorescein-conjugated anti-CD8 mAb on day 14 after a single in vitro stimulation with peptide CAMEL$_{1–11}$ (1 μM). Dot plots are shown for three responder (LAU 50, 143, and 342) and one nonresponder patient (LAU 212). Numbers in the *upper right quadrant* indicate the percentage of A2/CAMEL$_{1–11}$ tetramer$^+$ cells within CD8$^+$ lymphocytes. *B*, Histograms of CAMEL$_{1–11}$ tetramer-sorted populations from patients LAU 50 and 342. A highly enriched A2/CAMEL$_{1–11}$ tetramer$^+$ cell population (LAU 50 tetramer$^+$) and a representative clone (clone LAU 50 CAMEL/4) from patient LAU 50 are shown in the *upper panels*. The *lower panels* show a tetramer$^-$ and a polyclonal monospecific tetramer$^+$ population from patient LAU 342 (line LAU 342 CAMEL). Numbers indicate the mean fluorescence of gated populations. *C*, TIL, cultured for 2 wk in the presence of exogenously added cytokines only, were stained as described in *A*. Dot plots are shown for patients LAU 50 and 56.

in the unsorted LAU 342 culture displayed a bright fluorescence signal (Fig. 1*A*), the sorted line was tetramer dull (Fig. 1*B*). This phenotype was maintained over several cycles of in vitro stimulation with PHA. One representative clone from patient LAU 50 (clone LAU 50 CAMEL/4) and the line LAU 342 CAMEL (Fig. 1*B*) were selected for further analysis. A2/CAMEL$_{1–11}$ tetramer$^+$ CD8$^+$ T cells were also detected among tumor-infiltrated lymph node cells (TILN) from HLA-A2 melanoma patients cultured for 2 wk in the presence of exogenously added cytokines only (Fig. 1*C*). These results underline the existence of natural CAMEL-specific CD8$^+$ T cell responses in several melanoma patients.

A2/CAMEL$_{1–11}$ peptide tetramer^{bright} and tetramer^{dull} T cells recognize MHC/peptide complexes with high and low avidity

To assess whether the difference in the intensity of T cell staining by specific MHC/peptide tetramers correlated with different avidity of the Ag-specific T cells, as suggested by a recent study (22), we performed the experiments illustrated in Fig. 2. Both clone LAU 50 CAMEL/4 (bright tetramer staining) and line LAU 342 CAMEL (dull tetramer staining) specifically lysed T2 cells in the presence, but not in the absence, of a high dose (1 μM) of peptide CAMEL$_{1–11}$. However, lysis by clone LAU 50 CAMEL/4 was slightly more efficient than that by line LAU 342 CAMEL at high E:T cell ratios (Fig. 2*A*). Moreover, peptide titration showed that clone LAU 50 CAMEL/4 recognized the peptide CAMEL$_{1–11}$ about 10,000-fold more efficiently than it did line LAU 342 CAMEL (Fig. 2*B*). Using a single specific T cell clone, amino acid sequence 1–11 from CAMEL has been previously identified as the optimal antigenic peptide (4). To determine the length requirements for TCR recognition by the CAMEL$_{1–11}$-specific T cells described in this study we tested both N- and C-terminally truncated peptide analogues (Fig. 2*C*). Indeed, the results of this analysis confirmed the findings of Aarnoudse et al. (4). Truncation of the N-terminal methionine residue resulted in ~10-fold decreased recognition by clone LAU 50 CAMEL/4 and loss of recognition by line LAU 342 CAMEL. Further truncation of the leucine at position 2 resulted in loss of T cell recognition by clone LAU 50 CAMEL/4. In addition, truncations at the C terminus of the peptide completely abolished recognition by both T cell populations.

Analysis of NY-ESO-1/LAGE expression by melanoma tumors and cell lines

A highly correlated expression of NY-ESO-1 and LAGE-1 genes in a small number of melanoma tumors has been reported, with five of six positive tumors coexpressing the two genes (8). We investigated extensively the expression of the two genes in a large series of melanoma metastases (100 tumors from 63 patients) by RT-PCR analysis. On a patient basis, 14 (23%) expressed NY-ESO-1, and 20 (33%) expressed LAGE-1 in their tumors at a level >1% of that found in the melanoma cell line SK-Mel-37, which was used as a reference. In addition, six and one patients expressed very low levels (equivalent to <1% of those in SK-Mel-37) of NY-ESO-1 and LAGE-1, respectively. Table I summarizes the PCR results for the patients with NY-ESO-1- and/or LAGE-1-positive tumors. Ten of the 14 patients whose tumors clearly expressed NY-ESO-1 were also positive for LAGE-1 expression. However, tumors from several patients (e.g., LAU 4, 119, 156, 203, 331, and 332) selectively expressed only one of the two genes (4 and 10 for NY-ESO-1 and LAGE-1, respectively). Multiple simultaneous and/or subsequent metastases were available for analysis from 26 of the patients studied. A conserved pattern of expression of NY-ESO-1 and LAGE-1 was observed in the tumors of the majority of these patients, as found for 16 NY-ESO-1- and LAGE-1-negative patients (not shown) and for LAU 4, 53, and 156 (Table I). However, heterogeneous expression was clearly observed in some patients (e.g., LAU 242 and 321). A correlation between expression of NY-ESO-1/LAGE-1 and MAGE-1 and -3 has been reported (8). RT-PCR analyses for MAGE-1 and -4 (the

Table I. *Analysis of NY-ESO-1 and LAGE-1 expression in melanoma tumors*

Patient	Sample[a]	NY-ESO-1	LAGE-1	MAGE-1	MAGE-4	Cell Line[b]
LAU 50	Cut, 93	+[c]	++	(+)	++	
LAU 50	LN, 95	+	–	++	++	Me 275
LAU 53	Cut, 96	+	++	–	+++	
LAU 53	Cut, 96	+	+	–	++	
LAU 53	Cut, 97	+	+	–	++	Me 312/Me 325
LAU 53	Cut, 98	+++	–	–	+++	Me 333
LAU 86	LN	+++	+++	+++	+++	Me 237
LAU 148	LN	+	+	++	+++	
LAU 193	LN	+	++	+++	++	
LAU 194	LN	+	++	+++	–	Me 285.A
LAU 202	LN	++	+	+++	+++	
LAU 205	Primary	(+)	++	–	–	
LAU 205	LN	(+)	–	–	–	
LAU 242	LN, Feb. 97	–	–	++	–	Me 311
LAU 242	LN, Oct. 97	+	+++	++	+	Me 324
LAU 302	Cut	+++	+	++	+	Me 342
LAU 333	LN	++	+++	++	–	
LAU 4	Cut, 94	+++	–	+++	–	
LAU 4	Cut, 94	+++	–	+++	–	
LAU 4	Cut, 95	+++	–	+++	(+)	
LAU 4	Cut, 95	+++	–	+++	–	
LAU 119	LN	+++	–	+++	++	Me 252
LAU 156[d]	Bone, 97	+++	–	–	+	
LAU 156[d]	Bone, 99	+++	–	+	++	
LAU 266	Cut	+++	–	++	+++	Me 329.M2
LAU 56	Cut, 93	–	–	–	–	
LAU 56	LN, 97	(+)	–	–	–	Me 323
LAU 92	LN, May 94	–	–	–	–	Me 242.B.1
LAU 92	Muscle, Jul. 94	–	+	++	–	
LAU 149	Sub-cut, 98	–	+	–	–	
LAU 149	Paravert, 99	–	–	–	–	
LAU 181	LN	–	+	+++	–	
LAU 203	LN	–	+++	+	–	Me 290
LAU 289	Sub-cut	–	+	+++	++	Me 343
LAU 321	Cut	–	–	–	–	
LAU 321	LN	–	++	++	+++	
LAU 331	LN	–	+++	++	–	
LAU 332	LN	–	+++	–	–	
LAU 350	LN	–	+	–	–	
LAU 362	LN	–	++	–	–	
LAU 145	Sub-cut	–	–	–	–	
LAU 145	Sub-cut	–	–	–	++	Me 257
LAU 343	LN	–	–	+	–	T343A
LAU 343	LN	–	–	+	–	

[a] Except for one primary tumor (LAU 205), all lesions were metastatic. LN, Lymph node; cut, cutaneous; paravert, paravertebral. When multiple metastases were not removed simultaneously, dates of surgery are indicated.

[b] Tumors from which cell lines were derived are indicated.

[c] RT/PCR analyses and semiquantitative assessment of mRNA expression were performed as described in *Materials and Methods*, using SK-Mel-37 (for NY-ESO-1 and LAGE-1) and MZ2-MEL-3.0 (for MAGE genes) melanoma cells as reference. Expression levels were scored as follows: +++, 50–200%; ++, 10–50%; +, 1–10%; (+), <1% of the levels found in the reference cell line.

[d] This patient had an ocular melanoma.

latter being expressed in melanoma at a frequency similar to that reported for NY-ESO-1 and LAGE-1) (11) were thus performed. MAGE-1 and -4 were expressed in 57 and 50% of the NY-ESO-1- and/or LAGE-1-positive tumor samples ($n = 42$), respectively, but only in 38 and 14% of the negative ones ($n = 58$; Table I and data not shown).

To study the correlation between expression of NY-ESO-1 and LAGE-1 by tumor cells and recognition by CAMEL $_{1–11}$- and NY-ESO-$1_{157–165}$-specific CTL, we characterized in detail the expression of these genes in 35 melanoma cell lines derived from the patients analyzed above. Expression of one or both genes was detected by RT-PCR in 17 cell lines, while the remainder were negative. In the majority of cases (>75%) the gene expression profile of cultured cells reflected that of the original tumor. The remaining cases probably reflect a heterogeneous expression of NY-ESO-1/LAGE-1 within the tumor lesion. Indeed, staining of melanoma metastases with mAb B9.8 revealed intratumor heterogeneity, both as the percentage of positive cells and as staining intensity, in a fraction of the samples analyzed (21). Table II summarizes the results obtained for the positive melanoma cell lines. Cell lines SK-Mel-37, 518/IL-2.14, and FM6, used in previous reports (4, 9), were also included as a reference. Western blot analyses of lysates of melanoma cell lines with Abs recognizing NY-ESO-1 only or both NY-ESO-1 and LAGE-1 proteins confirmed the expression of the genes at the protein level (Fig. 3). Overall, there appeared to be a correlation between the degree of expression at the RNA and protein levels (Fig. 3 and Table II).

Assessment of tumor killing by CAMEL$_{1–11}$- and NY-ESO-1-specific CTL

We initially compared high and low avidity CAMEL$_{1–11}$-specific CTL for their capacity to kill melanoma cells expressing NY-

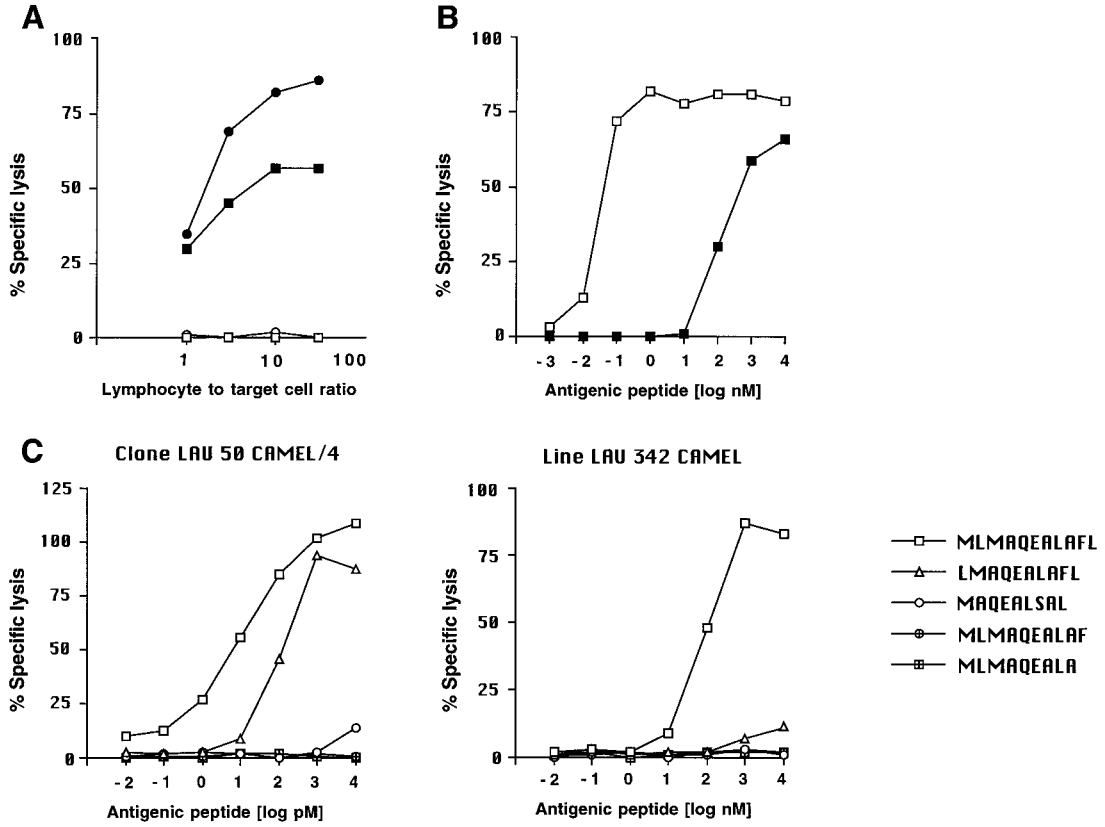

FIGURE 2. A2/CAMEL$_{1–11}$ tetramer^{bright} and tetramer^{dull} T cell populations recognize CAMEL$_{1–11}$ peptide with high and low avidity, respectively. Clone LAU 50 CAMEL/4 (tetramer^{bright}) and line LAU 342 CAMEL (tetramer^{dull}) were tested for Ag recognition in a 4-h chromium release assay as detailed in *Materials and Methods*. Lysis of T2 cells was assessed either *A*) at the indicated lymphocyte/target (L/T) cell ratio in the absence (open symbols) or presence (solid symbols) of peptide CAMEL$_{1–11}$ (1 μM); or *B*) at the L/T ratio of 10:1 in the presence of serial CAMEL$_{1–11}$ peptide dilutions. □, clone LAU 50 CAMEL/4; ■, line LAU 342 CAMEL. *C*, Recognition of CAMEL-truncated peptides by clone LAU 50 CAMEL/4 and line LAU 342 CAMEL was assessed in a 4-h chromium release assay at the L/T ratio of 10:1.

ESO-1/LAGE-1. Remarkably, only high avidity CTL were able to specifically lyse Ag-expressing tumors, although both high and low avidity cells efficiently lysed HLA-A2$^+$ NY-ESO-1/LAGE-1$^-$ targets in the presence of exogenously added peptide CAMEL$_{1–11}$ (Fig. 4 and data not shown).

We next analyzed the capacity of high avidity CAMEL$_{1–11}$/ORF2-specific CTL (clone LAU 50 CAMEL/4) to lyse the melanoma cell lines listed above. Tumor recognition by a CTL clone (clone LAU 156 NY-ESO-1/5) (10) specific for the NY-ESO-1/LAGE-1$_S$ peptide 157–165 derived from the primary ORF product (see Fig. 5) was tested in parallel. The two T cell clones showed similar patterns of tumor recognition (Table II). The reference melanoma cell lines SK-Mel-37, FM6, and 518/IL2.14 (NY-ESO-1$^+$, LAGE-1$^+$, HLA-A2$^+$) were efficiently lysed in both the absence and the presence of the antigenic peptides. The melanoma cell line NA8-MEL (NY-ESO-1$^-$, LAGE-1$^-$, HLA-A2$^+$) was efficiently lysed only in the presence of antigenic peptide, while no significant lysis was detected on melanoma line Me 242.B.1 (NY-ESO-1$^+$ and LAGE-1$^+$ but HLA-A2$^-$).

Among the melanoma cell lines generated in our laboratory, the majority of the NY-ESO-1/LAGE-1$^+$ lines were specifically lysed by both the NY-ESO-1/LAGE-1 ORF1- and ORF2-specific CTL in the absence of exogenously added peptide, although the level of specific lysis varied from line to line. An exception was represented by cell line Me 257, which was not significantly lysed in the absence of antigenic peptide despite clearly positive NY-ESO-1 and LAGE-1 gene expression (Fig. 3). However, Me 257 cells (which express the melanocyte differentiation Ags Melan-A/MART-1 and tyrosinase) were specifically lysed by Melan-A/MART-1 and tyrosinase-specific CTL clones in the absence of exogenously added antigenic peptides, thus excluding a general defect in the presentation of endogenously derived peptides (data not shown). Two melanoma cell lines expressing NY-ESO-1 but not LAGE-1 (Me 333 and Me 252) were also specifically lysed by both CTL clones, although to a limited extent. Finally, of six melanoma lines expressing LAGE-1 but not NY-ESO-1, three were efficiently lysed (Me 237, Me 285.A, Me 290), while the remainders were not (Me 323, Me 324, T343A). It should be noted that among the latter, Me 323 and T343A cells, although they were LAGE-1 PCR positive, expressed very low levels of LAGE-1 protein (Fig. 3). In addition, Me 323 cells (Melan-A/MART-1$^+$, tyrosinase$^+$) also failed to present endogenously derived Melan-A/MART-1 and tyrosinase peptides to the corresponding CTL clones (data not shown). Finally, all the HLA-A2$^+$ melanoma lines tested (expressing, or not, NY-ESO-1 and LAGE-1) were efficiently lysed by either CTL clone in the presence of the appropriate antigenic peptide.

Recognition of NA8-MEL and COS-7 cells transiently transfected with NY-ESO-1- and LAGE-1-encoding constructs by specific CTL

To unambiguously assess recognition of NY-ESO-1- or LAGE-1-expressing cells by ORF1- and ORF2-specific CTL, melanoma cells NA8-MEL (NY-ESO-1$^-$, LAGE-1$^-$, HLA-A2$^+$) and COS-7 cells (NY-ESO-1$^-$, LAGE-1$^-$, HLA-A2$^-$) were transiently transfected with plasmids encoding full-length NY-ESO-1, LAGE-1$_S$,

Table II. *Correlation between NY-ESO-1 and LAGE-1 expression and tumor recognition by CAMEL$_{1–11}$/ORF2- and NY-ESO-1$_{157–165}$/ORF1-specific CTL*

		Ag Expression					Lysis by Specific CTL Clones[b]			
		PCR[c]		Western[d]			LAU 50 CAMEL/4		LAU 156 NY-ESO-1/5	
Patient	Cell Line	NY-ESO-1	LAGE-1	NY-ESO-1	NY-ESO-1/LAGE-1	HLA-A2[a]	–P	+P	–P	+P
LAU 50	Me 275/CI2	+++	++	+++	+++	+	+++	+++	+++	+++
LAU 53	Me 312	++	++	+++	+++	+	++	++	++	+++
LAU 53	Me 325	++	++	++	++	+	+++	+++	+++	+++
LAU 53	Me 333	++	–	+	+	+	+	+++	+	+++
LAU 289	Me 343	–/+	++	–	++	+	–	+++	+	+++
LAU 302	Me 342	+++	++	++	+++	+	++	+++	+++	+++
LAU 145	Me 257	++	++	++	+++	+	–	+++	–	+++
LAU 92	Me 242.B.1	+++	++	+++	+++	–	–	–	–	–
LAU 119	Me 252	++	–	+++	+++	+	+	+++	+++	+++
LAU 343	T343A	–	+	–	(+)	+	–	+++	–	+++
LAU 56	Me 323	–	++	–	(+)	+	–	+++	–	+++
LAU 86	Me 237	–	++	–	+	+	++	+++	+++	+++
LAU 194	Me 285.A	–	+	–	+	+	+++	+++	+++	+++
Me 203	Me 290	–	++	–	+	+	++	+++	++	+++
LAU 242	Me 324	–	++	–	++	+	–	+++	–	+++
	NA8-MEL	–	–	–	–	+	–	+++	–	+++
	SK-Mel-37	+++	+++	+++	+++	+	+++	+++	++	++
	518/IL-2.14	+++	+++	+++	+++	+	+++	+++	+++	+++
	FM6	+++	+++	+++	+++	+	+++	+++	+++	+++

[a] HLA-A2 surface expression was confirmed by flow cytometric analysis.

[b] Tumor lysis by specific CTL clones was assessed in a standard 4-h chromium release assay as detailed in *Materials and Methods*. Results obtained at E:T ratio of 30 are shown. Percentage of specific lysis is indicated as –, <20%; +, 20–40%; ++, 40–60%, and +++, >60%. –P and +P, Without and with the addition of 1 μM peptide.

[c] NY-ESO-1 and LAGE-1 expression by melanoma lines was assessed by PCR analysis. Scoring was performed as described in the legend to Table I.

[d] Western blotting was performed as described in *Materials and Methods* with Ab ES121 (detecting NY-ESO-1) and B9.8 (detecting both NY-ESO-1 and LAGE-1). Semiquantitative scoring was made by visual assessment of Western blots similar to that shown in Fig. 4.

and LAGE-1$_L$ cDNAs. COS-7 cells were cotransfected with a plasmid encoding HLA-A2. Transfected cells were then tested for their ability to specifically stimulate TNF release by NY-ESO-1$_{157–165}$ (ORF1) and CAMEL$_{1–11}$ (ORF2)-specific CTL. As summarized in Table III and in good agreement with the tumor recognition data shown above, both NY-ESO-1 and high avidity CAMEL-specific CTL efficiently recognized NA8-MEL and COS-7 cells transfected with plasmids encoding NY-ESO-1, LAGE-1$_S$, or LAGE-1$_L$. Importantly, both NY-ESO-1 and CAMEL-specific CTL clones exhibited a similar pattern of recognition, indicating that the relevant antigenic peptides were simultaneously generated with comparable efficiency. In addition, these results, together with those on tumor cells shown above, formally prove that the epitope recognized by NY-ESO-1$_{157–165}$-specific CTL is also generated from the homologous LAGE-1$_S$ protein. Interestingly, the comparable efficiencies in stimulating TNF secretion observed with LAGE-1$_S$ and LAGE-1$_L$ transfectants suggest that most of the primary LAGE-1$_L$ transcripts undergo complete splicing (see Fig. 5). Low avidity CAMEL-specific CTL specifically secreted TNF only upon stimulation with transfected COS-7, but not NA8-MEL, cells. Because COS-7 cells are more efficiently transfected and express higher levels of the transfected gene than NA8-MEL cells (23) (our data, not shown), these results indicate that low avidity CAMEL-specific CTL can indeed recognize the endogenously expressed Ag, but only when present at very high (and presumably not physiological) levels.

We also analyzed the ability of CAMEL-specific CTL to recognize the product of plasmids encoding minimal epitopes. As shown in Table III, CAMEL-specific CTL secreted TNF above background levels only upon stimulation with NA8-MEL transfected with plasmids encoding the CAMEL$_{1–11}$ pGFP/Ub minigene. Cells transfected with a NY-ESO-1$_{157–165}$ pGFP/Ub minigene, used as an internal control, failed to stimulate specific TNF secretion by clone LAU 50 CAMEL/4, but were efficiently recognized by clone LAU 156 ESO/5 (Table III).

Discussion

The gene encoding NY-ESO-1 has been recently identified by immunoscreening of cDNA expression libraries from tumors (7). LAGE-1 was independently identified by representational difference analysis of genes that are expressed in human melanoma cell lines and not in normal skin (8). NY-ESO-1 and LAGE-1 display 94% identity at the nucleotide level and are expressed by different types of tumors, including melanoma. In this study we have extensively analyzed the expression of the two genes in melanoma lesions. On a patient basis, 23% expressed NY-ESO-1, and 33% expressed LAGE-1 in their tumors. Although we found that the two genes were frequently coexpressed, thus confirming results reported by others on smaller tumor series (4, 8), melanoma lesions from a significant proportion of patients selectively expressed only one gene. Altogether, these results indicate that the proportion of melanoma patients expressing detectable levels of either NY-ESO-1 or LAGE-1 in at least one lesion, and thus eligible for specific immunotherapy, is relatively high (45%).

The identification of T cell epitopes from tumor-associated Ags recognized by CD8$^+$ lymphocytes in association with frequently expressed HLA class I alleles is a prerequisite for the design of peptide-based immunotherapy of cancer. Several T cell epitopes derived from the primary ORF of melanoma-associated Ags have been identified, including a number derived from CT Ags. Among the latter, NY-ESO-1 has recently received attention, because Ab responses, indicative of strong immunogenicity, have been detected in ~50% of late stage melanoma patients bearing NY-ESO-1-positive tumors (20). An NY-ESO-1/ORF1-derived CTL epitope recognized by HLA-A31-restricted TIL derived from a melanoma lesion has been described previously (3). More recently, an epitope

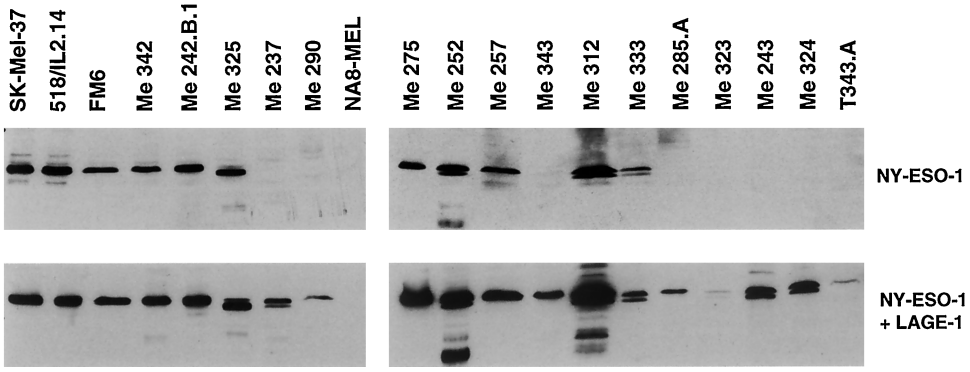

FIGURE 3. Expression of NY-ESO-1 and LAGE-1 proteins in melanoma cell lines. Lysates of the indicated melanoma cell lines (~50 μg protein/lane) were analyzed by Western blotting with mAbs ES121 (recognizing NY-ESO-1; *top panel*) and B9.8 (recognizing both NY-ESO-1 and LAGE-1; *bottom panel*). Note that NY-ESO-1 and LAGE-1 proteins appear to comigrate.

located in region 157–165 of NY-ESO-1 has been shown to be recognized by a tumor-reactive CTL line from a melanoma patient in association with HLA-A2 (an allele expressed by ~50% of Caucasians) (9). We have recently studied $CD8^+$ T cell responses to this epitope in HLA-A2 melanoma patients by using fluorescent HLA-A2/NY-ESO-$1_{157–165}$ peptide tetramers. Specific $CD8^+$ T cell responses were readily detected in peptide-stimulated $CD8^+$ T cell cultures from 50% (5 of 10) of the patients analyzed (10). Similarly, Jäger et al. have detected $CD8^+$ T cell responses to the same epitope (as measured by IFN-γ enzyme-linked immunospot assay) in 10 of 11 patients with NY-ESO-1 Abs, but not in seronegative patients or patients with NY-ESO-1-negative tumors (24).

The HLA-A2-restricted CAMEL-derived CTL epitope (aa 1–11) encoded by ORF2 of both LAGE-1 and NY-ESO-1 was originally identified as the target of a tumor-reactive CTL clone isolated from PBMC of a melanoma patient stimulated with IL-2-transfected autologous tumor cells (4). Few other human CTL epitopes derived from gene products encoded by alternative ORF have been described to date. These include a CTL epitope encoded by ORF3 of the wild-type TRP1/gp75 differentiation Ag and an epitope from NY-ESO-1 ORF2 (2, 3), both restricted by HLA-A31, an allele expressed by only 6% of Caucasians. More recently, an HLA-B*702-associated epitope derived from ORF2 of intestinal carboxyl esterase mRNA recognized by TIL from a renal cell carcinoma patient has been identified (5). Little is known on CTL responses to these alternative ORF-derived epitopes. In fact, most of the information available is still limited to single patients. In the present study we have detected A2/$CAMEL_{1–11}{}^+$ $tetramer^+$ $CD8^+$ T cells in peptide-stimulated PBMC from 3 of 33 (9%) $A2^+$ patients as well as in TILN. Thus, a response to this alternative ORF-derived epitope is not an exception resulting from a unique treatment/stimulation procedure (25). The proportion of patients with circulating $CAMEL_{1–11}$-specific $CD8^+$ T cells appeared to be lower than that for HLA-A2 NY-ESO-1 ORF1-specific $CD8^+$ T cells observed by us and others (10, 24). Nonetheless, this proportion might have been underestimated due to the stimulation protocol used in this study. This involved a single in vitro peptide stimulation in the presence of autologous APC, which is thus likely to give detectable responses only when a relatively high frequency of specific CTL precursors is present in the patient's PBMC. At any rate, the low percentage of responders to in vitro peptide stimulation does not necessarily imply the inability of nonresponders to mount specific anti-CAMEL CTL responses upon vaccination. While studying responses to the primary ORF-derived NY-ESO-$1_{157–165}$ epitope, we have observed that a specific CTL response could also be detected, albeit at a low level, in melanoma patients whose tumor lesion had no evidence of NY-ESO-1 expression (10). Future studies are planned to investigate, in a comparative setting, the incidence of responses to NY-ESO-$1_{157–165}$ and $CAMEL_{1–11}$ in groups of patients with NY-ESO-1/LAGE-1-positive or -negative tumors.

Upon tetramer-guided cell sorting, we derived dull and bright A2/$CAMEL_{1–11}$ $tetramer^+$ lymphocytes. The two populations exhibited a 10-fold difference in tetramer staining intensity at saturating tetramer concentrations. In agreement with a recent report (22), a correlation was observed between tetramer staining and T cell recognition avidity. Indeed, to obtain 50% maximal lytic activity on HLA-A2-positive target cells, A2/$CAMEL_{1–11}$ $tetramer^{dull}$ lymphocytes required a dose of antigenic peptide 10,000-fold higher than that required by A2/$CAMEL_{1–11}$ $tetramer^{bright}$ lymphocytes. Importantly, only A2/$CAMEL_{1–11}$ $tetramer^{bright}$ lymphocytes were able to efficiently recognize tumor cells endogenously expressing NY-ESO-1 and/or LAGE-1. These data indicate that fluorescent A2/$CAMEL_{1–11}$ peptide tetramers can be used to selectively identify high avidity tumor-reactive CAMEL-specific CTL.

The availability of isolated CTL clones specific for NY-ESO-1 and LAGE-1 sequences encoded by ORF1 and -2 made it possible to functionally assess the capacity of tumor cells to generate the corresponding epitopes. We characterized and tested a large panel of A2-positive NY-ESO-1- and/or LAGE-1-expressing melanoma

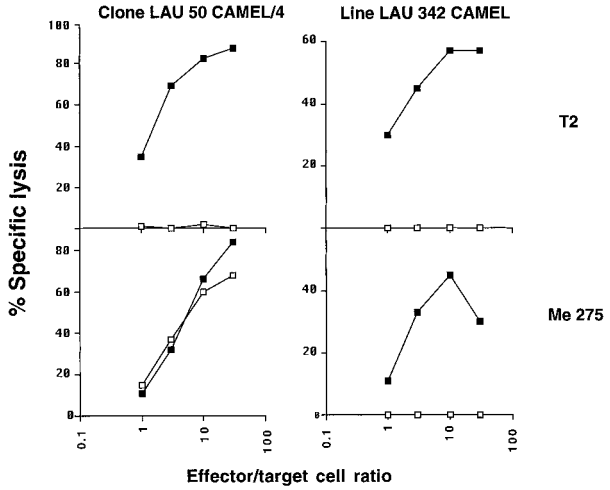

FIGURE 4. Tumor recognition by high and low avidity A2/$CAMEL_{1–11}$ $tetramer^+$ T cell populations. Specific lysis by clone LAU 50 CAMEL/4 ($tetramer^{bright}$) and line LAU 342 CAMEL ($tetramer^{dull}$) was assessed in a 4-h chromium release assay on T2 ($A2^+$, NY-ESO-1^-, LAGE-1^-) and Me 275 ($A2^+$, NY-ESO-1^+, LAGE-1^+) cells in either the absence (open symbols) or the presence (filled symbols) of exogenously added CAMEL peptide 1–11 (1 μM).

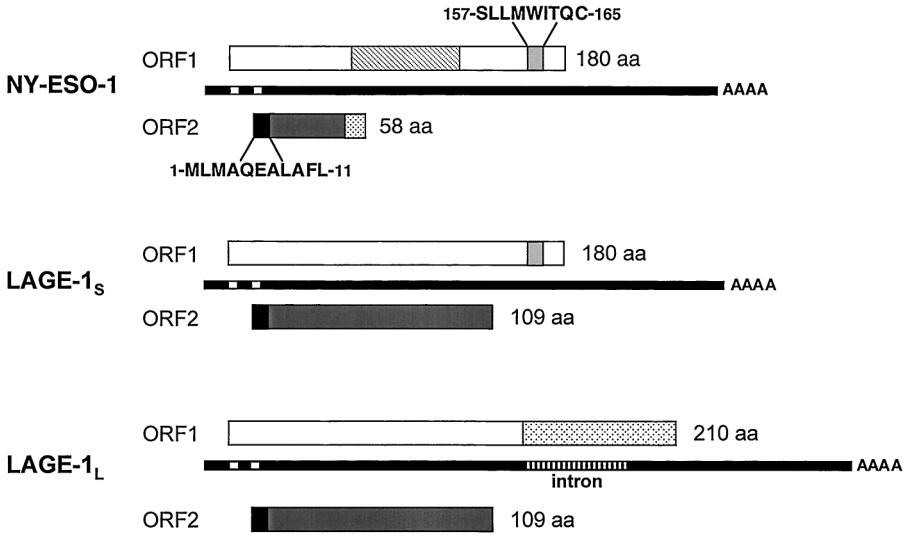

FIGURE 5. Schematic representation of NY-ESO-1/LAGE-1 transcripts and protein products. Translated proteins in ORF1 and putative polypeptides in ORF2 from NY-ESO-1 and the two alternatively spliced forms of LAGE-1 mRNA are indicated (modified from Ref. 4). ORF1 and -2 start at the first and second AUG codons, respectively (indicated in the transcripts as white boxes). A plain color is used for identical/highly homologous sequences, while patterned segments indicate more divergent regions. The location and sequence of the described HLA-A2-restricted CTL epitopes are shown.

cell lines. Several conclusions could be drawn from these experiments. 1) For the majority of the cell lines tested, there was a good correlation between NY-ESO-1/LAGE-1 mRNA and protein expression and specific CTL lysis. However, a few exceptions of melanoma cells expressing detectable levels of NY-ESO-1 or LAGE-1 but not recognized by specific CTL in the absence of exogenous antigenic peptides were detected. Lack of recognition of two of these lines could be explained by a particularly low level of protein expression, whereas other mechanisms are probably involved in the remaining cases. Such mechanisms could include a general defect in the generation of antigenic peptides by the intracellular processing machinery as well as specific alterations in NY-ESO-1/LAGE-1 protein metabolism and remain to be studied. 2) ORF1- and ORF2-specific CTL showed a remarkably similar pattern of tumor recognition, both qualitatively and quantitatively. Thus, although it is generally assumed that nonprimary ORF-encoded products are produced much less efficiently than primary ORF-derived proteins, we observed that NY-ESO-1/LAGE-1 ORF2-derived A2/peptide complexes are produced at a level functionally detectable to a comparable extent to that of NY-ESO-1/LAGE-1 ORF1-derived A2/peptide complexes. 3) The $CAMEL_{1-11}$/ORF2-derived epitope is efficiently generated by melanoma cells individually expressing NY-ESO-1 and LAGE-1, as shown by chromium release assay. This finding confirms, in a physiological context, the original observation by Aarnoudse et al. that COS-7 cells transfected with either LAGE-1 or NY-ESO-1 cDNAs can stimulate TNF release by a specific T cell clone (4). 4) The HLA-A2-restricted NY-ESO-1/ORF1-derived epitope (aa 157–165) is efficiently generated by melanoma cells expressing LAGE-1 only. Although not surprising, given the sequence identity of NY-ESO-1 and LAGE-1 in the region containing this epitope, this finding provides formal proof that a similar processing occurs for the two homologous products. It also provides the rationale for the inclusion of patients with LAGE-1-positive but NY-ESO-1-negative tumors in future $NY\text{-}ESO\text{-}1_{157-165}$-specific immunization protocols.

Several mechanisms have been described for the generation of alternative ORF polypeptides, including translational initiation codon

Table III. *Simultaneous generation of ORF1- and ORF2-derived epitopes by transfection of NA8-MEL and COS-7 cells with plasmids encoding NY-ESO-1 and LAGE-1 sequences*

Cell Line	Plasmid[a]	TNF Production by Specific CTL[b]		
		Clone LAU 156 NY-ESO-1/5	Clone LAU 50 CAMEL/4	Line LAU 342 CAMEL
NA8-MEL	Control	6	2	14
	Control + P	198	864	570
	$LAGE\text{-}1_S$	101	26	30
	$LAGE\text{-}1_L$	38	67	19
	NY-ESO-1	60	116	21
	CAMEL-mini GFP	2	680	10
	NY-ESO-1-mini GFP	60	3	4
COS-7	Control	4	3	7
	Control + P	200	1016	200
	$LAGE\text{-}1_S$	285	472	40
	$LAGE\text{-}1_L$	84	752	24
	NY-ESO-1	186	800	65
	CAMEL-mini	2	1184	888
	NY-ESO-1-mini	384	8	4

[a] Cells were transfected with plasmids containing full length cDNAs for NY-ESO-1, $LAGE\text{-}1_S$, and $LAGE\text{-}1_L$ or mini-gene versions of the $CAMEL_{1-11}$ and $NY\text{-}ESO\text{-}1_{157-165}$ epitopes (CAMEL- and NY-ESO-1-mini). Cells transfected with empty vector alone in the absence (control) or presence (control + P) of the relevant antigenic peptide were used as negative and positive controls, respectively. COS-7 cells were cotransfected with an HLA-A2 containing plasmid.

[b] Transfected cells were incubated with clone LAU 156 NY-ESO-1/5 (specific for $NY\text{-}ESO\text{-}1_{157-165}$/ORF1), clone LAU 50 CAMEL/4, and line LAU 342 CAMEL (specific for $CAMEL_{1-11}$/ORF2), and TNF release was measured by a bioassay as described in *Materials and Methods*. Values greater than 3-fold the corresponding background value were considered positive and are shown underlined.

scan-through, ribosomal frameshifting, reinitiation of translation, and abnormal splicing events (1, 5, 26). The specific mechanism responsible for the efficient production of NY-ESO-1 and LAGE-1 ORF2-derived peptides remains to be elucidated, as is the actual extent of CAMEL synthesis. Defective ribosomal products have been recently shown to constitute up to 30% of all newly synthesized proteins and have been proposed to be a major source for MHC class I antigenic peptides (27). To what degree alternative ORF polypeptides, such as CAMEL, can be assimilated to defective ribosomal products is unknown.

In conclusion, our findings demonstrate that both primary and nonprimary ORF-derived CTL epitopes can simultaneously and efficiently be generated by tumor cells and support the use of nonprimary ORF-derived CTL epitopes as vaccines to induce tumor specific cell-mediated immunity against cancer.

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